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ORIGINAL ARTICLE

Effects of alendronate on bone healing after tooth extraction in rats

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OBJECTIVES: Tooth extraction has been identified as an important risk factor for bisphosphonate-induced osteonecrosis of the jaw. Therefore, the main goal of this study was to determine the effects of alendronate on healing of the extraction socket and on interdental alveolar bone after tooth extraction in rats.

MATERIALS AND METHODS: Animals were injected subcutaneously with vehicle or alendronate for 3–4 weeks before the first mandibular molar was extracted and these treatments were continued during post-extraction periods of 10, 21, 35 and 70 days. Mandibles were processed to evaluate healing of the extraction socket and adjacent alveolar bone by assessing bone formation, bone resorption and vascularity by histomorphometric techniques.

RESULTS: Alendronate decreased new woven bone formation, blood vessel area, perimeter and number in the extraction socket at 10 days postextraction, but not at later time points. Furthermore, alendronate-treated rats had increased interdental alveolar bone volume and height only at 10 days postextraction. In addition, a 2.5-fold increase in the percentage of empty osteocyte lacunae was found in alveolar bone of alendronate-treated rats only at 10 days postextraction.

CONCLUSIONS: Alendronate transiently decreases bone formation and vascularity in the extraction socket and delays the removal of interdental alveolar bone after tooth extraction in rats.

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Keywords: osteonecrosis of the jaw; alendronate; tooth extraction; bone resorption; bone formation; alveolar bone

Introduction

Bisphosphonates (BPs) are stable analogs of inorganic pyrophosphate that are well established as antiresorptive drugs for over 30 years. BPs have proven to be important for the treatment of osteoporosis since the initial approval of alendronate (ALN) by the FDA more than 14 years ago. These drugs bind to bone surfaces and are 'ingested' by osteoclasts, altering their resorptive capacity and survival (Sato *et al*, 1991; Rodan and Fleisch, 1996). The potent inhibition of bone resorption by BPs prevents cancellous bone loss and even induces a modest increase in bone mass in elderly women with established osteoporosis (Devogelaer *et al*, 1996). Most importantly, BPs decrease the incidence of hip and vertebral fractures in osteoporotic patients (Cranney *et al*, 2002). More recently, BPs have been used to reduce malignancy-associated hypercalcemia and to inhibit bone metastases in patients with breast and prostate cancer and multiple myeloma (Lipton *et al*, 2000; Berenson *et al*, 2001).

Significant adverse side effects of BP treatment were limited to gastrointestinal intolerance (with oral administration) or an influenza-like syndrome (with intravenous administration) (Bilezikian, 2006) until 2003, when several independent investigators reported a possible association between patients treated with BPs and an atypical bone disorder named osteonecrosis of the jaw (ONJ) (Marx, 2003; Hellstein and Marek, 2004; Ruggiero *et al*, 2004; Marx *et al*, 2005). BP-associated ONJ is defined as an area of exposed bone in the maxillofacial region that does not heal within 8 weeks after identification by a health care provider, in a patient who is receiving or had been exposed to a bisphosphonate and had not had radiation therapy to the craniofacial region (Khosla *et al*, 2007). The incidence of ONJ is most frequently observed after dental interventions that involve bone, in particular after tooth extraction (Marx, 2003; Marx *et al*, 2005; Bilezikian, 2006; Jadu *et al*, 2007; Mavrokokki *et al*, 2007; King and Umland, 2008), in evident periodontitis (Marx *et al*, 2005; Bilezikian, 2006), and in patients receiving corticosteroid treatment (Marx, 2003; Marx *et al*, 2005; Bilezikian, 2006; Ruggiero *et al*, 2006; Jadu *et al*, 2007; King and Umland, 2008). Other predisposing factors include oral tori and exostoses, trauma to oral tissues and lingual mandibular sequestration. The occurrence of ONJ is two-fold greater in the

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mandible than in the maxilla (Bilezikian, 2006; Migliorati *et al*, 2006; Woo *et al*, 2006). It has been most frequently observed, with an incidence of 6–10%, in patients with multiple myeloma and breast or prostate cancers treated intravenously with high doses of nitrogen-containing BPs (N-BPs) such as zoledronic acid (ZOL) or pamidronate for inhibition of bone metastases (Marx, 2003; Ruggiero *et al*, 2004; Marx *et al*, 2005; Migliorati *et al*, 2005, 2006; Bilezikian, 2006; Woo *et al*, 2006). ONJ has also been reported to occur in postmenopausal women undergoing treatment for osteoporosis with lower doses of the less potent N-BPs ALN, ibandronate, and risedronate (Ruggiero *et al*, 2004; Marx *et al*, 2005; Woo *et al*, 2006; Yarom *et al*, 2007). Although the incidence is much less than in cancer patients, there are concerns that ONJ will be more frequently observed in osteoporotic patients as more of these elderly women are treated with BPs for longer periods of time.

Since tooth extraction has been identified as one of the most important risk factors associated with ONJ, the main goal of this study was to determine the effects of ALN on healing of the extraction socket and on interdental alveolar bone after tooth extraction. An essential component of normal healing after tooth extraction is an adequate blood supply. During the early phases of wound healing, angiogenic capillary sprouts invade the wound clot and within a few days organize into a microvascular network forming part of the granulation tissue. Besides their well-known antiresorptive activity, BPs have also been shown to have antiangiogenic effects (Meunier *et al*, 1979; Fournier *et al*, 2002; Santini *et al*, 2002; Wood *et al*, 2002; Santini *et al*, 2003; Hashimoto *et al*, 2007). Therefore, if drugs such as BPs reduce vascularity and bone resorption, they could compromise the early stages of bone healing after tooth extraction. Based on this, our central hypothesis is that BPs are involved in the pathogenesis of ONJ by: (i) reducing osteogenesis and vascularity and hence, delaying healing of the extraction socket; and (ii) suppressing the removal of alveolar bone, which will progressively accumulate in the oral cavity and may eventually become necrotic.

Materials and methods

Animals and experimental groups (study 1)

Seventeen 10 week-old intact female Sprague–Dawley rats (Charles River Laboratory, Wilmington, MA, USA) were used for this study. These animals were injected subcutaneously (SC) twice weekly with phosphate-buffered saline (vehicle) or with two different doses of ALN: 15 or 150 μg kg^{-1} body weight (bw). The 15 μg kg^{-1} dose used in this study was based on the dosage used by Fuchs *et al* (2007). It represents an intermediate dosage between the lowest effective dose used in preclinical studies in ovariectomized (OVX) rats (2.8 μg kg^{-1} SC twice weekly) (Toolan *et al*, 1992; Rodan *et al*, 1993) and the lowest dose used in the original study by Seedor *et al* (1991) [0.07 mg phosphorus (P) kg^{-1} SC twice a week (1 mg P kg^{-1} = 4 mg kg^{-1}), which is equivalent to 28 μg kg^{-1} SC twice a week].

The dose of 15 μg kg^{-1} SC twice weekly corresponds to a clinically relevant dose. The oral bioavailability of ALN is about 0.9–1.8% (Porras *et al*, 1999). Hence, a 15 μg kg^{-1} SC twice weekly dose (30 μg kg^{-1} week^{-1}) would be comparable to an oral dose of 1.6–3.3 mg kg^{-1} week^{-1}. For a 50 kg woman, this would represent an oral dose of 80–165 mg week^{-1} or 11.4–23.5 mg day^{-1}. The recommended dosage of ALN for the treatment of ostoporosis in postmenopausal women is one 70 mg tablet once weekly or one 10 mg tablet once daily but 20 mg has also been used (Chavassieux *et al*, 1997; Bagger *et al*, 2003; Bone *et al*, 2004; Emkey, 2004).

The rationale for the highest dose (150 μg kg^{-1}) used in this study was based on the principle of the ten-fold safety factor (Freed, 2006). This factor is used in toxicology as a reasonable starting point for the risk assessment of a new drug. It considers the potential interspecies differences in sensitivity to a drug when investigating a new endpoint, as in this case healing of the extraction socket. A 10-fold safety factor was also used by Toolan *et al* (1992) as a comparative dose of ALN in their study in OVX rats.

The BP ALN was obtained from Merck & Co. Inc. (Merck Research Laboratories, Rahway, NJ, USA) and dissolved in a vehicle of phosphate-buffered saline. Each of the three groups was composed of four or five rats. Treatments were initiated at 3 weeks before tooth extraction and continued for 10 days postextraction (PE).

Study 2

For this longitudinal assessment, we used 73 9-week-old intact female Sprague–Dawley rats (Charles River Laboratory, Wilmington, MA, USA). Animals were injected SC twice weekly with either phosphate-buffered saline (vehicle) or with ALN at a dose of 15 μg kg^{-1} bw. Treatments were administered for 4 weeks (instead of the 3-week period in study 1) before the first mandibular molar was extracted and continued during the PE period. Groups of vehicle- and ALN-treated rats were sacrificed at 10, 21, 35, and 70 days PE (n = 5–10 per group).

Tooth extraction and euthanasia procedures

All rats were anesthetized with an IP injection of ketamine (50 mg kg^{-1}) and xylazine (8 mg kg^{-1}). The left first mandibular molar (M1) was extracted in each rat with a dental explorer (#23). The tip of this instrument was first placed at the disto-buccal gingival margin between the first and second molars. The dental explorer was repeatedly rotated in a dorsal and mesial direction to loosen the first molar. The tip was then removed from its original position, placed at the bifurcation between the mesial and distal roots of the first molar and repeatedly rotated dorsally until extraction was achieved. In some cases, an apical portion of the root broke off and remained within the root socket. For the 17 rats in study 1, the entire molar was successfully extracted in 13 rats, whereas in four rats

(23.5%), part of a root broke and remained within the socket. In study 2, tooth extractions were successfully performed in 62 rats, whereas in 11 rats (15%), part of a root broke off. These rats with unsuccessful tooth extractions were excluded from the study.

To label bone-forming surfaces, all rats were injected SC with demeclocycline (Sigma Chemical Co., St Louis, MO, USA) and calcein (Sigma Chemical Co.) on the 10th and 3rd days prior to sacrifice, respectively. Both fluorochrome markers were administered at a dose of 15 mg kg^{-1} body weight. All rats were sacrificed by exsanguination from the abdominal aorta under ketamine and xylazine anaesthesia. All procedures involving use of rats were approved by the Institutional Animal Care and Use Committee at the University of Florida (Gainesville, FL, USA) and adequate measures were taken to minimize pain and discomfort in the animals.

Bone and blood vessel histomorphometry

The left mandible was stripped of musculature, cut cross-sectionally with a hand-held saw to isolate the region of the body with the extraction site and mandibular molars and placed in phosphate-buffered formalin for 24 h for tissue fixation. The bone samples were then dehydrated in ethanol, embedded undecalcified in modified methyl methacrylate (Baron *et al*, 1983) and sectioned longitudinally with Jung 2065 and 2165 microtomes (Leica Corp., Rockleigh, NJ) at thicknesses of 4 and 8 μm. The thinner sections were stained according to the von Kossa method with a tetrachrome counterstain (Polysciences, Warrington, PA, USA) whereas the 8 μm thick sections remained unstained for collection of fluorochrome-based data.

Histomorphometric parameters were measured in a blinded manner with the Osteomeasure/Trabecular Analysis System (OsteoMetrics, Inc., Atlanta, GA, USA). Analyses were performed within the distal root socket of M1 and in the interdental alveolar bone between this root socket and the second mandibular molar (M2). In study 1, histomorphometry was performed only in the distal root socket of M1. Figure 1 shows a schematic view of the two regions of interest (ROI) where quantitative histomorphometric analyses were conducted. ROI 1 is located in the distal root socket of M1 and extended from the alveolar crest to the apical end of the root socket and between the mesial and distal alveolar bone surfaces. ROI 2 was used to quantify variables in the interdental alveolar bone. It was defined as a rectangular area 2.5 mm in height and 0.75 mm in width positioned in the interdental alveolar bone between M2 and the distal root socket of M1, and centered at the middle axis of the alveolar bone. The lowest part of this rectangular area was approximated by a horizontal line (dotted line in Figure 1) that connects the tips of the M2 roots and the apical end of the root socket of M1. The variables assessed in ROI 1 included the percentage of the root socket filled with new woven bone in study 1 (woven bone volume) or woven or lamellar bone in study 2 (bone volume), and the percentage of surfaces of the new bone with osteoid, osteoblasts, osteoclasts and scalloping (osteoid, osteoblast, osteoclast and eroded surfaces, respectively) at a magnification of 200 $\times$. Fluorochrome-based indices of bone formation, including mineralizing surface (percentage of cancellous bone perimeter with double fluorochrome labels) and mineral apposition rate, were measured at 200 $\times$ in the thicker, unstained sections. Bone formation rate (surface referent) was calculated by multiplying mineralizing surface by mineral apposition rate (Frost, 1983). In addition, the number of blood vessels mm^{-2}, the percentage of area occupied by blood vessels and the perimeter of blood vessels mm^{-2} were also measured within this region at a magnification of 200 $\times$.

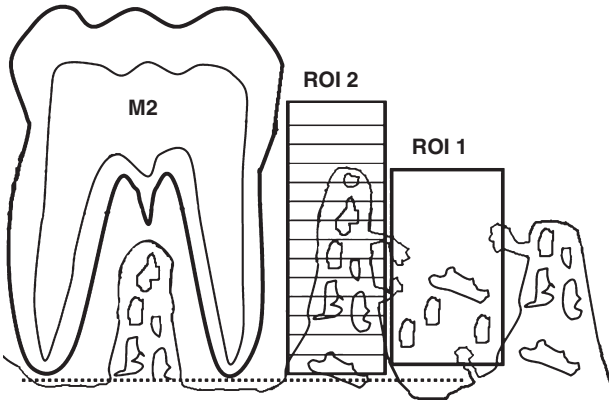

Figure 1 Schematic view of the regions of interest (ROI) for quantitative histomorphometric analyses. Histomorphometric analyses were performed within the distal root socket of the first mandibular molar (M1) and in the interdental alveolar bone, as depicted in ROI 1 and ROI 2, respectively. In study 1, histomorphometry was performed only in ROI 1, which extended from the alveolar crest to the apical end of the root socket and between the mesial and distal alveolar bone surfaces of the distal root socket of M1. ROI 2 was defined as a rectangular area 2.5 mm in height and 0.75 mm in width positioned in the interdental alveolar bone between the second mandibular molar (M2) and the distal root socket of M1 and centered at the mid-axis of the alveolar bone. The lowest part of this rectangular area was approximated by a horizontal line (dotted line) from the tips of the M2 roots to the apical end of the root socket of M1

The variables assessed in ROI 2 included the percentage of alveolar bone present within the area (alveolar bone volume), alveolar bone height, dynamic histomorphometric parameters (mineralizing surface, mineral apposition rate and bone formation rate), the percentage of alveolar bone surfaces with osteoid, osteoblasts, osteoclasts and scalloping (osteoid, osteoblast, osteoclast and eroded surfaces, respectively) at a magnification of 200 $\times$. The percentage of empty osteocyte lacunae was also measured in ROI 2 at a magnification of 400 $\times$. The terminology used, when applicable, was based on recommendations by the Histomorphometry Nomenclature Committee of the American Society of Bone and Mineral Research (Parfitt *et al*, 1987).

In addition to the mandibular analyses, several indices of bone mass were measured in the proximal tibial metaphysis to confirm the antiresorptive activity of ALN in these growing rats. ALN is well known to increase bone mass and mineralization by inhibiting osteoclast resorption and reducing bone turnover

(Reszka and Rodan, 2003). Therefore, we measured cancellous bone volume by bone histomorphometry and total BMC and BMD by peripheral quantitative computed tomography (pQCT) in the right and left proximal tibial metaphyses, respectively, to indirectly assess the antiresorptive activity of ALN.

The right proximal tibiae were processed for bone histomorphometry as previously described (Iwaniec *et al*, 2007). Bone volume was determined in cancellous bone tissue of the proximal tibial metaphysis beginning at a distance of 1 mm from the growth plate-metaphyseal junction to exclude the primary spongiosa. For the pQCT analysis, left tibiae were scanned using a Stratec XCT Research M instrument (Norland Medical Systems, Fort Atkinson, WI, USA) with software version 5.40. Scans were performed at a distance of 5 mm distal to the proximal end of the tibia. This site is at the level of the secondary spongiosa of the proximal tibial metaphysis. Volumetric content and density were determined for both trabecular and cortical bone as previously described (Ke *et al*, 2001).

Statistical analysis

Data are expressed as mean ± SD for each group. The PRISM 3.02 statistical package (GraphPad Inc., San Diego, CA, USA) was used for the statistical analysis. In study 1, the histomorphometric and pQCT data were evaluated by ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. When ANOVA assumptions regarding normality of data were not met, the non-parametric Kruskal–Wallis test was used. In study 2, changes in histomorphometric variables between vehicle-treated and ALN-treated rats were evaluated specifically at each time point using the unpaired Student's *t*-test. Regardless of the test employed, P values less than 0.05 were considered to be statistically significant.

Results

Study 1: effects of ALN on bone healing of the extraction socket at 10 days PE

In this study, we performed the histomorphometric analyses within the distal root socket of M1 in the specified area (ROI 1) described in Figure 1. We found that both doses of ALN decreased woven bone volume, compared to vehicle-treated rats, by approximately 55% at 10 days PE (Figure 2a). In addition, ALN induced a significant decrease in osteoid surface with the 15 μg kg^{-1} dose and tended to decrease osteoid surface with the 150 μg kg^{-1} dose. Strong trends for decreased osteoblast surface were also observed with both doses of ALN (Figure 2c). In contrast, no differences were observed in eroded and osteoclast surfaces between the vehicle-treated group and the groups treated with either dose of ALN (Figure 2d,e). As expected, the new bone within the distal root socket at 10 days PE was woven in nature. Therefore, we were unable to collect dynamic histomorphometric data due to diffuse fluorochrome labeling. Remarkably, both doses of ALN decreased blood vessel area (Figure 2f), blood vessel number

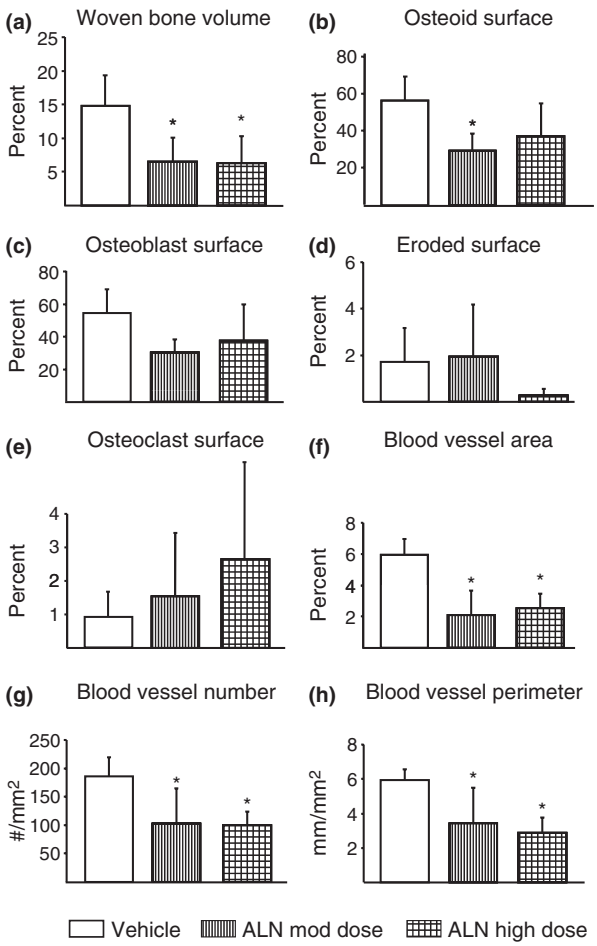

Figure 2 Alendronate reduces bone formation and vascularity within the distal root socket of the first mandibular molar at 10 days PE in rats. Bone and blood vessel histomorphometric data from the distal root socket of the first mandibular molar, including woven bone volume (**a**), osteoid surface (**b**), osteoblast surface (**c**), eroded surface (**d**), osteoclast surface (**e**), blood vessel area (**f**), blood vessel number (**g**) and blood vessel perimeter (**h**) (see ROI 1 in Figure 1 for details). Each bar represents the mean of four to five rats ± s.d. The animals were treated with vehicle or with two different doses of alendronate (ALN): 15 or 150 μg kg^{-1} bw for 3 weeks prior to the extraction of the tooth and during the PE period. All animals were sacrificed at 10 days PE. An asterisk denotes a significant difference from the vehicle-treated group ($P < 0.05$)

(Figure 2g) and blood vessel perimeter (Figure 2h) by approximately 60%, 45% and 40%, respectively. The effects of ALN on vascularity and new bone formation within the root socket are seen in Figure 3. Finally, both doses of ALN increased cancellous and cortical bone mass presumably due to their inhibitory effect on bone resorption (Table 1).

Study 2: longitudinal assessment of the effects of ALN on healing of the root socket and interdental alveolar bone after tooth extraction

Based on the results obtained in study 1, we investigated the longer-term effects of the lower dose of ALN (15 μg kg^{-1} bw) on the progression of healing of the root socket after tooth extraction. The histomorphometric assessment within the extraction socket was

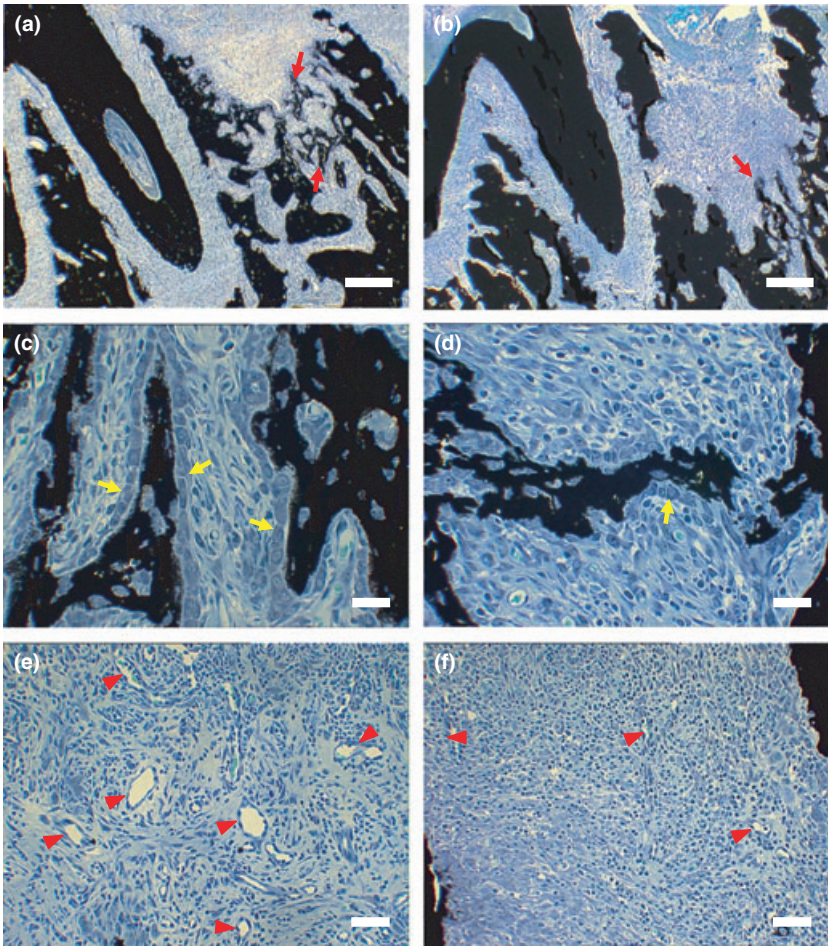

Figure 3 Alendronate reduces the amount of woven bone and the number of blood vessels within the root socket at 10 days PE in rats. This figure shows histologic sections of the distal root socket of the first mandibular molar from a vehicle-treated rat and a rat treated with a 150 μg kg^{-1} dose of ALN at 10 days PE (see ROI 1 in Figure 1 for details). Note the greater amount of black-stained woven bone (red arrows) within the root socket of the vehicle-treated rat (**a**) compared to the alendronate-treated rat (**b**). At higher magnification, increased numbers of osteoblasts (yellow arrows) lining the surfaces of woven bone can be seen in the vehicle-treated rat (**c**) compared to the alendronate-treated rat (**d**). Blood vessels (red arrowheads) are more numerous in the root socket of the vehicle-treated rat (e) compared to the alendronate-treated rat (**f**). Von Kossa/tetrachrome stain. Bars = 250 μm (**a, b**), 25 μm (**c, d**), or 50 μm (**e, f**)

Table 1 Moderate and high doses of alendronate increase bone mineral content, bone mineral density and cancellous bone volume in the proximal tibial metaphysis

	Vehicle	*ALN mod dose*	*ALN high dose*
tBMC (mg mm^{-1})	9.02 ± 0.83	12.70 ± 1.76*	12.44 ± 1.23*
tBMD (mg cm^{-3})	568.60 ± 64.57	730.88 ± 48.04*	737.38 ± 56.79*
Cancellous bone volume (%)	19.61 ± 4.32	35.94 ± 10.71*	36.01 ± 10.08*

Total bone mineral content (tBMC) and total bone mineral density (tBMD), by pQCT and cancellous bone volume, by bone histomorphometry, were measured in the proximal tibial metaphysis of rats from study 1 as indirect methods to assess the antiresorptive effect of this BP at the tissue level. These rats were treated with vehicle (vehicle), a moderate dose of alendronate (ALN mod dose), or a high dose of alendronate (ALN high dose) for 3 weeks prior to tooth extraction and during the PE period. All animals were sacrificed at 10 days PE. Data are the mean of four to five rats ± s.d. Data were analyzed by ANOVA followed by the Newman–Keuls multiple comparison test. *Significant difference from the vehicle-treated group ($P < 0.05$).

performed in ROI 1 as in study 1 (Figure 1). In addition, the interdental alveolar bone in ROI 2 (Figure 1) was subjected to histomorphometric analysis. A summary of the histomorphometric findings for study 2 is shown in Figure 4. As observed in study 1, we found that ALN decreased woven bone volume by approximately 75% compared to vehicle treatment of rats at 10 days PE (Figure 4a). However, after this time, the extraction socket was progressively filled at a comparable rate in both vehicle- and ALN-treated rats. By 70 days, the socket was almost completely filled with mature lamellar bone in both vehicle- and ALN-treated rats (Figure 5). In addition, a trend for decreased osteoblast surface (Figure 4c), and a significant decrease in eroded surface (Figure 4d), but not in osteoclast surface (Figure 4e), were also observed in ALN-treated rats at 10 days PE. Except for eroded surface, which showed a trend for a significant decrease (Figure 4d), none of these parameters were affected by ALN treatment during the remainder of the PE period. As previously observed in study 1, ALN reduced the number of blood vessels and blood vessel perimeter and tended to decrease the

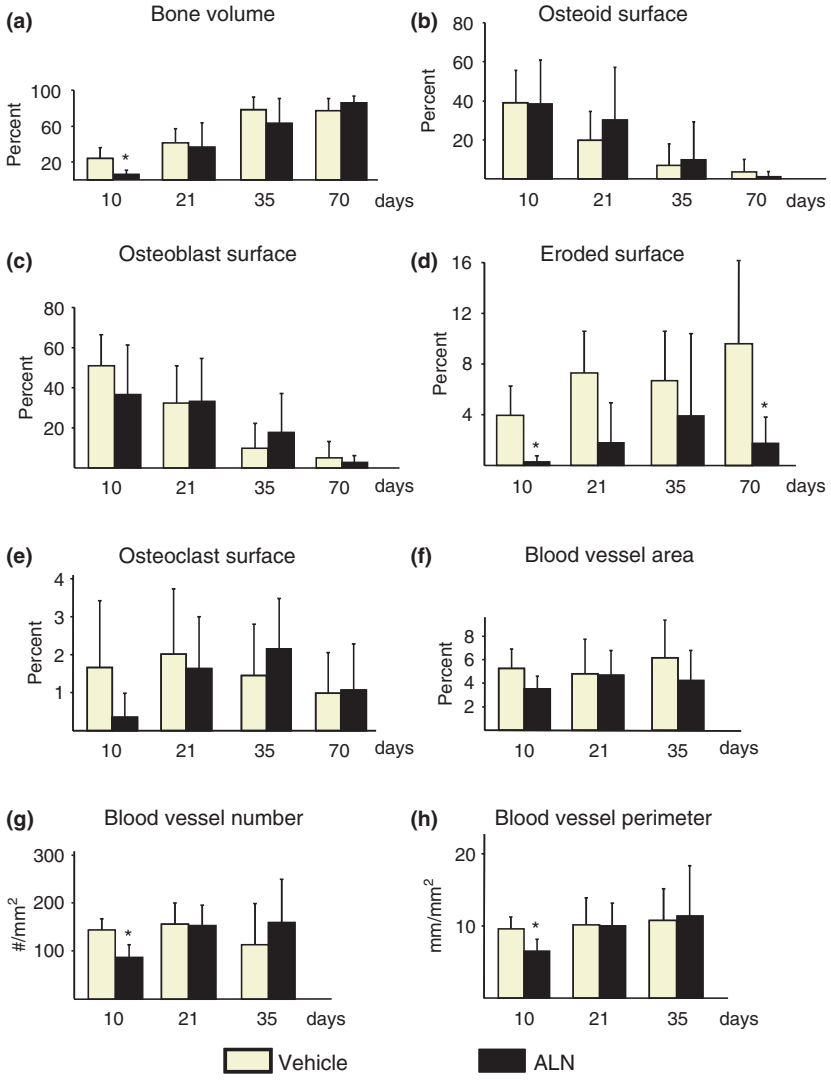

Figure 4 Alendronate transiently decreases new bone formation and blood vessel number in the extraction socket in rats. Bone histomorphometric data from the distal root socket of the first mandibular molar, including woven bone volume (**a**), osteoid surface (**b**), osteoblast surface (**c**), eroded surface (**d**) and osteoclast surface (**e**) from vehicle- and alendronate-treated rats (15 μg kg^{-1} dose) are presented for the different time points (10, 21, 35 and 70 days PE) (see ROI 1 in Figure 1 for details). Histomorphometric data for blood vessel area (**f**), blood vessel number (**g**) and blood vessel perimeter (**h**) are presented for 10, 21 and 35 days PE. The bars represent the mean of five to ten rats $\pm$ s.d. at each time point. Animals were treated with vehicle or a moderate dose of alendronate (ALN) for 4 weeks prior to tooth extraction and the treatments continued throughout the PE period. An asterisk denotes a significant difference from its respective time-matched, vehicle-treated group ($P < 0.05$)

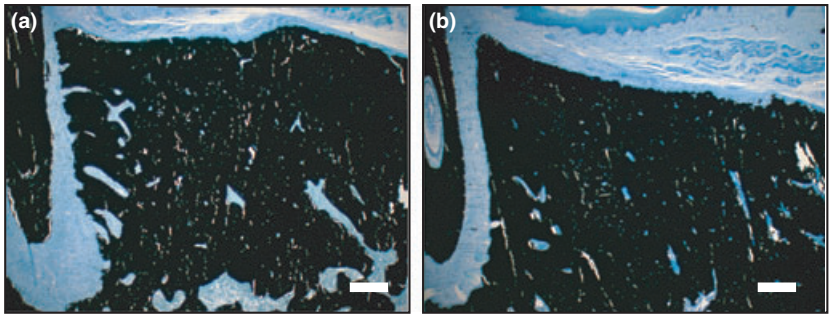

Figure 5 The effect of alendronate on filling of the extraction socket with bone is transient. This figure shows histologic sections of the distal root socket of the first mandibular molar at 70 days PE in vehicle-treated (**a**) and alendronate-treated (**b**) rats (15 μg kg^{-1} dose) (see ROI 1 in Figure 1 for details). The former root socket is entirely filled with lamellar bone in both animals, which shows that the adverse effects of alendronate observed during the early stages of bone healing are transient. Von Kossa/tetrachrome stain. Bars = 250 μm (**a, b**)

percentage of area of blood vessels in the extraction socket at 10 days PE (Figure 4g, h and f, respectively). The decline in these parameters was approximately 30% compared to vehicle-treated rats. In contrast, these effects were not observed at 21 and 35 days PE. The histomorphometric assessment of vasculature parameters was not performed at 70 days PE because by this time, most of the extraction socket was filled with bone. As in study 1, we were unable to collect dynamic histomorphometric data in rats at 10 days PE due to

diffuse fluorochrome labeling of woven bone. Nevertheless, we evaluated dynamic histomorphometric parameters at the later time points and found that ALN did not affect mineralizing surface, mineral apposition rate or bone formation rate at or after 21 days PE (data not shown).

The histomorphometric analysis conducted in the interdental alveolar bone revealed that ALN-treated rats had higher alveolar bone volume and height, compared to vehicle-treated rats, at 10 days PE (Figure 6a and b, respectively). In addition, we observed histologically that in two of five ALN-treated rats (40%) at 10 days PE from study 2, and in one of four ALN treated rats (25%) from study 1, the interdental alveolar bone exceeded the superficial surface of the socket, which was occupied by inflammatory tissue and not covered by oral epithelium. In these cases, the interdental bone appeared to be exteriorized in the oral cavity. However, at later time points, there were no significant differences in these variables between vehicle- and ALN-treated rats. Furthermore, ALN treatment induced a significant decrease in osteoblast surface (50%), mineralizing surface (45%), bone formation rate (90%) and eroded surface (90%), but not osteoclast surface, in the interdental alveolar bone at 10 days but not at 21 days PE (Figure 6). We did not perform histomorphometry in interdental alveolar bone at 35 and 70 days PE because after 21 days PE, the alveolar bone fused with the new bone in the extraction socket. Finally, we found that the interdental alveolar bone present in ROI 2 of ALN-treated rats at 10 days PE displayed a 2.5-fold increase in the percentage of empty osteocyte lacunae compared to alveolar bone from vehicle-treated rats (Figure 6h). These empty osteocyte lacunae were more frequently observed in the alveolar crest area. Differences in the interdental alveolar bone height and the number of empty osteocyte lacunae between vehicle- and ALN treated rats are shown in Figure 7.

Discussion

The effect of ALN on healing of the extraction socket and the adjacent alveolar bone after tooth extraction in rats has not been adequately studied by histomorphometric techniques. Therefore, we used these techniques to assess the bone healing process. We observed a transient decrease in vascularity and woven bone volume within the root socket as well as a transient retention of interdental bone at the alveolar crest in ALN-treated rats. Consistent with our findings, Hikita *et al* (2009) recently used μCT to show that local administration of 50 μl (1 mg kg^{-1}) of ALN directly into the maxilla every 4 days inhibited bone resorption in the alveolar septum and delayed new bone formation in the extraction socket during the first 7 days, but not at 14 days postextraction. Taken together, these findings suggest that ALN delays the bone healing process in the early stages after tooth extraction.

The sequence of events that occurs after tooth extraction in rats has been described in detail (Huebsch *et al*, 1952; Johansen, 1970; Smith, 1974; Lin *et al*, 1994). Briefly, the socket is filled with a blood clot, which is then replaced by a richly-vascularized granulation tissue. At the same time, intense osteoclastic bone resorption occurs in the adjacent buccal and lingual alveolar plates and within the socket to remove necrotic

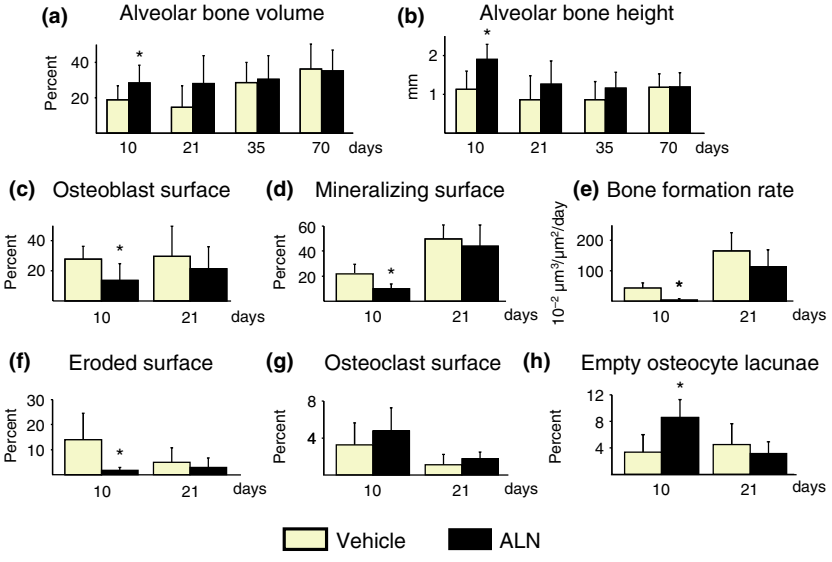

Figure 6 Alendronate transiently decreases alveolar bone turnover and increases the percentage of empty osteocyte lacunae in the interdental alveolar bone. Quantitative histomorphometric measurements were performed in the alveolar bone located at the interdental area between the second mandibular molar and the distal root socket of the first mandibular molar (see ROI 2 in Figure 1 for details). Each bar represents the mean of five to ten rats ± s.d. Animals were treated with vehicle or a 15 μg kg^{-1} dose of alendronate (ALN) for 4 weeks prior to tooth extraction and treatments continued for 10, 21, 35 and 70 days PE. Histomorphometric data for alveolar bone volume (**a**) and alveolar bone height (**b**) are presented for the different time points. Histomorphometric data for osteoblast surface (**c**), mineralizing surface (**d**), bone formation rate (**e**), eroded surface (**f**), osteoclast surface (**g**) and empty osteocyte lacunae (**h**) are presented for the vehicle and alendronate-treated rats at 10 and 21 days PE

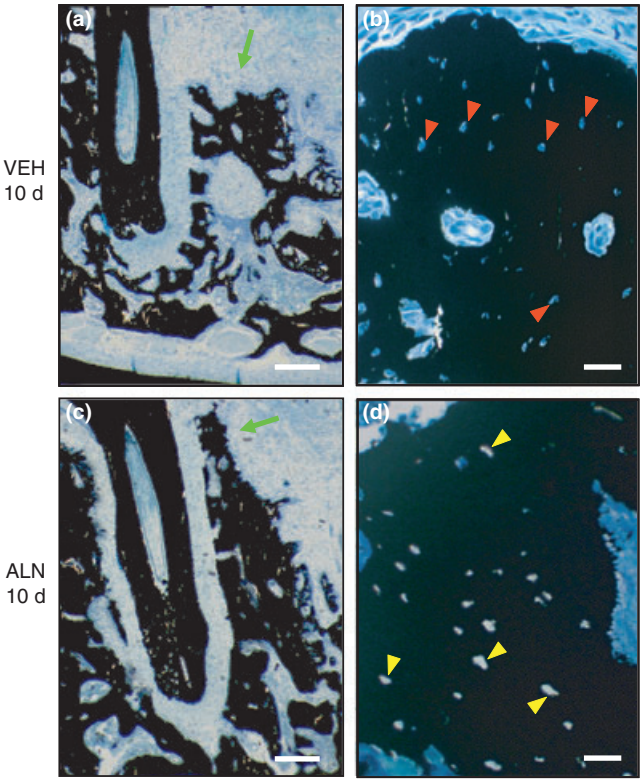

Figure 7 Alendronate-treated rats have higher alveolar bone volume and a higher percentage of empty osteocyte lacunae in the interdental alveolar bone at 10 days PE. This figure shows histologic sections of the interdental alveolar bone between the second mandibular molar (M2) and the distal root socket of the first mandibular molar from vehicle- and alendronate-treated rats (15 μg kg^{-1} dose) at 10 days PE (see ROI 2 in Figure 1 for details). Note the lower height of the black-stained interdental alveolar bone (green arrows) in the vehicle-treated rat (**a**) compared to the alendronate-treated rat (**c**). Also note the greater number of empty osteocyte lacunae present in the alendronate-treated rat (**d**) (yellow arrowheads) compared to the lacunae in the vehicle-treated rat (**b**), which regularly contained osteocytes (red arrowheads). Von Kossa/tetrachrome stain. Bars = 250 μm (**a, c**) or 25 μm (**b, d**)

bone and bone debris. Simultaneously, osteoblast differentiation and proliferation starts on the lateral alveolar walls and apical region of the socket. New bony trabeculae are initially observed in the apical region by day 5. Filling of the extraction socket occurs by apposition of bone along the lateral alveolar walls and fundus of the socket and by the formation of new woven bone projecting into the socket until it is entirely filled by days 20–28 PE. At later times, bone remodeling occurs within the socket to replace the newly-formed woven bone with mature lamellar bone. Although rodents regenerate oral tissues after tooth extraction much faster than humans, the sequence of events described above for rats is consistent with observations in higher mammals (Cardaropoli *et al*, 2003) and humans (Steiner *et al*, 2008).

We used relatively young rats instead of aged rats more comparable to elderly ONJ patients due to the fact that cementum deposition along the apices of the roots makes tooth extraction difficult in rats exceeding 60 days of age (Pietrokovski and Massler, 1967). It has been shown that increased age is associated with a decreased rate of bone repair and fracture healing (Hee *et al*, 2001; Naik *et al*, 2009). In fact, wound healing after tooth extraction is delayed in patients in the sixth decade and older compared to those in the second decade of life (Amler, 1977, 1993). In addition, younger rats were found to have higher VEGF serum levels compared to older rats after tooth extraction (Lin *et al*, 2008). Taken together, these data suggest that the observed decrease in bone formation and vascularity in the extraction socket of ALN-treated rats may have been even more pronounced in older animals.

A transient decrease in vascularity was observed in ALN-treated rats during the early stages of bone healing after tooth extraction. The anti-angiogenic effects of BPs have already been demonstrated by other investigators *in vivo* and *in vitro* (Meunier *et al*, 1979; Fournier *et al*, 2002; Santini *et al*, 2002; Wood *et al*, 2002; Santini *et al*, 2003). Fournier *et al* (2002) found that BPs reduced cultured endothelial cell proliferation, induced apoptosis and decreased formation of capillary-like tubes. Similarly, ZOL decreased the proliferation of endothelial cells *in vitro*, which were stimulated with foetal calf serum, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (Wood *et al*, 2002). There is also some *in vivo* evidence that BPs have anti-angiogenic properties. In patients with Paget's disease, treatment with the BP clodronate decreased the number of blood vessels within the bone marrow in histologic sections of iliac crest biopsies (Meunier *et al*, 1979). BPs also decreased testosterone-stimulated revascularization of the prostate gland in castrated rats (Fournier *et al*, 2002), and suppressed angiogenesis in subcutaneous implants impregnated with bFGF in mice.

The transient nature of the effects of ALN on vascularity and bone healing within the extraction socket is difficult to explain. As mentioned above, Hikita *et al* (2009) also found that the ALN-induced delay in bone healing after tooth extraction in rats was transient. After oral or SC administration, ALN enters the blood circulation and is deposited primarily in bone matrix. Because of the decreased vascularity within the extraction socket of ALN-treated rats, one would expect lower ALN bioavailability in this microenvironment during the later stages of the postextraction period. Moreover, because ALN inhibits bone resorption, there would presumably be decreased release of this drug from the alveolar bone surfaces adjacent to the extraction socket. Therefore, as the postextraction period progresses, the lower concentration of ALN within the extraction socket would allow bone healing and vascularity to approach the levels of vehicle-treated rats. This may be a plausible explanation for the transient delay in bone healing that we and others have observed after tooth extraction in ALN-treated rats.

In contrast to the probable direct effects of BPs on angiogenesis, their effects on osteoblastogenesis are contentious. Several investigators have reported that BPs exert a direct positive effect on osteoblast

differentiation (Reinholz *et al*, 2000; Fromigue and Body, 2002; von Knoch *et al*, 2005). In contrast, Khokher and Dandona (1989) found that BPs inhibit human osteoblast proliferation and differentiation. Conversely, Iwata *et al* (2006) found that ALN suppresses osteoblast activity without affecting osteoblast proliferation or differentiation at the periosteal surface. Another study shows that ALN has no effect on the viability, proliferation or mineral deposition activity of normal human osteoblasts (Garcia-Moreno *et al*, 1998). Whether the ALN-induced reduction in new bone formation within the root socket found in our study was due to a direct effect on osteoblastogenesis or indirectly through the decreased vascularity is a matter of conjecture at this time. It has been shown that neoangiogenesis plays a critical role in osteogenesis during bone healing (Glowacki, 1998; Carano and Filvaroff, 2003; Brandi and Collin-Osdoby, 2006; Kanczler and Oreffo, 2008). In fact, this notion is emphasized by the findings that angiogenic inhibitors prevent callus formation, deposition of woven bone along the periosteal surface near the fracture site and fracture healing (Street *et al*, 2002). In addition, animal models with defective or delayed angiogenesis have impaired healing during distraction osteogenesis (Choi *et al*, 2004). VEGF is a polypeptide which promotes primarily endothelial cell proliferation and angiogenesis (Gospodarowicz *et al*, 1989; Leung *et al*, 1989). Administration of VEGF after fracture stimulates neovascularization and improves bone healing and the biomechanical properties of the callus in rats (Street *et al*, 2002). In addition to its potent mitogenic effect on blood vessels, VEGF also stimulates bone formation by promoting osteoblast chemotaxis, differentiation and matrix mineralization (Midy and Plouet, 1994; Deckers *et al*, 2000; Mayr-Wohlfart *et al*, 2002; Street *et al*, 2002). ALN was found to inhibit VEGF-induced endothelial cell migration, capillary tube formation and Rho activation (Hashimoto *et al*, 2007). Furthermore, ZOL was found to decrease significantly serum levels of VEGF in cancer patients (Santini *et al*, 2003, 2007). Therefore, it is possible that ALN reduces angio- and osteogenesis by suppressing VEGF production.

Regarding interdental alveolar bone, we found that ALN transiently decreased bone turnover, reducing bone resorption and formation at 10 days PE, but not at later time points. As observed in the extraction socket, ALN did not affect osteoclast numbers but significantly affected osteoclast activity, resulting in a transient reduction in interdental alveolar bone resorption. Furthermore, the alveolar bone of ALN-treated rats contained an increased number of empty osteocyte lacunae at 10 days PE. The method we employed to determine osteocyte viability and to assess necrotic bone is not optimal because of the vagaries of sampling and sectioning. Therefore, our data on osteocyte viability must be interpreted with caution. However, because these differences are so striking, our data are suggestive that the accumulated bone at the alveolar crest in the ALN-treated rats is necrotic tissue. More reliable methods for quantification of osteocyte viability, in particular *in situ* histochemical analysis of lactate dehydrogenase (LDH) activity (Noble and Stevens, 2003; Mann *et al*, 2006) and identification of necrotic areas by bulk staining with basic fuchsin (Burr and Hooser, 1995; Allen, 2007; Allen and Burr, 2007) are preferable. Unfortunately, the limited availability of only one mandible with an extraction site per animal, which was processed for quantitative bone histomorphometry, did not allow additional methods of analysis.

Previous studies have demonstrated the inhibitory effect of ALN on bone resorption associated with dental interventions or periodontal disease (Weinreb *et al*, 1994; Shoji *et al*, 1995; Altundal and Guvener, 2004). Altundal and Guvener (2004) reported that ALN suppressed bone resorption along the alveolar crest after extraction of the first mandibular molar in rats. Furthermore, studies in rats and higher mammals have shown that BPs prevent alveolar bone loss during experimental periodontitis (Weinreb *et al*, 1994; Shoji *et al*, 1995). In these studies, BP treatment was examined for its potential application to prevent alveolar bone destruction during periodontal disease and to maintain alveolar bone width and height following tooth loss for dental implantation or for other prosthodontic approaches. Although preservation of alveolar bone is a rational goal in these circumstances, the persistence of necrotic alveolar bone after tooth extraction may make the jaw more vulnerable to ONJ in BP-treated patients.

The present study represents, to our knowledge, the first histomorphometric study showing that ALN transiently reduces vascularity and bone formation in the root socket following tooth extraction specifically in rats (Aguirre *et al*, 2007, 2008). In line with our findings, Kobayashi *et al* (2007) showed that the potent N-BP ZOL also induced inhibition of bone formation and angiogenesis after tooth extraction in mice. Several preclinical models of ONJ-like lesions induced by BP treatment have been studied (Gotcher and Jee, 1981a,b; Allen, 2007; Kobayashi *et al*, 2007; Allen and Burr, 2008; Sonis *et al*, 2009). Gotcher and Jee (1981a,b) used rice rats (*Oryzomys palustris*), which are extraordinarily susceptible to periodontal disease (Gupta and Shaw, 1956; Gotcher and Jee, 1981b), another well-documented risk factor for the development of ONJ (Marx *et al*, 2005; Bilezikian, 2006). In this study, rice rats were treated with dichloromethylene diphosphonate (Cl_2MDP), and this first generation BP was found to successfully prevent loss of interdental alveolar bone. However, from the perspective of ONJ, the most striking finding was the unusual amount, location and morphology of abnormal alveolar bone in these animals. In the histopathological description, the authors stated that alveolar bone in Cl_2MDP-treated rice rats was exposed in the oral cavity and some portions were devoid of bone cells and 'devitalized'. This description has strong similarities to the features of the necrotic alveolar bone observed in BP-treated patients with ONJ. Furthermore, it also has similarities to our observations in interdental alveolar bone of ALN-treated rats at 10 days PE. More recently, Sonis *et al* (2009) found that concurrent treatment with ZOL and dexamethasone

(DX) over a 1- to 3-week period induced some ONJ-like changes following molar extraction in rats. The majority of the rats receiving this concurrent treatment, but not ZOL alone, showed clinical and histological evidence of ulceration overlying areas of necrotic bone at day 14 PE. In addition, Allen and Burr (2008) found that 3 years of ALN administration in beagle dogs reduced alveolar bone turnover and increased the incidence of matrix necrosis in the mandible, but did not observe exposed bone lesions in the oral cavity. These preclinical studies suggest, in agreement with strong epidemiological evidence in patients, that BPs are involved in the pathogenesis of ONJ.

In conclusion, we present evidence that ALN transiently decreases bone formation and vascularity within the root socket and resorption of interdental alveolar bone after tooth extraction in rats. Such effects could also occur in patients treated for longer periods or with more potent BPs, which may contribute to the development of ONJ lesions.

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Conflict of interest

The authors have no conflicts of interest.

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