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Removal of pamidronate from bone in rats using systemic and local chelation

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Abstract

Objectives—Bisphosphonates become adsorbed on hydroxyapatite crystals in the bone matrix.

In case of side-effects, stopping the treatment would not affect the bisphosphonates already deposited in bone. This study tests the feasibility of in-vivo targeted removal of bisphosphonates from bone using chelating agents.

Design—32 Sprague Dawley rats were given an injection of fluorescent pamidronate (OsteoSense EX; 0.16 nmol/g). They were treated with either systemic (cadmium) or local [ethylenediaminetetraacetic (EDTA) or citric acid (CA)] chelating agents to induce the removal of the bisphosphonate from bone. We evaluated the decrease in fluorescence in the alveolar bone, femur, tibia, and vertebrae. We also analyzed the systemic effects of treatment.

Results—Systemic chelation reduced the pamidronate signal universally. However, the maximum reduction was observed in the alveolar bone and femur (22% and 21%, p values 0.008 and 0.028, respectively). Systemic chelation did not impair calcium homeostasis. The chelation effect was not due to a systemic toxic effect on the liver or kidney. On the other hand local chelation at the extraction site significantly (p=0.011) decreased the pamidronate signal at bony surfaces of the socket.

Conclusions—Systemic and local chelating agents can remove bisphosphonate from bone. This study establishes a new concept for the prevention of side effects of bisphosphonates during high-risk situations.

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Keywords

Osteonecrosis; mandible; pamidronate; complications; Bisphosphonates; Bone; Chelating agents; Osteonecrosis of the jaw; Feasibility study

INTRODUCTION

A unique feature of bisphosphonates compared to other anti-resorptive compounds is their ability to physically accumulate in the bone matrix due to high affinity to hydroxyapatite crystals.^{1,2} The core structure of bisphosphonates includes two phosphate groups flanking a non-hydrolyzable central carbon and a hydroxyl group in the R1 position.³⁻⁶ The hydroxyl group is essential for bisphosphonates' affinity for the calcium in the bone matrix,⁷ creating a tertiary interaction between the bisphosphonate and the hydroxyapatite crystals within the bone matrix.^{3,8}

Nitrogen-containing bisphosphonates have an additional nitrogen or amino group in the R2 position, which increases the bisphosphonate's anti-resorptive potency by a factor of 10 to 10,000, relative to non-nitrogen-containing bisphosphonates.^{3,9,10} Once the bisphosphonate attaches to a bone formation surface, it is only liberated when the bone in which it was deposited is resorbed.^{7,9-11} Therefore, the expected half-life of nitrogen-containing bisphosphonates in humans has been estimated around 10 years,^{3,10-12} although the true half-life of bisphosphonates depends largely on the rate of bone turnover, which is variable within as well as between individuals. As normal remodeling of bone occurs, the accumulated bisphosphonates are gradually liberated and excreted through the kidneys for up to eight years after the cessation of treatment.^{9,11}

Bisphosphonates accumulate at preferential sites that included the alveolar bone, distal end of the femur, proximal end of the tibia, and vertebral bodies.^{13,14} The role of locally-deposited bisphosphonates in the development of complications, such as bone necrosis, remains unknown. While such bone necrosis has been largely limited to the alveolar bone in human patients, reports have emerged of atypical fractures and other complications at other sites.^{15,16} Very limited information currently exists regarding the role of locally deposited bisphosphonates in the pathogenesis of bisphosphonate-related osteonecrosis.

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) has emerged as a significant health problem in recent years, affecting around 7% of patients receiving long-term Zoledronic Acid (Zol) treatments, 67% of which were preceded by tooth extraction.^{6,17} Even though other anti-resorptive medications, such as RANKL antibodies, have also been associated with similar side effects, these drugs do not accumulate in the bone matrix and their effect can therefore be reversed by the cessation of treatment. Thus far, stopping the intravenous treatment of bisphosphonates has not been shown to ameliorate the condition, probably because it does not remove the drug that has already accumulated in bone.¹⁸⁻²⁰

The aim of this study was to test the feasibility of targeted removal of bisphosphonates from bone using chelating agents in an attempt to improve remodeling and healing in the alveolar bone whenever early signs of BRONJ manifest, or prior to invasive dental treatments. We

tested both systemic (Cadmium; Cd) and local (Ethylenediaminetetraacetic acid (EDTA), and citric acid (CA)) chelating agents.

Using ^{45}Ca isotope administration to rats, Ando and colleagues first reported in 1978 that Cd increased the excretion of bone-derived calcium within the first few days of exposure, well before the onset of Cd-induced kidney dysfunction.²¹ Ten years later, our group conducted a second study using calcium isotope technology that showed a similar finding in mice.²² A separate study showed that the calcium excreted in feces in response to Cd exposure in this model was bone-derived.²³ It is important to note that there was no hyperkalemia, since the excretion of calcium in feces proportionately increased. The same effect was also evident in the presence of pre-existing osteoclast deficiency, with evidence of osteoclast recovery after cadmium exposure.²⁴ In the present study, we expected that this significant and transient release of calcium from bone would dislodge the adsorbed bisphosphonate and cause the bisphosphonate to be released from bone after cadmium gavage (systemic chelation). The concept of using systemic chelating agents to remove heavy metals that deposit to bone, such as mercury and lead, is well established.²⁵⁻²⁹

Previous studies also showed that ex-vivo demineralization of bone samples with EDTA decreased their bisphosphonate content.¹³ Both citric acid and EDTA are soluble in their ionized form. At a pH of 7.4, citric acid and EDTA should be deprotonated (ionized; negatively charged), and both can bind to Ca^{++} so tightly. Therefore, local application with CA or EDTA should remove bisphosphonates from the bone surface by removing the calcium of hydroxyapatite, to which bisphosphonates are bound. Our group used EDTA to remove calcium oxalate and gallium nitrate from mineralized dentin in previous studies.^{30,31}

In this study, we tested the dynamics of in-vivo chelation of far-red fluorescent pamidronate (FRFP) from the mandible, femur, tibia and vertebrae following systemic chelation (cadmium); and from the alveolar bone of the mandible following localized (EDTA or citric acid) chelation. We hypothesized that chelating agents, whether systemic or local, would reduce the FRFP signal from alveolar bone, and that such effect will not be due to systemic effects on the liver or kidney or disturbance in calcium homeostasis. Transient release of bisphosphonate from bone can be the basis for a novel concept in the prevention of BRONJ in high-risk conditions, such as before necessary dental procedures.

METHODS

A. Systemic Chelation

Animals—The experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Georgia Regents University (Protocol #2012-0496; Date: 10/25/2012). The timeline of the experiment is outlined in figure 1. Twenty-two female Sprague Dawley rats (average 190g in weight) were placed into metabolic cages and fed a standard powdered diet (Mod TestDiet AIN-76a; St. Louis, MO) containing 0.53% Ca. After a 1-week acclimation period, urine samples were collected for a total of 7 days to establish a baseline. On the 8th day, each animal was placed under isoflurane anesthesia [Butler Schein; Lot B106G12A; Dublin, OH] and given an intra-peritoneal injection of far-red fluorescent pamidronate (FRFP; 0.16 nmol/g OsteoSense 750

EX; PerkinElmer Lot# 1651527; Santa Clara, CA). The dosage of FRFP given was calculated based on the recommended dosage for mice (4 nmol/100 μ L purified buffered saline for a 25g-mouse) scaled to the weight of 190g-rats (30.4 nmol/100 μ L PBS).

At 1 and 2 weeks after injection, a 15 gauge feeding tube [Instech Solomon; Lot 188; Plymouth Meeting, PA] was used to give the rats a gavage of either sterile water (control; n=11) or 2 mg (17.79 μ mol of a 1.0M Cadmium Chloride; Hampton Research; lot 271517; Aliso Viejo, CA) of cadmium chloride (treatment; n=11) under isoflurane anesthesia. At the time of anesthesia, the animals were weighed. A gavage volume of 2 mL (below the 3.4 mL stomach capacity of rats) was chosen with a concentration of 8.9 μ mol/mL of the 1.0M Cadmium Chloride administered to each rat. This dosage has been shown to mobilize calcium from the skeleton without causing significant reduction in local bone mineral density, hypercalcemia, or kidney toxicity.^{24,32}

Animals were imaged twelve hours after FRFP injection, twelve hours before gavage, and one and three days after gavage, as outlined below. Animals were sacrificed using CO₂ gas followed by thoracotomy, one week following the second gavage administration. At time of sacrifice, the mandible, femur, tibia and vertebra were collected, soft tissue removed, and ex-vivo fluorescent images taken. In addition, the kidneys, liver, and blood were also collected for analysis. The kidneys and liver were fixed in 10% formalin. Whole blood was collected at time of sacrifice by inserting a cannula into the left ventricle and collecting the blood into a lithium-heparin tube. Serum was collected for urea, calcium, creatinine, and PTH level analysis.

Fluorescent Imaging—Fluorescent imaging was completed using VivoVision Ivis Lumina small-animal imaging system (Xenogen Corp; Alameda, CA) with an excitation and emission ICG filters, f stop of 2, and binning at medium. Animals were imaged twelve hours following FRFP injection, twelve hours before gavage, one and three days after each gavage, and after sacrifice (ex-vivo) for the collected bones. Fluorescent images were taken twelve hours before the FRFP injection to correct for any auto-fluorescence or background at the 750nm excitation wavelength, and again twelve hours prior to the first cadmium administration to correct for the loss of fluorescence due to normal decay. The hair overlying the jaw area was removed to minimize background auto-fluorescence. After hair removal, Quadritop [Butler Schein; Dublin, OH] was applied to ease irritation caused by the regrowth of hair. Imaging was done under continuous administration of isoflurane. The intensity of the fluorescents was measured in max radiant efficiency ratio for the regions of interests that included; the mandibles (in-vivo), tibial plateau, 3 vertebrae, femoral epicondyles, and the alveolar bone of the mandible (ex-vivo). Regions of interest were standardized and positioned before the application of the fluorescent signals to prevent observational bias. Measurements were taken blindly using the machine's software.

Bone Density and Calcium Homeostasis—Dual Energy X-Ray Absorptiometry (DEXA) scans using the Lunar PISIImus2 2.10 [Inside Outside Sales, LLC; Fitchburg, WI] were performed on the harvested mandibles to determine bone mineral density. PIXImus2 uses lower x-ray energy than that used for peripheral densitometry in humans in order to achieve contrast in the extremely low density bone, as would be seen in small animal models

including rat.³³ During scanning, the mandibles were placed in the specimen sticky tray [Inside Outside Sales, LLC; Fitchburg, WI]. The position of the samples was standardized and the mandibles were oriented such that X-rays penetrated the mandibles in a bucco-lingual direction. Three parameters were measured; the bone mineral content (BMC), which is the total mass of bone mineral content expressed in grams (g), the area of the bone which is determined by edge detection algorithms in the software and expressed in cm² and the bone mineral density (BMD) which is the quotient of the BMC and area and is expressed in g/cm².

Blood levels of parathyroid hormone (PTH) were analyzed using Rat Intact PTH ELISA kit [Immutorpics; Lot 2391307; San Clemente, CA] according to the manufacturer's directions. Blood was collected for serum calcium analysis via an intracardiac cannula into a glass collection vial at the time of sacrifice and stored at 4°C overnight. The samples were spun at 1600 RPM for 10 minutes and the serum collected for analysis. Serum calcium levels were analyzed by adding 50 µL of the serum samples to 4mL potassium chloride (KCl). Signals were read by flame Atomic Absorption Spectroscopy using a calcium lamp, and data were calculated using a standard curve.

Liver and Kidney Histopathology—Livers and kidneys were fixed in 10% formalin, followed by dehydration in increasing concentrations of ethanol and xylene, with subsequent paraffin embedding using a semi-enclosed Bench-top Tissue Processor (Leica Microsystems, Buffalo Grove, IL) and sectioned 5 microns. Slides were deparaffinized in xylene, hydrated in serial ethanol, stained with hematoxylin for 5 minutes, washed, and stained with eosin for 2 minutes. After staining the slides were dehydrated and cover slipped. The coded micrographs were blindly examined for signs of cadmium-induced damage by histologic evaluation of proximal tubules morphology in the kidney tissue as well as enumeration of apoptotic and necrotic cells in 10 random high power fields in kidney and liver tissue.

Kidney functions—Serum urea levels were measure with Urea Assay Kit [Abcam; Lot GR138519-2; Cambridge, MA] according to manufacturer's guidelines. Creatinine levels within the serum were analyzed through the addition of 50uL picric acid to the samples and reading them with a plate reader at 490nm.

Total protein levels in urine were analyzed using Pierce BCA protein assay kit [Thermo Scientific; Rockford, IL; Lot OD187370]. After the 1-week acclimation period, urine samples were collected every 12 hours (to account for the activity cycle of the animal) for a total of 7 days to establish a baseline and continued to be collected every 12 hours for the duration of the experiment. Total urine volumes were measured using a graduated cylinder, and then aliquots were stored at -80°C. Weights of the animals were recorded at the time of FRFP injection, both gavages, and at sacrifice.

B. Local Chelation

Animals—Ten female Sprague Dawley rats (average weight of 190 gram) received a tail vein injection of 0.16 nmol/g OsteoSense 680 EX (FRFP; PerkinElmer Lot 1775255; Waltham, MA), while under isoflurane anesthesia. The animals were anesthetized with an

intraperitoneal injection of a cocktail composed of ketamine (100 mg/mL) and xylazine (20 mg/mL) at a dosage of 0.1 mL/kg. After reaching anesthesia two rubber bands were used to keep the mouth open and to immobilize the tongue. The rubber bands were attached to a rubber dam frame to allow appropriate access to the mandibular molar area. The left mandibular first and second molars were extracted using an Adson surgical forceps. Any remaining root segments were removed and the defect reshaped using a 1mm round carbide burr to a standardized depth of 2 mm from the cemento-enamel junction. At the completion of the reshaping, a standardized Q-tip cotton swap soaked in EDTA (n=5) or citric acid (n=5) was placed in the extraction site for 10 or 3 minutes, respectively, with freshly soaked swaps being used every 2 minutes. An identical extraction protocol was completed on the right mandible, with the extraction site being rinsed with sterile water, to act as an internal control. Animals were euthanized using CO₂ gas the following day. A second treatment of EDTA, citric acid or sterile water was repeated after harvesting the mandible to ensure the consistency of chelation regardless of the size of oral opening and eliminate the possibility of cross contamination between the right and left extraction sites. The mandibles were fixed in methyl methacrylate plastic for histological analysis of the fluorescent signal.

Histology—The mandibles were dehydrated and embedded in methyl methacrylate plastic and sectioned with an isomet low speed saw with a diamond wafering blade [Buehler, Lake Bluff, IL]. Sections containing the extraction site were mounted on histology slides and fluorescent images taken using a Zeiss LSM 510 Meta Inverted Confocal [Pleasanton, CA] microscope mounted on an Axiovert 200m base stand using AIM 3.2 SP2 software. The samples were excited with a 633nm laser at 50% of the maximum with the pinhole set at 713 at 10x. The integrated density of the fluorescence within the basal bone below the extraction site was measured using ImageJ software (<http://imagej.nih.gov/ij/>).

To measure the depth of demineralization, additional sections of the extraction site were deplasticized in acetone, hydrated in serial ethanol, stained with silver nitrate for 10 minutes, sodium carbonate-formaldehyde for 2 minutes, farmer's diminisher for 30 seconds and finally MacNeil's tetrachrome for 5 minutes. After staining the slides were dehydrated and cover slipped. Three randomly chosen 20x fields within extraction site were examined and the depth of the surface demineralized bone area was measured at 3 random points, allowing for a total of 9 measurements per sample.

Statistical analysis

Statistical analysis was done using SPSS Statistics® software for Windows (v. 20; IBM, Corp, New York, USA). Values were represented as mean±SD. Normality and equal variances assumptions for each test were evaluated using the Shapiro-Wilk test and Levene's Test, respectively. Comparisons between groups in the systemic treatment experiment were done using an unpaired, two-tailed student's t-test with equal variances with significance defined as $p < 0.05$, unless otherwise stated. Comparisons between the right and left extraction sites in the local treatment experiment were done using a paired, two-tailed student's t-test with equal variance and significance level of $p < 0.05$.

RESULTS

Systemic Chelation

Fluorescence measurements—The femoral epicondyles showed the highest radiant efficacy (4.01 ± 0.28) followed by the tibial plateau (3.18 ± 0.32), alveolar bone in the mandible (2.75 ± 0.17) and finally the lumbar vertebrae (2.54 ± 0.21) (figure 2). On the other hand, the chelation-induced removal of pamidronate was highest in the alveolar bone and the femoral epicondyles, with a 21% ($p=0.008$) and 22% ($p=0.028$) decline, respectively, compared to 19% ($p=0.046$) in the lumbar vertebrae and only a 12% decline in the tibial plateau ($p=0.17$).

The in-vivo time course of reduction in pamidronate fluorescence in the rat mandibles is illustrated in figure 3, which shows the percent decline in-vivo FRFP signal, normalized to the 12 hour pre-gavage time point of each individual rat. During the first 24 hours, both the control and treatment groups had a percent decline in FRFP of 13.3 ± 1.95 and 14.4 ± 2.69 , respectively, with no significant differences ($p=0.38$). However, at 3-day post-cadmium administration there was a drastic decline ($p=0.031$) in the fluorescence's of the treatment group when compared to the control, which continued at day 8 ($p=0.001$) and day 11 ($p=0.019$). At the 11-day time point, the reduction in FRFP levels reached a plateau, with a slight increase in the control signal.

Bone mineral density, and calcium and PTH levels—To determine whether systemic chelation caused excessive demineralization in the mandible, the bone mineral density was compared between groups by DEXA. There was no difference ($p=0.16$) in the BMD between the control ($0.13 \pm 0.0011 \text{ g/cm}^2$) and treatment ($0.13 \pm 0.0014 \text{ g/cm}^2$) groups. The chelation group showed slightly lower levels of plasma PTH, but the difference was not significant ($p=0.21$). However, the serum calcium levels for the control (10.3 mg/dL) and treatment (9.88 mg/dL) fell within the normal range of 9.5-10.5 mg/dL and were not significantly different, ($p = 0.576$). (figure 4).

Liver and kidney pathology—Histologically, there was no evidence of renal or hepatic toxicity in cadmium-treated animals. The proximal tubular epithelium from both control as well as cadmium treated kidney tissues exhibited cuboidal shapes, well-defined nuclei, and a uniform cytoplasm, with no spaces or gaps between the cells and without evidence of tubular necrosis. In addition, no difference was noted between groups in either the kidney or livers regarding morphology or the number of necrotic or apoptotic nuclei (figure 5). We evaluated blood urea nitrogen (BUN) and creatinine levels as indicators of kidney damage that could confound the bone effect. No differences were found in either the BUN (17.5 ± 0.42 control and $17.09 \pm 0.053 \text{ mg/dL}$) or creatinine (0.26 ± 0.013 control and $0.28 \pm 0.009 \text{ mg/dL}$) levels $p=0.17$ and $p=0.139$, respectively. Both the BUN and creatinine levels for both groups were well within the normal ranges found in rats, 15-21 mg/dL BUN and 0.2-0.8 mg/dL creatinine. (figure 5) Total protein levels in urine were analyzed daily starting 5 days prior to the second gavage and continuing through 5 days post gavage. A repeated measures analysis was applied and no significant difference between groups was found, $p=0.916$.

The rats' weights and diurnal urinary output were also monitored to determine if the treatment caused weight loss or polyuria. Figure 5 shows that while the treatment group did have a more pronounced decline in their weights as compared to the control that both groups of animals experienced weight loss indicating that the weight loss could have been due to the stress of the experiment itself. Diurnal urinary output levels for the rats were normal with a higher volume being collected during the morning time point, which was expected due to the rat's overnight active period.

Local Chelation

Treatment caused a significant decrease in the integrated density of the fluorescence for both the citric acid treatment ($2.36e^6 \pm 1.27e5$, $p=0.024$) and EDTA ($1.39e^6 \pm 1.08e5$, $p=0.031$) when compared to the internal controls (figure 6). Histological analysis of the depth of decalcification due to each of the treatments was also analyzed to determine if there was a direct correlation between the amount of decalcification occurring and the reduction of bisphosphonate fluorescence. Citric acid significantly increased the depth of the post-extraction decalcification along the socket walls from $0.153 \pm 0.53 \mu\text{m}$ for the controls to $5.21 \pm 1.20 \mu\text{m}$ ($p<0.001$). The same significant difference ($p<0.001$) was found in the EDTA treatment with $6.23 \pm 2.51 \mu\text{m}$ of decalcification of the socket wall, compared to $0.831 \pm 1.15 \mu\text{m}$ on the control side.

Discussion

The aim of this study was to test the feasibility and dynamics of acute, targeted removal of bisphosphonates from bone in-vivo using systemic (cadmium) and local (Ethylenediaminetetraacetic acid (EDTA) or citric acid) chelating agents. Our results showed significant reduction in pamidronate content in alveolar bone using either method. Such reduction was not part of a toxic reaction to the chelating agent but rather through a direct demineralizing effect on bone. The removal of calcium from bone surfaces was associated with liberation of the pamidronate. To date, this is the first study that proved this principle in live animals.

The delivery of FRFP to the skeleton rapidly occurred within 24 hours of administration and its distribution within the skeleton was consistent with previous studies, with the long bones containing higher levels of attached bisphosphonate than the mandibles or vertebrae.¹⁴ The subsequent systemic removal of the bound FRFP showed a greater effect in the mandibles, which experienced one of the largest declines in bisphosphonate content. In-vivo monitoring of the reduction of FRFP signal in the mandible due to the systemic administration of cadmium indicated that the effect of the systemic chelating agent was most evident 3 days after systemic chelation, in concordance with previous results that showed that calcium release from bone after cadmium exposure reached a maximum during the third day.²² Interestingly, we observed that while a continued reduction of FRFP signal could be seen after the second administration, the signal reached a plateau where no further decline was achieved. However, this observation can be due to sensitivity limitations of the imaging equipment. At day 11, the control group experienced an insignificant increase in the fluorescent signal. This increase in the maximum radiant efficiency was most likely caused

by the redistribution of bisphosphonate to this area of the mandible, during the natural remodeling cycle of bone.³

Cadmium is a known toxin in humans, affecting both the kidney and the liver. Therefore, it was necessary to ensure that the bone effect was not confounded by any impairment in kidney or liver functions. In this experiment, the gavage dose of 200 µg Cd should produce a bone response starting at 2-5 ng Cd/ml blood, a concentration range that is below the OSHA standard of 5 ng/ml for industrial exposures (USOSHA92) and is at the upper end of blood cadmium concentrations reported for smokers.^{34,35} While we did find that the treatment group lost weight during the course of the experiment, the control group also experienced weight loss, albeit slightly less than the treatment group. The declining weights observed in the animals was most likely due to stress from being held in metabolic cages for a period of 1 month, as well as, being fed a powdered diet and handled almost daily creating a stressful environment.

We found that the diurnal urinary cycle was preserved in both groups, although some significant differences between groups were found. These differences were always within 48 hours of either the FRFP injections or cadmium administration, which can be explained by the increased fluid intake, either through the IV injections or the 2mL gavage, in addition to the fact that the animals were removed from the metabolic cages for a period of time to be imaged and frequently urinated in their temporary cages skewing the results. Blinded histological examination by an independent pathologist showed no pathological changes in the animals' kidneys and liver suggestive of tissue damage. There were no differences between the groups in BUN, creatinine, or calcium levels, all of which were within normal range (15-21 mg/dL, creatinine levels 0.2-0.8 mg/dL, and calcium 9.5-10.5 mg/dL).

There were no differences in the mandibular BMD or PTH between groups, indicating that the systemic cadmium dosage used, while enough to effectively remove bisphosphonate, did not cause excessive demineralization of bone. Due to the nature of bisphosphonate treatment, the lack of significant demineralization is important, as it doesn't further compromise the integrity of the bone structure, which could complicate existing conditions. However, one of the indications for using intravenous bisphosphonate in cancer patients is the prevention of malignancy-induced hypercalcemia. Therefore, the use of systemic chelating agents, even as a single dose, in these cases would not be advisable until the risk of hypercalcemia can be completely eliminated. Further studies are underway to address this issue.

The purpose of the cadmium use in this study was to prove the concept that a systemic agent has the capability of removing bisphosphonates from bone without causing systemic toxicity. Further studies using other systemic chelating agents are underway to determine if they will similarly remove bisphosphonates from bone without disturbing calcium homeostasis or BMD.

We then tested the efficacy of localized chelating agents at extractions sites. Most BRONJ cases occurred after an invasive dental procedure. The American Association of Oral and Maxillofacial Surgeons (AAOMS) advised against performing these procedures in patients

receiving long-term intravenous bisphosphonates.²⁰ In cases where such procedures are necessary, stopping treatment by itself does not reduce the bisphosphonate content in the bone matrix. We tested the use of EDTA and citric acid, which are the most frequently used chelating agents in dental clinics,³⁶⁻³⁸ to remove bisphosphonates from the healing surfaces and restore osteoclast attachment and function. These demineralizing agents are known to also erode away necrotic tissue, remove smear layers, and have a broad antimicrobial efficacy to prevent infection after the completion of the procedure.³⁹ Furthermore, EDTA and citric acid react with dentin's primary inorganic element, hydroxyapatite crystals, to remove the calcium ions from the bone and dentin and increase their permeability and solubility.^{36,40} Our data demonstrated that both chelating agents successfully removed the FRFP from bone.

Reports on the ability of these agents to remove calcium ions have been variable, with some studies indicating that EDTA removes more calcium than citric acid,³⁹ while other studies prove the opposite.⁴¹ Such conflicting results could be attributed to the differences in irrigation methods.³⁶ Our results demonstrated that EDTA had a slightly higher yield in removing calcium from bone, compared to citric acid, as determined through the measured depth of decalcification of the bone within the treated socket walls. However, citric acid had a more pronounced effect than EDTA on the release of FRFP from its attachment to bone, causing a greater decrease in the fluorescent signal found within the extraction site. Further studies are underway to optimize the application time of each agent to achieve the demineralization depth required to remove the accumulated bisphosphonate within the alveolar bone, as well as, an irrigation method to rinse the extraction site.

The removal of bisphosphonates from bone is influenced by the pH, with about 50% release of bound bisphosphonate at a pH of 3.5, seen during the acidification produced by osteoclasts during resorption.² This acidic environment is created through the transport of H^+ and Cl^- ions across the osteoclast ruffled border membrane. The bisphosphonates undergo protonation of the phosphate groups, reducing their charge, within the acidified conditions, which decreases their affinity for Ca^{2+} ions and facilitates their release from bone surfaces into solution.⁴² During the acidification and subsequent release of bisphosphonates, the localized concentration rises to approximately 0.8mM, however, as soon as acidification stops, some of the liberated bisphosphonates is redistributed to the available hydroxyapatite and the local concentration drops.^{2,42,43}

Our results prove the principle that chelating agents can acutely remove bisphosphonates from the bone matrix in live animals. One limitation of the study is the fact that chelation was done shortly after bisphosphonates were administered. It is anticipated that recently deposited bisphosphonates would be present at the bone surface, accessible to fluid exchange, and thus the chelating agents. There is no evidence that chelation would be effective in removing bisphosphonates that have accumulated over long-term treatment and become situated deep into the matrix. However, those "buried" bisphosphonate molecules are believed to be of no consequence to remodeling, because they are inaccessible to osteoclasts.⁷ It is when these buried bisphosphonate molecules become exposed, by trauma or otherwise, that they can influence active remodeling and/or socket healing, and possibly contribute to the pathogenesis of bone necrosis. Chelating agents are targeting those exposed

molecules. Therefore, this technique would be mostly effective in preventing bone necrosis that follows a traumatic event, such as dental extraction.

Another limitation of the study is the need test a non-toxic systemic chelating agent in a large animal study, further validation of the immediate and time-wise effects of systemic chelating agents on the serum calcium levels, testing the effect of chelating agents on long-term deposition of bisphosphonates, and testing whether the acute removal of bisphosphonates from alveolar bone can prevent BRONJ in a disease model. Studies are underway to address these limitations based on the results of the current study.

In conclusion, this study illustrates the validity of using calcium-chelating agents to remove bisphosphonate from its attachment to the hydroxyapatite crystals. Such an effect was not due to a systemic effect on the liver and kidney and was not part of a disturbance in calcium homeostasis. The transient release of the bisphosphonates from bone and their subsequent removal can be used in the context of alleviating the drug's burden on bone within specific sites without interfering with its desired systemic effects or causing complication to existing conditions or bone integrity.

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References

1. Weiss HM, Pfaar U, Schweitzer A, et al. Biodistribution and plasma protein binding of zoledronic acid. *Drug Metab Dispos.* 2008; 36:2043–2049. [PubMed: 18625688]
2. Sato M, Grasser W, Endo N, et al. Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. *The Journal of Clinical Investigation.* 1991; 88:2095–2105. [PubMed: 1661297]
3. Drake MT, Clarke BL, Khosla S. Bisphosphonates: Mechanism of action and role in clinical practice. *Mayo Clin Proc.* 2008; 83:1032–1045. [PubMed: 18775204]
4. Coxon FP, Thompson K, Rogers MJ. Recent advances in understanding the mechanism of action of bisphosphonates. *Curr Opin Pharmacol.* 2006; 6:307–312. [PubMed: 16650801]
5. Russell R. Determinates of structure - function relationships among bisphosphonates. *Bone.* 2007; 40(Suppl2):S21–25.
6. Pazianas M. Osteonecrosis of the jaw and the role of macrophages. *J Natl Cancer Inst.* 2011; 103:232–240. [PubMed: 21189409]
7. Kimmel DB. Mechanism of action, pharmacokinetic and pharmacodynamic profile, and clinical applications of nitrogen-containing bisphosphonates. *J Dent Res.* 2007; 86:1022–1033. [PubMed: 17959891]
8. Russell R. Bisphosphonates: from bench to bedside. *Ann N Y Acad Sci.* 2006; 1068:367–401. [PubMed: 16831938]
9. Cremers S, Papapoulos S. Pharmacology of bisphosphonates. *Bone.* 2011; 49:42–49. [PubMed: 21281748]
10. Lin JH. Bisphosphonates: a review of their pharmacokinetic properties. *Bone.* 1996; 18:75–85. [PubMed: 8833200]
11. Papapoulos S, Cremers S. Prolonged Bisphosphonate Release after Treatment in Children. *N Engl J Med.* 2007:356.

12. Gertz B, Holland S, Kline W, et al. Clinical pharmacology of alendronate sodium. *Osteoporos Int*. 1993; 3:S13–16. [PubMed: 8298197]
13. Kozloff KM, Weissleder R, Mahmood U. Noninvasive optical detection of bone mineral. *J Bone Miner Res*. 2007; 22:1208–1216. [PubMed: 17488196]
14. Kozloff KM, Volakis LI, Marini JC, et al. Near-infrared fluorescent probe traces bisphosphonate delivery and retention in vivo. *J Bone Miner Res*. 2010; 25:1748–1758. [PubMed: 20200982]
15. Armamento-Villareal R, Napoli N, Panwar V, et al. Suppressed bone turnover during alendronate therapy for high-turnover osteoporosis. *N Engl J Med*. 2006; 355:2048–2050. [PubMed: 17093260]
16. Odvina C, Zerwekh J, Rao D, et al. Severely suppressed bone turnover: a potential complication of alendronate therapy. *J Clin Endocrinol Metab*. 2005; 90:1294–1301. [PubMed: 15598694]
17. Reid IR, Cornish J. Epidemiology and pathogenesis of osteonecrosis of the jaw. *Nat Rev Rheumatol*. 2012; 8:90–96. [PubMed: 22124271]
18. Ruggiero SL, Mehrotra B, Rosenberg TJ, et al. Osteonecrosis of the jaws associated with the use of bisphosphonates: a review of 63 cases. *J Oral Maxillofac Surg*. 2004; 62:527–534. [PubMed: 15122554]
19. Ruggiero SL, Dodson TB, Assael LA, et al. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws—2009 update. *J Oral Maxillofac Surg*. 2009; 67:2–12. [PubMed: 19371809]
20. Ruggiero, S.; Dodson, TB.; Fantasia, J., et al. Special Committee on Medication-Related Osteonecrosis of the Jaws. American Association of Oral and Maxillofacial Surgeons (AAOMS); 2014. Medication-Related Osteonecrosis of the Jaw—2014 Update.. [http://www.aaoms.org/docs/position_papers/mronj_position_paper.pdf?pdf=MRO NJ-Position-Paper](http://www.aaoms.org/docs/position_papers/mronj_position_paper.pdf?pdf=MRO%20NJ-Position-Paper)
21. Ando M, Sayato Y, Osawa T. Studies on the disposition of calcium in bones of rats after continuous oral administration of cadmium. *Toxicol Appl Pharmacol*. 1978; 46:625–632. [PubMed: 746551]
22. Bhattacharyya MH, Whelton B, Peterson D, et al. Skeletal changes in multiparous mice fed a nutrient-sufficient diet containing cadmium. *Toxicology*. 1988; 50:193–204. [PubMed: 3388439]
23. Wang C, Bhattacharyya MH. Effect of cadmium on bone calcium and ⁴⁵Ca in nonpregnant mice on a calcium-deficient diet: evidence of direct effect of cadmium on bone. *Toxicol Appl Pharmacol*. 1993; 120:228–239. [PubMed: 8511792]
24. Wilson AK, Bhattacharyya MH. Effects of cadmium on bone: an in vivo model for the early response. *Toxicol Appl Pharmacol*. 1997; 145:68–73. [PubMed: 9221825]
25. Hoet P, Buchet JP, Decerf L, et al. Clinical evaluation of a lead mobilization test using the chelating agent dimercaptosuccinic acid. *Clin Chem*. 2006; 52:88–96. [PubMed: 16239340]
26. O'Donohue NV. Lead poisoning in childhood treated by the subcutaneous administration of a chelating agent. *Arch Dis Child*. 1956; 31:321–323. [PubMed: 13363476]
27. Saric M, Beritic T, Mimica M. [A case of severe lead encephalopathy in an adult treated with a chelating agent]. *Arh Hig Rada Toksikol*. 1962; 13:107–113. [PubMed: 13991492]
28. Sidbury JB Jr, Bynum JC, Fetz LL. Effect of chelating agent on urinary lead excretion; comparison of oral and intravenous administration. *Proc Soc Exp Biol Med*. 1953; 82:226–228. [PubMed: 13037852]
29. Tandon SK, Singh S, Prasad S, et al. Reversal of lead-induced oxidative stress by chelating agent, antioxidant, or their combination in the rat. *Environ Res*. 2002; 90:61–66. [PubMed: 12359191]
30. Liewehr FR, Craft DW, Primack PD, et al. Effect of bisphosphonates and gallium on dentin resorption in vitro. *Endod Dent Traumatol*. 1995; 11:20–26. [PubMed: 7641611]
31. Ghazi AM, Shuttleworth S, Angulo SJ, et al. Gallium diffusion in human root dentin: quantitative measurements by pulsed Nd:YAG laser ablation combined with an inductively coupled plasma mass spectrometer. *J Clin Laser Med Surg*. 2000; 18:173–183. [PubMed: 11573509]
32. Bhattacharyya MH. Cadmium osteotoxicity in experimental animals: mechanisms and relationship to human exposures. *Toxicol Appl Pharmacol*. 2009; 238:258–265. [PubMed: 19463839]
33. Elsubeihi E, Heersche J. Quantitative assessment of post-extraction healing and alveolar ridge remodeling of the mandible in female rats. *Archives of Oral Biology*. 2004; 49:401–412. [PubMed: 15041488]

34. Elinder CG, Kjellstrom T, Lind B, et al. Cadmium exposure from smoking cigarettes: variations with time and country where purchased. *Environ Res.* 1983; 32:220–227. [PubMed: 6617614]
35. Pocock SJ, Delves HT, Ashby D, et al. Blood cadmium concentrations in the general population of British middle-aged men. *Hum Toxicol.* 1988; 7:95–103. [PubMed: 3378814]
36. Covankara F, Erdogan H. Effects of chelating agents on the mineral content of root canal dentin. *Surg Oral Med Oral Pathol Oral Radiol Endod.* 2011; 11:149–154.
37. Hulsmann M, Heckendorff M, Lennon A. Chelating agents in root canal treatment: mode of action and indications for their use. *Int Endod J.* 2003; 36:810–830. [PubMed: 14641420]
38. Gonzalez-Lopez S, Camejo-Aguilar D, Sanchez-Sanchez P, et al. Effecto of CHX on the decalcifying effect of 10% citric acid, 20% citric acid, or 17% EDTA. *J Endod.* 2006; 32:781–784. [PubMed: 16861082]
39. Zehnder M, Schmidlin P, Sener B, et al. Chelation in root canal therapy reconsidered. *J Endod.* 2005; 31:817–820. [PubMed: 16249726]
40. Thangaraj D, Ballal V, Acharya S. Determination of calcium loss and its effect on microhardness of root canal dentin follwing treatment with 17% EDTA solution at different time intervals - an in vitro study. *Endodontology;Secretary's Message.* :4–9.
41. Spano J, Siva R, Guedes D, et al. Atomic absorption spectrometry and scanning electron microscopy evaluation of concentration of calcium ions and smear layer removal with root canal chelators. *J Endod.* 35:727–730. 209. [PubMed: 19410093]
42. Coxon FP, Thompson K, Roelofs AJ, et al. Visualizing mineral binding and uptake of bisphosphonate by osteoclasts and non-resorbing cells. *Bone.* 2008; 42:848–860. [PubMed: 18325866]
43. Russell R. Bisphosphonates: mode of action and pharmacology. *Pediatrics.* 2007; 119:S150–162. [PubMed: 17332236]

Highlights

1. This study proved the feasibility of removing bisphosphonates from bone *in-vivo*.
2. Systemic agent reduced the bisphosphonate signal from the skeleton.
3. This effect was due to a direct action on bone.
4. Local application produced a similar effect on extraction sites in the mandible.
5. Results suggest a new strategy to prevent BRONJ following dental procedures.

Figure 1.
Experimental timeline for the cadmium experiment. Arrows refer to the times of imaging.

Figure 2.

Decrease in FRFP signal after cadmium treatment in different bones collected at the end of the experiment (ex-vivo). A) Normalized Fluorescent Signal was less in cadmium treated than control animals at the alveolar bone ($p=0.008$), lumbar vertebrae ($p=0.45$), femoral epicondyles ($p=0.029$), and tibial plateau (non-significant). B) Representative fluorescent images of control and treated animal mandibles, vertebrae, femurs, and tibial plateau showing the diminished fluorescence in the bones of cadmium-treated animals.

Figure 3.

Cadmium-induced decrease in the in-vivo FRFP signal within the mandible. A) Time course of the percent of FRFP signal decline for control and 2 mg cadmium treated animals 1 and 3 days post each gavage (red arrows). FRFP signals were normalized to the 12 hour pre-treatment measurement. A significant decline in the FRFP signal was seen in the treatment animals at day 3 ($p=0.016$), 8 ($p=0.0015$), and 11 ($p=0.018$). B) Fluorescent images of the reduced FRFP signal in the cadmium animals compared to the controls after the second gavage.

Figure 4.

Effect of cadmium on calcium homeostasis. A) The BMD of the mandible was measured by DEXA and showed no significant difference between the treated and control animals. There was also no difference in the serum levels of B) calcium and C) PTH level.

Figure 5.

Systemic (off-target) effects of cadmium. A) H&E images taken at magnifications of 10X and 20X were used to analyze the kidney and liver health to assess if the cadmium treatment caused systemic toxicity. No differences in the morphology could be identified. B) BUN levels and D) creatinine levels within the serum were used to determine if kidney function was impaired by the treatment. Both groups fell within normal levels for both measurements with no difference between control and treated animals. C) Urine was collected every 12 hours to monitor kidney output and determine if the 2mg cadmium treatment was causing polyuria. No differences were found between groups either before or after the gavages (red arrows). F) Total Protein levels within urine samples collected daily for 10 days starting 5 days prior to the second gavage (black arrow). E) Weights were collected at the time of injection (day 0), gavage (red arrows), and sacrifice. No differences were found between groups for any of the analysis done.

Figure 6.

Histological analysis of treatment-induced FRFP decline and increased decalcification. A) Measurement of the depth of decalcification by citric acid and EDTA compared to their internal controls. Both treatments were found to have a significant ($p < 0.001$) amount of decalcification after treatment. Micrographs show the layer of the decalcified bone (blue stain; black arrows) caused by local chelation. Depth measurements were made from the outer boarder of the decalcified tissue (blue) to the outer boarder of the calcified bone (black). B) Measurement of the integrated density of the FRFP signal found within the alveolar bone analyzed at 10X. Significant differences were found between the internal controls and citric acid ($p = 0.025$) and EDTA ($p = 0.031$) treated mandibles. Images of the FRFP signal within the extraction site for a control and EDTA sample (white arrows) show a significant reduction in fluorescence after chelation.