Published in final edited form as:

Bone. 2018 May ; 110: 141–149. doi:10.1016/j.bone.2018.01.030.

Removal of matrix-bound zoledronate prevents post-extraction osteonecrosis of the jaw by rescuing osteoclast function

Ranya Elsayed, DDS^{*}, Pheba Abraham, DDS, MSc^{*}, Mohamed E. Awad, MBBS^{*}, Zoya Kurago, DDS, PhD^{**}, Balasudha Baladhandayutham, BDS, MSc^{***}, Gary M. Whitford, DDS, PhD^{*}, David H. Pashley, DDS, PhD^{*}, Charles E. McKenna, PhD[‡], and Mohammed E. Elsalanty, MBBS, PhD^{*}

^{*}Department of Oral Biology, Dental College of Georgia, Augusta University, Augusta, GA, USA

^{**}Department of Diagnostic Sciences, Dental College of Georgia, Augusta University, Augusta, GA, USA

^{***}Department of Restorative Sciences, Dental College of Georgia, Augusta University, Augusta, GA, USA

[‡]Department of Chemistry, Dana and David Dornsife College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, CA, USA

Abstract

Unlike other antiresorptive medications, bisphosphonate molecules accumulate in the bone matrix. Previous studies of side-effects of anti-resorptive treatment focused mainly on systemic effects. We hypothesize that matrix-bound bisphosphonate molecules contribute to the pathogenesis of bisphosphonate-related osteonecrosis of the jaw (BRONJ). In this study, we examined the effect of matrix-bound bisphosphonates on osteoclast differentiation *in vitro* using TRAP staining and resorption assay, with and without pretreatment with EDTA. We also tested the effect of zoledronate chelation on the healing of post-extraction defect in rats. Our results confirmed that bisphosphonates bind to, and can be chelated from, mineralized matrix *in vitro* in a dose-dependent manner. Matrix-bound bisphosphonates impaired the differentiation of osteoclasts, evidenced by TRAP activity and resorption assay. Zoledronate-treated rats that underwent bilateral dental extraction with unilateral EDTA treatment showed significant improvement in mucosal healing and micro-CT analysis on the chelated sides. The results suggest that matrix-bound bisphosphonates are accessible to osteoclasts and chelating agents and contribute to the pathogenesis of BRONJ. The use of topical chelating agents is a promising strategy for the prevention of BRONJ following dental procedures in bisphosphonate-treated patients.

Corresponding Author: Mohammed Elsalanty, MBBS, PhD, Department of Oral Biology, Dental College of Georgia, Augusta University, 1120 15th Street, Augusta, GA, USA 20912, melsalanty@augusta.edu, 706-721-2585.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Zoledronate; bisphosphonates; osteonecrosis; bone matrix; alveolar bone; osteoporosis treatment; chelating agents

INTRODUCTION

Bisphosphonates are effective drugs for the management of bone-wasting conditions (1). They reduce the complications of bone metastatic lesions and minimizes skeletal-related events (SRE) in several malignancies, such as breast and prostate carcinomas, and multiple myeloma. Bisphosphonates inhibit osteoclastic bone resorption, thus decreasing the risk of bone fractures in elderly cancer patients, improving the quality of life, and increasing the survival rates in lung cancer with bone metastasis (2), breast cancer (3, 4), and prostate cancer (5). Their direct anti-tumor effect is being investigated in clinical trials (6), after it has been proven in several preclinical trials (7–9).

However, bisphosphonate use has declined by more than 50% from 2008 to 2012, due to concerns over uncommon side effects, such as atypical femur fractures and osteonecrosis of the jaw (10, 11). The highest incidence of bisphosphonate-related osteonecrosis of the jaw (BRONJ) was reported in 5–10% of cancer patients on high doses of zoledronic acid (Zol) treatment (12, 13). Patient incompliance (14), as well as reduction of drug prescription, have led to increase in osteoporosis-related complications in recent years (10).

Newer anti-resorptive medications were also associated with ONJ (12). On the other hand, since the majority of cases followed an invasive dental procedure, prevention strategies have so far depended on stopping the treatment before such procedures (drug holiday). However, unlike other anti-resorptive medications, bisphosphonates deposit in the bone matrix, due to their two phosphonate groups that form the bone hook (15, 16). The hydroxyl group on the R1 side-chain and even the R2 side-chain may also increase binding affinity, as well as therapeutic potency (17–20). These properties cause bisphosphonates to accumulate in the bone matrix for years after cessation of treatment, with an average half-life of approximately ten years (21). Therefore, while a drug holiday can reverse the systemic effect of bisphosphonates, as well as other anti-resorptive medications, it is unlikely to impact the activity of matrix-bound bisphosphonates. Previous studies have confirmed the accumulation of high levels of bisphosphonates in alveolar bone and periodontal ligament surrounding teeth (15, 22). The systemic effects of bisphosphonates have been extensively studied, including abnormal bone remodeling, systemic inflammation, and immune modulation (12). Yet, the role of matrix-bound bisphosphonate molecules in the development of BRONJ has not been elucidated. The development of an effective preventive strategy for BRONJ, a largely untreatable and devastating condition, depends on addressing both the systemic and any potential local effect of the drug.

Ethylenediaminetetraacetic acid (EDTA) is widely-used for dental as well as medical applications as a chelating agent that can bind to metals via four carboxylate and two amine group (23). It has been FDA approved and is used regularly to treat heavy metal poisoning in patients worldwide (24–26). In dentistry, it is routinely used for removal of smear layer, root

canal treatment, and root surface conditioning during periodontal treatments (23, 27, 28), where EDTA 17% is the most commonly used concentration in clinical dentistry as well as dental research (29–34). A previous study has shown a significant decrease in bisphosphonate content from bone using EDTA decalcification ex-vivo (35).

We have previously shown that bisphosphonates accumulate in alveolar bone and that they continue to be susceptible to both systemic and local chelating agents (15). In the current study, we hypothesized that matrix-bound bisphosphonates are both biologically accessible and active, and that they play a significant role in the pathogenesis of bisphosphonate-related osteonecrosis. Unlike other anti-resorptive medications, this property extends the availability of the drug beyond stopping the systemic treatment. We tested this overall hypothesis through the following specific aims: 1) we tested the hypothesis that matrix-bound zoledronate inhibited osteoclast differentiation and function in-vitro, using dentin discs, synthetic bone-mimicking matrix, and human bone, and 2) we tested the hypothesis that localized chelation of zoledronate from alveolar bone dental extraction sites could remove sufficient zoledronate to improve post-extraction socket healing and prevent the occurrence of osteonecrosis.

METHODS

Preparation of dental discs

Extracted non-pathological human 3rd molars were collected (under human assurance committee exemption #94-02-194, 2009, Augusta University) and stored in sodium azide at 4°. Teeth were cut to generate 1 mm-thick dentin slices using an IsoMet® Low Speed Saw (Buehler, Inc. Lake Bluff, Illinois, USA). The dentin surfaces were ground and polished using grinding discs (CarbiMet Abrasive paper plain 320 grit, Buehler Inc., Lake Bluff, Illinois, USA). The resulting smear layer was removed by acid etching the surface using 37% phosphoric acid for 15 seconds to the discs before application of the bisphosphonate treatment.

Fluorescent imaging of AF647-Zol bound to dentin discs before and after chelation with EDTA

Dentin discs were treated with varying doses (0, 0.1µM, 1µM) of AF647 Zol (Biovinc, LLC, Los Angeles, CA, USA) in a 24 well plate for 24 hours. The discs were then washed thoroughly with sterile phosphate –buffered saline (PBS) three times. Then discs were imaged using an Ami-X optical fluorescence imaging system (Spectral Instruments Imaging, LLC, Tucson, AZ, USA) with an excitation and emission Indocyanine green (ICG) filters. The fluorescent images were taken at 640nm and 670nm excitation and emission wavelength respectively. Regions of interest were standardized before the application of the fluorescent signals to prevent observational bias. Mean fluorescent intensity of the selected region of interest was plotted. Equal volumes of 0.5mL EDTA 17wt% (ethylenediaminetetraacetic acid and disodium salt dihydrate, Fisher Scientific Co, Aiken Rd, Asheville, NC, USA) was then applied to the discs for 30 minutes. The chelated solution was collected and its fluorescence was assessed by a plate reader (BioTek micro plate reader, BioTek instruments

Inc., Winooski, VT, USA), and the discs were re-imaged to compare fluorescent intensity before and after EDTA application.

Mass spectrometry of dentin discs

Dentin discs were treated with vehicle (PBS) or increasing doses of Zol (10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} moles) for 24 hours, then EDTA was applied for 30 minutes after which, the supernatant was collected, additionally the dentin discs were centrifuged in spin columns and the fluid from the dentinal tubules was collected and added to the supernatant. Zoledronic acid standards and samples were first derivatised before the liquid chromatography-mass spectrometry (LC-MS) analysis as follows: First, the sample (100uL) was diluted in isopropanol (400uL). Then, trimethylsilyl diazomethane (TMS-DAM; 2.0mol/L solution in hexane, 50ul, Sigma-Aldrich, Inc., St. Louis, MO, USA) was added. After 1 hour, the samples were evaporated to dryness under vacuum at 40°C and subsequently re-dissolved in methanol:water (1:9, 100uL) prior to injection.

Separation of the molecules was performed on a Shimadzu Nexera UHPLC system equipped with a Phenomenex Kinetex C18 column (100×2.1mm, 1.7um) at a flowrate of 0.2ml/min and column oven temperature of 40°C. A gradient elution between 5% acetonitrile in 10mM ammonium acetate (buffer A) and 90% acetonitrile in 10mM ammonium acetate (buffer B) was used for separation with the following steps: 2% buffer B for 2 minutes; 2%–80% buffer B in 4 minutes; 80% buffer B for 1 minute; 80% to 2% buffer B in 1 minute and 2% buffer B for 7 minutes. The effluent was ionized using positive ion electrospray on an AB-SCIEX 4000 QTRAP mass spectrometry (Sciex, Ltd., Gurugram, Haryana 122015, India) with the following instrument settings: ion spray voltage 5500V, curtain gas 20, temperature 450, gas1 30, gas 2 20, and low/unit resolution for Q1/Q3. The optimal collision energy, de-clustering potential, entrance potential and exit potential were determined using standards. The MS was running in MRM mode monitoring two transitions of 329.1/203.1 and 329.1/135.0 for derivatized zoledronic acid to improve the selectivity of the assay. After the samples were analyzed, the integrated peak areas for the two transitions were calculated using Multiquant software (version 2.0; Sciex. Ltd, Gurugram, Haryana 122015, India).

Cell culture, Osteoclast differentiation and resorption pit formation

Murine monocyte/macrophage lineage cell line (RAW264.7; American Type Culture Corporation (ATCC; Manassas, VA, USA) were cultured in Dulbecco's modified eagle medium (DMEM; ATCC) with 10%FBS and 1% Penicillin/Streptomycin, and incubated in 37°C and 5% CO₂ and passaged at a low number to be used for the differentiation assay. Osteoassay plates (Corning Inc., New York, NY, USA) were pretreated with increasing doses (1 μM, 10 μM) of zoledronic acid (Zol; Selleck Chemicals, Inc.; Houston, TX, USA), for 24 hours, after which they were thoroughly washed to remove any unbound Zol. RAW 264.7 cells were seeded onto the OsteoAssay plates, and grown until 80% confluency. Osteoclast differentiation and resorption pit formation were assessed according to the manufacturer protocol. Briefly, differentiation medium was prepared using Minimum Essential Medium (MEM-Alpha), 10% FBS, 1% Pen/Strep and 50 ng/mL receptor activator of nuclear factor kappa-B ligand (Recombinant mouse RANKL; Abcam, Inc., Cambridge, MA, USA). Differentiation medium was changed every other day for 7 days, then the cells

were TRAP stained with (Acid Phosphatase, Leukocyte (TRAP) Sigma Aldrich, Inc. St. Louis, MO, USA) according to manufacturer's instructions. The percentage of TRAP-positive cells was calculated using Image-J software (<https://imagej.nih.gov/ij/>), by counting the number of TRAP-positive cells and the total number of cells. The results were reported as a percentage: $\frac{\text{TRAP} - \text{positive cell}}{\text{Total number of cells}} \times 100$. Resorption pits were assessed after 21 days of incubation, by bleaching plates to remove the cells followed by staining with toluidine blue to stain the resorption pits. For counting the TRAP-positive cells or the resorption pits, each well was divided into 4 quadrants and 8 random pictures were taken, 2 within each quadrant, using a light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). An average of the 8 images was used for the statistical analysis. Images were coded and blind analysis was done. Three independent trials were done.

EDTA chelation of zoledronate-treated matrix

To investigate the effect of chelation-induced removal of Zol on osteoclast differentiation, we repeated the osteoclast differentiation experiment on the osteoassay plate as described above, with addition of EDTA 17% (Ethylenediaminetetraacetic Acid, Disodium Salt Dihydrate, Fisher Scientific, Inc., Aiken Rd, Asheville, NC, USA) for 30 minutes after the 24-hour Zol treatment. The plate was then thoroughly washed with phosphate buffered saline (PBS) to remove the EDTA; the RAW264.7 cells were then seeded onto the plate.

Measurement of adhesion of RAW264.7 cells on mineralized matrix pre-treated with zoledronate

To exclude any intrinsic effects of EDTA pretreatment on the initial adhesion of cells to the matrix, EDTA 17%, or PBS as control was applied onto 24 well osteoassay plates (Corning, Inc., New York, NY, USA) for 30 minutes, the plates were then washed and 50,000 cells/well were seeded and incubated for 24 hours at 37°C and 5% CO₂. The plates were then washed to remove any unattached cells and 8 microscopic images at 10x were taken randomly per well, and counted blindly. The average of those 8 images per well was used for statistical analysis.

Gene expression

RAW264.7 cells were treated with increasing doses of Zol and differentiated into osteoclasts as previously mentioned for 5 days; then total RNA was extracted using the triazole/RNAeasy spin columns (Qiagen RNeasy plus Mini kit, Qiagen, Inc., Valencia, CA, USA). Reverse transcription of RNA to cDNA was done using (Bio Rad iScript™ Reverse Transcription Supermix for RT-qPCR). Relative gene expression of RANK, DC-STAMP, Cathepsin-K was assessed with Syber green qPCR (Applied Biosystem, Foster City, CA, USA) using primers with the following sequences: **RANK: fwd** GCA TCC CTT GCA GCT CAA CA **rev** ATG GAA GAG CTG CACC AC, **Cathepsin-K: fwd** CCA GTT TTA CAG CAA AGG TGT GT **rev** TGC CAC AGG CGT TGT TCT TA **DC Stamp: fwd** TCC TCA TCG CAG CAG TTG TT **rev** CGC ATC ACA GGC CAA AGA AG. Fold-change and fold-regulation were calculated using delta-delta CT method.

Quantification of resorption byproducts (pyridinoline, PYD, and calcium) levels in Human bone plate

A human bone plate (OsteoAssay Human bone plate, Lonza, Inc., Walkersville, MD, USA) was treated with 2 different doses of Zol (1 μ M and 10 μ M) for 24 hours with or without subsequent chelation with EDTA 17% (repeated thorough washing with PBS followed each of the Zol and EDTA treatment). Then, RAW264.7 cells were cultured on the plate and differentiated into osteoclasts, as described above, for 21 days. The supernatant was collected at 7, 14, and 21 days-time points for detection of PYD and Ca levels. The PYD levels were detected in the supernatants with a human Pyridinoline (PYD) ELISA kit (Biotang, Inc., Lexington, MA, USA) at the three indicated time points. Calcium concentrations were determined by Atomic absorption spectrometry (Varian SpectraAA atomic absorption spectrometer; Varian Medical Systems, Palo Alto, CA, USA) and direct aspiration following the addition of equal volumes of 5000 μ g/mL KCl to suppress ionization of calcium in the acetylene/nitrous oxide flame.

Proliferation Assay

To investigate the effect of Zol on the cell proliferation, we used the CyQuant cell proliferation assay kit (Invitrogen, Inc., Carlsbad, CA, USA). OsteoAssay plates (Corning, Inc., New York, NY, USA) were treated with increasing doses of Zol (1 μ M, 5 μ M, 10 μ M, 50 μ M and 100 μ M) for 24 hours. After thorough washing, RAW264.7 were added to the plate for 24 hours and incubated in 37°C and 5% CO₂, after which the assay was performed according to the manufacturer's instructions, where the amount of DNA content was compared between all groups based on created DNA standard curve.

Animals

The experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Augusta University (Protocol #2012-0496; Date: 10/25/2012). Twenty female Sprague-Dawley rats (ages 10–12 months) were treated with either intravenous Zol 80 μ g/kg/week (experimental; n=10) or saline (control; n=10) for 13 weeks, as previously described (36). On week 13, bilateral extraction of first and second mandibular molars was done under general anesthesia using intraperitoneal injections of ketamine (100 mg/mL) and xylazine (20 mg/mL). On each side, after achieving hemostasis, a 2mm-tip cotton Q-tip was saturated with 17% EDTA (experimental side) or normal saline (control side) for 10 minutes, changing the tip every 2 minutes. The right versus left sides were randomized between EDTA and saline in both Zol and control groups. After 5 weeks of healing, the rats were euthanized and the two groups of mandibles were analyzed clinically, radiographically and histologically. The reasons for choosing female Sprague Dawley rats was that 1) they reached sufficient size for adequate control of dental extraction, allowing the safe removal of four molars without breaking the jawbone; and 2) to compare the results with our previous studies that showed consistent induction of BRONJ in this model, without having to modify the age of the animal, since males at the same size are usually older. In our ongoing studies, we will repeat the experiments using male rats.

Histological analysis

Histological analysis was performed as previously described (36). Briefly, samples were embedded in paraffin and sagittal sections of 5µm were cut through the extraction site, then stained with hematoxylin and eosin. Three random microphotographs were taken at 20x magnification of the region of interest (ROI) per animal (Zeiss AxioIma, Carl Zeiss Microscopy GmbH, Jena, Germany). The ROI was selected at the base of the extraction defect, using the mesial root of the third molar as a reference point, where the length of the ROI was determined by the beginning and the end of the root. The number of dead osteocytes (empty osteocyte lacunae) divided by the total number of cells were counted by a blinded investigator, and results were presented as percentage: $\frac{\text{Number of empty lacunae}}{\text{Total number of lacunae}} \times 100$.

The degree of alveolar bone necrosis, quantified as the percentage of empty lacunae at the extraction sites, was compared between the saline and EDTA sides in the control and Zol-treated animals.

Ex-vivo evaluation of mucosal closure and micro-CT analysis of alveolar bone

After euthanasia, photographs of extraction site were taken to assess the healing of the extraction sites and to identify exposed necrotic bone. The mandibles from the four groups were scanned by microCT using an ex vivo microCT system (Skyscan 1174; Skyscan, Vluchtenburgstraat 3, 2630 Aartselaar Belgium). Each sample was scanned in air using a 0.25-mm Aluminum filter, 22.6 µ isotropic voxels, 2000 millisecond integration time, 0.5° rotation step, and frame averaging of 3. For 3-D reconstruction (NRecon software, Skyscan), the grey scale was set from 70 to 255. The reconstructed images were then examined to identify evidence and extent of three-dimensional sequestration of the alveolar bone. A healing score (1–10, with 10 being the best healing) was given to each site, based on the degree of mucosal closure, with normal gingiva or granulation tissue, and the detection of sequestration in micro-CT, similar to previously published protocol (36). Briefly, photographs of unlabeled samples were taken the day after harvest. Same was done for the micro-CT images. Images were coded and scored blindly. To eliminate the possibility of identifying right versus left sides based on sample morphology, the study was designed so that the treatment (EDTA versus saline) was randomized between the right and left sides. A healing score was assigned to each sample as follows:

- 1–3: Exposed necrotic bone plus evidence of sequestration in the micro-CT
- 4–6: Mixed granulation tissue and mucosal coverage, some exposure, and no evidence of sequestration in micro-CT
- 7–9: Mixed granulation tissue and mucosal coverage with complete coverage, no sequestration
- 10: Normal mucosal coverage, no sequestration, evidence of normal bone filling of the defect.

Statistical analysis

Statistical analysis was done using GraphPad Prism software version 6 (GraphPad Software, La Jolla, CA, USA). Data values were reported as means ± SD. Normality assumption was

evaluated using the Shapiro-Wilk test. When normality assumptions were not met, an alternative non-parametric test was done. A one-way ANOVA test with significance defined as $p < 0.05$, a confidence level of 95% confidence interval and Bonferroni post-hoc comparison was used to compare multiple groups. Based on an estimated effect size of 1.64, based on our previous studies (36), a sample size of 8 animals was determined to yield a study power of 0.96 (G*Power v.3.1) for comparing means from dependent groups. Two-way ANOVA with Bonferroni post hoc multiple comparison test was done to compare treatment groups before and after chelation. A Wilcoxon signed-rank test was used to compare the clinical scores between EDTA and saline sides within each group; and a Mann-Whitney U test was used to compare the scores between the control and Zol-treated animals.

RESULTS

Quantification of zoledronate deposition and chelation in vitro

Fluorescence imaging of AF647-Zol-treated dentin discs showed a dose-dependent increase in the intensity of the fluorescent signal. The amount of AF647-Zol in dentin discs decreased after EDTA application with a statistical significance in the 0.1 μM ($p < 0.001$). (Fig 1A, 1B) The chelation supernatant showed an increase in the amount of released Zol in a dose dependent manner with statistical significance in both the 0.1 μM ($p < 0.043$) and 1 μM ($p < 0.001$; Fig 1C)

Chelation with EDTA of dentin discs pretreated with increasing doses of Zol (10^{-5} , 10^{-4} , 10^{-3} M) was further quantified using liquid chromatography coupled with tandem mass spectrometry (LC/MS). Results showed that Zol was detected in the chelated solution from the discs treated with 10^{-3} , 10^{-4} and 10^{-5} M of Zol, showing dose-dependence ($p = 0.049$; Fig. 1D).

Matrix pre-treatment with Zol inhibited osteoclast differentiation and resorption pit formation in-vitro

OsteoAssay plates pretreated with increasing doses of Zol (0.01 μM , 1 μM , 100 μM) for 24 hours, were used to grow RAW264.7 cells. TRAP staining at day 5 of incubation showed a dose-dependent decrease in the percentage of the TRAP-positive cells compared to the control group as shown in the representative images (Fig. 2A-upper panel), with a statistically significant decrease in the 1 μM and 100 μM concentrations ($p = 0.0093$ and $p = 0.0048$, respectively; Fig. 2B). Similarly, the percentage of the resorption pit areas at 21 days of incubation decreased, achieving statistical significance at the 100 μM Zol ($p = 0.0150$; Fig. 2A-lower panel, and Fig. 2C).

Proliferation assay showed that there was no significant effect on cell number in the treated cells compared with the control, except at the 100 μM Zol dose, where proliferation showed a significant decrease ($p = 0.01$; Fig. 2D). Furthermore, Zol treatment decreased relative expression of mRNA levels of RANK, Cathepsin-k and DC-STAMP, where direct pretreatment of RAW cells 264.7 with Zol (10 μM) for 24 hours caused statistically significant down-regulation of osteoclasts markers RANK, DC STAMP and Cathepsin K. ($p = 0.003$, $p = 0.001$, $p < 0.0001$) respectively (Fig. 2E–G).

Prior EDTA chelation-induced removal of Zol from the matrix rescues osteoclast differentiation and function in vitro

First, testing the effect of EDTA pretreatment on initial cellular adhesion showed no difference between the two groups (Fig. 3A). Osteoclast differentiation on human bone matrix pretreated with Zol (1 μ M, 10 μ M) showed a significant decrease in the percentage of TRAP-positive osteoclasts compared to untreated matrix ($p=0.0057$, $p<0.0001$, respectively). Interestingly, surface-chelating the bone matrix with 17% EDTA for 30 minutes, followed by washing, before the start of osteoclast differentiation led to a significant increase in the number of TRAP-positive osteoclasts in the 1 μ M ($p<0.0001$) and 10 μ M ($p=0.0009$) groups compared to non-chelated Zol-pretreated groups (Fig 3B).

The calcium levels in the supernatant showed an overall decrease with Zol treatment, statistically significant with the 10 μ M Zol treatment at the 14 and the 21 days' time-points ($p<0.0095$, $p<0.0055$, respectively) compared to the control (Fig. 3C). Furthermore, there was significant decrease in PYD levels with Zol treatment (1 μ M, 10 μ M) at all time-points ($p<0.0001$) (Fig 3D). Interestingly, pre-chelation of the matrix with EDTA caused a significant increase in the Calcium levels at 7 (both doses; $p<0.0001$) and 14 days-time points (1 μ M; $p<0.001$), compared to non-chelated groups. At 21 days, calcium values were comparable to the un-chelated groups. For PYD levels, chelation caused a significant increase at both the 1 μ M and 10 μ M ($p<0.0001$ and $p=0.01$, respectively) at 7 days, and maintained a significant increase in the chelated groups at 14 days (both doses; $p<0.0001$), and 21 days (1 μ M; $p=0.016$) (Fig 3C, D).

EDTA prevents BRONJ in vivo

Nine animals from the control and eight from the Zol groups reached the endpoint of the experiment. The remaining three animals were lost during, or shortly after, the surgical extraction procedure due to complications of anesthesia. There were no signs of BRONJ in the control animals with or without chelation (Fig 4A). Gingival coverage was deficient in Zol-treated animals on the sides treated with saline, with frequent exposure of necrotic bone (Fig 4A, second panel). Micro-CT analysis showed bone sequestration in a few defects on the saline-treated sides in Zol-treated animals (Fig. 4B). Healing scores at the extraction site, based on gingival coverage and micro-CT bone healing, confirmed significantly impaired healing in Zol-treated, compared to the control treated animals ($p=0.0033$; Fig 4C), and on the saline versus EDTA-treated sides in the Zol-treated group ($p=0.0156$; Fig 4C). There was no significant difference between the EDTA and saline-treated sides in the control animals (Fig 4C). Similarly, histomorphometric analysis (figure 4D–F) showed a significant increase in percentage of empty osteocyte lacunae in the Zol-treated, compared to control animals at the base of the defect, signifying an increase in bone necrosis ($p<0.0001$; Fig 4F). However, no significant difference was found in the percentage of empty osteocyte lacunae between the EDTA versus saline-treated sides in control animals. (Fig 4F). On the other hand, the percentage of empty lacunae was significantly lower on the EDTA versus saline-treated sides in the Zol treated group ($p<0.0001$; Fig 4F). To examine the effect of EDTA on the bone mineral density of the alveolar bone, we compared the bone mineral density (BMD) of alveolar bone apical to the defect on the EDTA versus saline-treated sides. No significant

difference was detected, excluding significant EDTA-induced demineralization in the alveolar bone (fig 4G).

DISCUSSION

In this study, we hypothesized that matrix-bound bisphosphonates were both biologically accessible and active, and that they played a significant role in the pathogenesis of bisphosphonate-related osteonecrosis. We tested this overall hypothesis through the following specific aims: 1) we tested the hypothesis that matrix-bound zoledronate inhibited osteoclast differentiation and function in-vitro, using dentin discs, synthetic bone-mimicking matrix, and human bone matrix, an effect that can be corrected by reducing matrix-bound bisphosphonate using EDTA; and 2) we tested the hypothesis that localized chelation of zoledronate after dental extraction could be sufficient to improve post-extraction socket healing and prevent the occurrence of osteonecrosis.

Our results demonstrated that matrix-bound bisphosphonate molecules inhibited osteoclast differentiation and resorption activity. Such inhibition was corrected when the matrix-bound bisphosphonates were removed using EDTA prior to seeding the cells. Zoledronate dose-dependent attachment and removal were confirmed with AF647-Zol and mass spectrometry. Pretreatment of Zol-free matrix with EDTA did not alter the attachment of monocytes to the surface, excluding a direct effect of EDTA on the affinity of the surface to cellular growth. Therefore, the observed improvement in cellular differentiation when the Zol-rich matrix was treated with EDTA prior to seeding of the cells was more likely due to removal of Zol molecules from the matrix and not an intrinsic effect of EDTA on the matrix itself. In addition, the observed Zol-induced inhibition on calcium and PYD released by osteoclast activity was significantly and consistently corrected when the zoledronate was removed from the matrix by EDTA prior to seeding of the cells.

These results provide strong support for the hypothesis that matrix-bound bisphosphonates elicited a biological effect on attached osteoclasts that is comparable to the well-established systemic effect. The implication of these finding is that bisphosphonates may continue to illicit a localized effect when bisphosphonate-laden surfaces are exposed, which occurs after dental extraction and periodontal disease. This mode of action, which is unique to bisphosphonates as opposed to other anti-resorptive medications, may play an important role in the induction of osteonecrosis following bone exposure, even after systemic treatment has been discontinued. After an intravenous dose of Zol in humans, about 40 % of the drug is excreted unchanged in the urine within the first 24 hours, while the remaining 60% becomes deposited in the bone matrix, where it persists bound to the bone for a long time, with minimal redistribution in the first few weeks (16).

To explore the role of localized bisphosphonates in the induction of osteonecrosis, we tested whether removing localized bisphosphonate after dental extraction could improve socket healing and prevent osteonecrosis in a BRONJ rat model (36), where an IV zoledronate of 80 ug/kg per week for 13 weeks, followed by extraction of the first and second molar consistently demonstrated gross, radiographic, and histologic features of BRONJ with no toxicity to the kidney or liver. The model also demonstrated the systemic therapeutic effects

of zoledronate, evidenced by the changes in vertebral bone quality (unpublished data). Bilateral extraction of 1st and 2nd molars were done to provide a trigger for BRONJ where invasive dental procedures are the number one precipitating event for osteonecrosis in bisphosphonate-treated human patients. We compared the post-extraction socket healing in Zol-treated rats between the side treated with EDTA and the contralateral side treated with saline. The results showed significant improvement in socket healing and reduction in osteonecrosis parameters on the EDTA-treated extraction sockets, compared to the saline-treated. Taken together, the results of this study showed, for the first time, that post-extraction osteonecrosis can be prevented by local removal of matrix-bound bisphosphonate molecules.

We have previously shown that bisphosphonates preferentially adhered to alveolar bone, compared to basal bone, and that treatment with systemic and localized chelating agents reduced the zoledronate content in alveolar bone, with minimal effect on serum calcium (15). Other studies have also shown that decalcification of bone, decreased the amount of bound Zol (35). The long-term accumulation of Zol in the bone after treatment discontinuity complicates the efforts to predict and/or prevent the occurrence of osteonecrosis, more so than other anti-resorptive drugs, which lack this characteristic (12). Thus, we believe that if an invasive dental procedure was necessary in patients on long-term bisphosphonates, stopping the treatment (drug holiday) should be coupled with post-extraction removal of Zol from the exposed surfaces of alveolar bone by local EDTA chelation. This strategy would target both the systemic and localized negative effects of Zol on the early healing response, and minimize the incidence of osteonecrosis. Further studies are needed to test such strategy in human patients.

The effects of direct Zol treatment of pre-osteoclast on their differentiation and functions are well established (37, 38). This study targeted for the first time the effects of residual Zol molecules in the matrix itself. Although matrix-bound bisphosphonate molecules may become buried under the surface, inaccessible to bone marrow cells, an invasive dental procedure, such as dental extraction, can expose matrix-bound Zol, some of which may become released into the oral environment, affecting nearby cells during the early phase of bone healing. Ongoing studies explore the mechanisms by which matrix-bound bisphosphonate molecules could tip the balance of bone healing, pushing it towards more tissue destruction than successful regeneration, ultimately resulting in osteonecrosis.

Our data suggest that the improved osteoclast function was not due to a direct EDTA effect on the cells or the matrix. However, a previous study demonstrated that calcium was crucial for the internalization of Zol into the osteoclasts (39). The release of both calcium and Zol from the matrix into the socket space could be an independent direct mechanism by which EDTA can affect osteoclasts. Another potential effect of EDTA can be through delaying the formation of the initial blood clot following dental extraction. Such a delay, which was not evident in our animals, can theoretically impact socket healing by altering the number of recruited cells from the circulation, and by modulating clot formation, which is the first step in bone healing. More detailed studies addressing these concerns are planned.

EDTA is Ethylene Diamine Tetra Acetic Acid, a strong metal chelator, which is soluble in water. It has 6 electrons to donate, thus forming hexadentate ligand that can chelate calcium via its 2 amine and 4 carboxylic acid groups. This forms a coordination complex that totally surrounds the calcium forming an octahedral structure. In this coordination structure, a heterocyclic ring is formed with the calcium ion in the center, totally enveloped by EDTA. Binding of EDTA to Ca keeps it in solution, while decreasing its reactivity. It has been shown that the affinity of EDTA for calcium (stability constant k_s $4.4 \times 10^7 \text{ M}^{-1}$) (40) is greater than that of Zol (k_s $9.74 \times 10^3 \text{ M}^{-1}$) (41) at pH7.4. Thus, calcium will preferentially bind to EDTA. Binding of Zol to EDTA is less likely, since they are both anions. Previous studies showed that adding EDTA to oral bisphosphonates enhanced their bioavailability in the gastrointestinal (GI) tract, due to the competitive EDTA-Ca chelation, inhibiting the formation of un-absorbable BP-Ca complexes inside the GI lumen (42–44). In similar way, applying EDTA to the hydroxyapatite crystals in bone will chelate calcium, releasing Zol into the supernatant. The free Zol is subsequently washed away with phosphate buffer saline, thereby reducing the amount of available Zol from the circumferential alveolar bone in the tooth socket. In figure 1, free Zol was detected in the supernatant after chelation. Further studies are underway to investigate the dynamics of such phenomenon and optimize the in-vivo chelation under different conditions and using different chelating agents.

In conclusion, matrix-bound Zol inhibit osteoclast differentiation and function, an effect that can be alleviated using localized treatment with EDTA. The removal of matrix-bound Zol improved socket healing and prevented osteonecrosis in Zol-treated animals. The results of this study may be the basis for a novel strategy to prevent BRONJ in patients on long-term zoledronate, who require invasive dental procedures.

Acknowledgments

Authors' roles: All authors included on the manuscript fulfil the authorship requirements set forth by Bone, as follows: study design: RE, ZK, DHP, and MEE; study conduct: RE, PA, MEA, BB, GMW, CEM; data collection: RE, PA, MEA; data analysis: RE, PA, MEA, MEE; data interpretation: RE, ZK, DHP, MEE; drafting manuscript: RE, PA, MEE; revising manuscript content: ZK, DHP, CEM, MEE; approving final version of manuscript: RE, PA, MEA, ZK, BB, GMW, DHP, CEM, MEE. Finally, RE and MEE take responsibility for the integrity of the data analysis. **Investigators who contributed to the study but did not fulfil authorship requirements:** We would like to thank Dr. Wenbo Zhi from the Department of Physiology, Medical College of Georgia, Augusta University, for conducting the zoledronate quantification using mass spectrometry.

Funding: This study was supported by NIDCR R15DE025134 and NIA 2P01AG036675-06A1

References

1. Russell RG. Bisphosphonates: the first 40 years. *Bone*. 2011; 49(1):2–19. [PubMed: 21555003]
2. Zarogoulidis K, Boutsikou E, Zarogoulidis P, Eleftheriadou E, Kontakiotis T, Lithoxopoulou H, et al. The impact of zoledronic acid therapy in survival of lung cancer patients with bone metastasis. *Int J Cancer*. 2009; 125(7):1705–9. [PubMed: 19521984]
3. Zhao X, Hu X. Dosing of zoledronic acid with its anti-tumor effects in breast cancer. *Journal of Bone Oncology*. 2015; 4(3):98–101. [PubMed: 26587376]
4. Early Breast Cancer Trialists' Collaborative G. Adjuvant bisphosphonate treatment in early breast cancer: meta-analyses of individual patient data from randomised trials. *The Lancet*. 386(10001): 1353–61.
5. Oades GM, Coxon J, Colston KW. The potential role of bisphosphonates in prostate cancer. *Prostate cancer and prostatic diseases*. 2002; 5(4):264–72. [PubMed: 12627210]

6. Coleman R, Cameron D, Dodwell D, Bell R, Wilson C, Rathbone E, et al. Adjuvant zoledronic acid in patients with early breast cancer: final efficacy analysis of the AZURE (BIG 01/04) randomised open-label phase 3 trial. *Lancet Oncol.* 2014; 15(9):997–1006. [PubMed: 25035292]
7. Guise TA. Antitumor effects of bisphosphonates: Promising preclinical evidence. *Cancer Treat Rev.* 34:S19–S24. [PubMed: 18486348]
8. Dedes PG, Kanakis I, Gialeli C, Theocharis AD, Tsegenidis T, Kleetsas D, et al. Preclinical evaluation of zoledronate using an in vitro mimetic cellular model for breast cancer metastatic bone disease. *Biochimica et Biophysica Acta (BBA) - General Subjects.* 2013; 1830(6):3625–34. [PubMed: 23395844]
9. Clezardin P, Ebetino FH, Fournier PG. Bisphosphonates and cancer-induced bone disease: beyond their antiresorptive activity. *Cancer Res.* 2005; 65(12):4971–4. [PubMed: 15958534]
10. Khosla S, Shane E. A Crisis in the Treatment of Osteoporosis. *J Bone Miner Res.* 2016; 31(8):1485–7. [PubMed: 27335158]
11. Kim SC, Kim DH, Mogun H, Eddings W, Polinski JM, Franklin JM, et al. Impact of the U.S. Food and Drug Administration's Safety-Related Announcements on the Use of Bisphosphonates After Hip Fracture. *J Bone Miner Res.* 2016; 31(8):1536–40. [PubMed: 26969902]
12. Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghaloo T, Mehrotra B, et al. American Association of Oral and Maxillofacial Surgeons Position Paper on Medication-Related Osteonecrosis of the Jaw—2014 Update. *J Oral Maxillofac Surg.* 72(10):1938–56. [PubMed: 25234529]
13. Reid IR, Cornish J. Epidemiology and pathogenesis of osteonecrosis of the jaw. *Nat Rev Rheumatol.* 2011; 8(2):90–6. [PubMed: 22124271]
14. Modi A, Siris ES, Tang J, Sen S. Cost and consequences of noncompliance with osteoporosis treatment among women initiating therapy. *Curr Med Res Opin.* 2015; 31(4):757–65. [PubMed: 25661017]
15. Howie RN, Bhattacharyya M, Salama M, El Refaey M, Isales C, Borke J, et al. Removal of pamidronate from bone in rats using systemic and local chelation. *Arch Oral Biol.* 2015; 60(12):1699–707. [PubMed: 26431826]
16. Weiss HM, Pfaar U, Schweitzer A, Wiegand H, Skerjanec A, Schran H. Biodistribution and plasma protein binding of zoledronic acid. *Drug Metab Dispos.* 2008; 36(10):2043–9. [PubMed: 18625688]
17. Pazianas M, van der Geest S, Miller P. Bisphosphonates and bone quality. *BoneKey Rep.* 2014;3.
18. Roelofs AJ, Stewart CA, Sun S, Błażewska KM, Kashemirov BA, McKenna CE, et al. Influence of bone affinity on the skeletal distribution of fluorescently labeled bisphosphonates in vivo. *J Bone Miner Res.* 2012; 27(4):835–47. [PubMed: 22228189]
19. Russell RGG, Watts NB, Ebetino FH, Rogers MJ. Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. *Osteoporos Int.* 2008; 19(6):733–59. [PubMed: 18214569]
20. Nancollas GH, Tang R, Phipps RJ, Henneman Z, Gulde S, Wu W, et al. Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite. *Bone.* 2006; 38(5):617–27. [PubMed: 16046206]
21. Papapoulos SE, Cremers SCLM. Prolonged Bisphosphonate Release after Treatment in Children. *N Engl J Med.* 2007; 356(10):1075–6.
22. Kozloff KM, Volakis LI, Marini JC, Caird MS. Near-infrared fluorescent probe traces bisphosphonate delivery and retention in vivo. *J Bone Miner Res.* 2010; 25(8):1748–58. [PubMed: 20200982]
23. Mohammadi Z, Shalavi S, Jafarzadeh H. Ethylenediaminetetraacetic acid in endodontics. *European Journal of Dentistry.* 2013; 7(Suppl 1):S135–S42. [PubMed: 24966721]
24. Smith SW. The role of chelation in the treatment of other metal poisonings. *Journal of medical toxicology: official journal of the American College of Medical Toxicology.* 2013; 9(4):355–69. [PubMed: 24113858]
25. Born T, Kontoghiorghes CN, Spyrou A, Kolnagou A, Kontoghiorghes GJ. EDTA chelation reappraisal following new clinical trials and regular use in millions of patients: review of

- preliminary findings and risk/benefit assessment. Toxicology mechanisms and methods. 2013; 23(1):11–7. [PubMed: 22991933]
26. Ouyang P, Gottlieb SH, Culotta VL, Navas-Acien A. EDTA Chelation Therapy to Reduce Cardiovascular Events in Persons with Diabetes. Current cardiology reports. 2015; 17(11):96. [PubMed: 26364188]
 27. Wu L, Mu Y, Deng X, Zhang S, Zhou D. Comparison of the effect of four decalcifying agents combined with 60 degrees C 3% sodium hypochlorite on smear layer removal. J Endod. 2012; 38(3):381–4. [PubMed: 22341079]
 28. Gamal AY, Mailhot JM. The effects of EDTA gel conditioning exposure time on periodontitis-affected human root surfaces: surface topography and PDL cell adhesion. J Int Acad Periodontol. 2003; 5(1):11–22. [PubMed: 12666951]
 29. Serper A, Calt S. The demineralizing effects of EDTA at different concentrations and pH. J Endod. 2002; 28(7):501–2. [PubMed: 12126374]
 30. Haapasalo M, Orstavik D. In vitro infection and disinfection of dentinal tubules. J Dent Res. 1987; 66(8):1375–9. [PubMed: 3114347]
 31. Guzel C, Uzunoglu E, Dogan Buzoglu H. Effect of Low-surface Tension EDTA Solutions on the Bond Strength of Resin-based Sealer to Young and Old Root Canal Dentin. J Endod. 2017
 32. Herrera DR, Martinho FC, de-Jesus-Soares A, Zaia AA, Ferraz CCR, Almeida JFA, et al. Clinical efficacy of EDTA ultrasonic activation in the reduction of endotoxins and cultivable bacteria. Int Endod J. 2017; 50(10):933–40. [PubMed: 27775835]
 33. Luque-Martinez I, Munoz MA, Mena-Serrano A, Hass V, Reis A, Loguercio AD. Effect of EDTA conditioning on cervical restorations bonded with a self-etch adhesive: A randomized double-blind clinical trial. J Dent. 2015; 43(9):1175–83. [PubMed: 25963586]
 34. Machado R, Garcia L, da Silva Neto UX, Cruz Filho AMD, Silva RG, Vansan LP. Evaluation of 17% EDTA and 10% citric acid in smear layer removal and tubular dentin sealer penetration. Microsc Res Tech. 2017
 35. Kozloff KM, Weissleder R, Mahmood U. Noninvasive Optical Detection of Bone Mineral. J Bone Miner Res. 2007; 22(8):1208–16. [PubMed: 17488196]
 36. Howie RN, Borke JL, Kurago Z, Daoudi A, Cray J, Zakhary IE, et al. A Model for Osteonecrosis of the Jaw with Zoledronate Treatment following Repeated Major Trauma. PLoS One. 2015; 10(7):e0132520. [PubMed: 26186665]
 37. Abe K, Yoshimura Y, Deyama Y, Kikuri T, Hasegawa T, Tei K, et al. Effects of bisphosphonates on osteoclastogenesis in RAW264.7 cells. Int J Mol Med. 2012; 29(6):1007–15. [PubMed: 22447156]
 38. Kimachi K, Kajiya H, Nakayama S, Ikebe T, Okabe K. Zoledronic acid inhibits RANK expression and migration of osteoclast precursors during osteoclastogenesis. Naunyn-Schmiedeberg's Archives of Pharmacology. 2011; 383(3):297–308.
 39. Thompson K, Rogers MJ, Coxon FP, Crockett JC. Cytosolic Entry of Bisphosphonate Drugs Requires Acidification of Vesicles after Fluid-Phase Endocytosis. Mol Pharmacol. 2006; 69(5):1624. [PubMed: 16501031]
 40. Henzl MT, Larson JD, Agah S. Estimation of parvalbumin Ca(2+)- and Mg(2+)-binding constants by global least-squares analysis of isothermal titration calorimetry data. Anal Biochem. 2003; 319(2):216–33. [PubMed: 12871715]
 41. Mostefa Side Larbi MA, Sauzet C, Piccerelle P, Cau P, Levy N, Gallice P, et al. Thermodynamic study of the interaction between calcium and zoledronic acid by calorimetry. The Journal of Chemical Thermodynamics. 2016; 97(Supplement C):290–6.
 42. Pazianas M, Abrahamsen B, Ferrari S, Russell RGG. Eliminating the need for fasting with oral administration of bisphosphonates. Ther Clin Risk Manag. 2013; 9:395–402. [PubMed: 24204155]
 43. Janner M, Mühlbauer RC, Fleisch H. Sodium EDTA enhances intestinal absorption of two bisphosphonates. Calcif Tissue Int. 1991; 49(4):280–3. [PubMed: 1836974]
 44. Kim JS, Jang SW, Son M, Kim BM, Kang MJ. Enteric-coated tablet of risedronate sodium in combination with phytic acid, a natural chelating agent, for improved oral bioavailability. Eur J Pharm Sci. 2016; 82(Supplement C):45–51. [PubMed: 26594027]

Highlights

- EDTA Chelation of matrix-bound zoledronate released quantifiable amounts in the supernatant
- Matrix-bound zoledronate inhibits osteoclast differentiation and function
- EDTA improved socket healing and prevented osteonecrosis in BRONJ rat model
- Chelating agents may be used to prevent BRONJ in human patients.

Fig. 1. Quantification of zoledronate chelation

A) Fluorescence imaging of AF647-Zol-treated dentin discs before (upper panel) and after (lower panel) EDTA chelation. **B)** Quantification of fluorescent intensity in the dentin discs showed significant decrease after EDTA application at the 0.1 μM ($***p < 0.001$). **C)** Quantification of endpoint fluorescence in the chelation supernatant solution showed an increase in the amount of released Zol in a dose dependent manner with statistical significance at both the 0.1 μM ($*p < 0.043$) and 1 μM ($***p < 0.001$). **D)** Quantification of zoledronate in supernatant solutions from zoledronate-treated discs using liquid chromatography coupled with tandem mass spectrometry (LC-MS) detected Zol molecules released into the supernatant solution after chelation.

Fig. 2. Localized Zoledronate within the bone matrix inhibits osteoclast differentiation and resorption pit formation in-vitro

A) Representative images of TRAP staining (upper panel) and resorption pit formation (lower panel) on OsteoAssay plate treated with PBS (control) or increasing Zol concentrations for 24 hours prior to RAW264.7 cell culture; B) Percentage of TRAP-positive cells to the total number of cells on day 5 declined as Zol concentration increased ($p=0.01$); C). Area of resorption pits formation at day 21 days declined with increasing concentration of Zol ($p=0.02$); D) Proliferation assay results showing that proliferation of RAW264.7 cells only declined when the matrix was treated with 100 μM ($p=0.01$); E–G) RT-PCR results showing decline in mRNA expression of Cathepsin-K ($p<0.0001$), RANK ($p=0.003$) and DC-STAMP ($p=0.001$) in RAW 264.7 in cells pretreated with Zol 10 μM relative to the controls.

Fig. 3. EDTA-induced removal of Zol rescues osteoclast differentiation and function in vitro
 A) No change in RAW264.7 cell adhesion to OsteoAssay plate after treatment of the Zol-free matrix with EDTA for 30 minutes prior to seeding; B) Percentage of TRAP positive cells increased in the Zol-treated matrix groups but not in controls when the matrix was treated with EDTA prior to seeding ($p < 0.001$ in both doses); C) Calcium levels in the supernatant fluid of RAW264.7 cells seeded on matrix treated with PBS (control) or zoledronate alone or with subsequent EDTA prior to seeding show consistent increase in the Zol+EDTA groups, compared to Zol only ($p < 0.0001$); D) PYD levels in the supernatants fluid of RAW264.7 cells seeded on matrix treated with PBS (control) or zoledronate alone or with subsequent EDTA prior to seeding show consistent increase in the Zol+EDTA groups, compared to Zol only ($p < 0.0001$)

Fig. 4. Localized chelation of zoledronate after dental extraction prevents BRONJ in vivo

A) Representative images of mucosal healing on the EDTA and saline-treated sides in Zol-treated and control animals, showing exposure of necrotic bone on the saline-treated side in Zol-treated animals (Zol-Saline). **B)** Representative micro-CT images of the alveolar bone at the sagittal, axial, and coronal planes (left-right) on the EDTA and saline sides in a Zol-treated animal taken in 3 planes of the reconstructed images (left-right: sagittal, axial, and coronal), showing sequestered bone on the saline but not the EDTA-treated side. **C)** Healing scores were significantly higher in the control versus the Zol-treated animals (** $p=0.003$) and on the EDTA versus saline-treated sides in Zol-treated animals (* $p=0.0156$). **D)** Representative 20x photomicrographs of H&E stained histologic sections from the extraction sites in the four groups showing prevalence of empty lacunae on the saline-treated sides in Zol-treated animals (Zol-Saline). **E)** 2.5 field outlining the ROI (red circle) guided by the beginning and end of the mesial root of the third molar (x). **F)** The percentage of empty osteocyte lacunae was higher in Zol-treated versus control animals, and on the saline

versus EDTA-treated sides in Zol-treated animals (** $p < 0.0001$). **G**) No difference in BMD of alveolar bone was detected by microCT between the EDTA versus saline-treated sides.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript