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Bisphosphonates inhibit pain, bone loss, and inflammation in a rat tibia fracture model of complex regional pain syndrome

Liping Wang, MD, Tian-Zhi Guo, MD, Tzuping Wei, PhD, Wen-wu Li, PhD, Xiaoyou Shi, MD, J David Clark, MD, PhD, and Wade S Kingery, MD

Corresponding Author: Wade S Kingery, MD, Physical Medicine and Rehabilitation Service (117), Veterans Affairs Palo Alto Health Care System, 3801 Miranda Ave., Palo Alto, CA 94304, Phone: 650-207-3733, FAX: 650-852-3470, wkingery@stanford.edu.
1 Liping Wang, MD

- Title: Research Scientist
- Affiliation: Physical Medicine and Rehabilitation Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, California
- lipingwang06@gmail.com
- Role: This author helped design the study, conduct the study, analyze the data, and write the manuscript
- Conflicts: Liping Wang reports no conflicts of interest
- Attestation: Liping Wang has seen the original study data and approves the final manuscript

2 Tian-Zhi Guo, MD

- Title: Research Associate
- Affiliation: Physical Medicine and Rehabilitation Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, California
- wxtguo@yahoo.com
- Role: This author helped design the study, conduct the study, and write the manuscript
- Conflicts: Tian-Zhi Guo reports no conflicts of interest
- Attestation: Tian-Zhi Guo has seen the original study data and approves the final manuscript

3 Tzuping Wei, PhD

- Title: Research Associate
- Affiliation: Physical Medicine and Rehabilitation Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, California
- tzupingwei@yahoo.com
- Role: This author helped design the study, conduct the study, and write the manuscript
- Conflicts: Tzuping Wei reports no conflicts of interest
- Attestation: Tzuping Wei has seen the original study data and approves the final manuscript

4 Wen-wu Li, PhD

- Title: Research Scientist
- Affiliation: Department of Anesthesiology, Stanford University School of Medicine, Stanford, California, Anesthesiology Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, California
- wenwu.li@gmail.com
- Role: This author helped design the study, conduct the study, and write the manuscript
- Conflicts: Wen-wu Li reports no conflicts of interest
- Attestation: Wen-wu Li has seen the original study data and approves the final manuscript

5 Xiaoyou Shi, MD

- Title: Research Associate
- Affiliation: Department of Anesthesiology, Stanford University School of Medicine, Stanford, California, Anesthesiology Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, California
- xyshi@stanford.edu
- Role: This author helped design the study, conduct the study, and write the manuscript
- Conflicts: Xiaoyou Shi reported no conflicts of interest
- Attestation: Xiaoyou Shi has seen the original study data and approves the final manuscript

6 J David Clark, MD, PhD

- Title: Professor
- Affiliation: Department of Anesthesiology, Stanford University School of Medicine, Stanford, California, Anesthesiology Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, California
- David.Clark5@va.gov
- Role: This author helped design the study and write the manuscript
- Conflicts: J David Clark reports no conflicts of interest
- Attestation: J David Clark has seen the original study data and approves the final manuscript

7 Wade S Kingery, MD

- Title: Assistant Chief of PM&R Service
- Affiliation: Physical Medicine and Rehabilitation Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, California
- Role: This author helped design the study, analyze the data, and write the manuscript
- Conflicts: Wade S Kingery reports no conflicts of interest
- Attestation: Wade S Kingery reviewed the analysis of the data, approves the final manuscript, and is the author responsible for archiving the study files

Physical Medicine and Rehabilitation Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, California, Department of Anesthesiology, Stanford University School of Medicine, Stanford California, Anesthesiology Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, California

Abstract

BACKGROUND—Bisphosphonates are used to prevent the bone loss and fractures associated with osteoporosis, bone metastases, multiple myeloma, and osteogenesis deformans. Distal limb fractures cause regional bone loss with cutaneous inflammation and pain in the injured limb that can develop into complex regional pain syndrome (CRPS). Clinical trials have reported that anti-resorptive bisphosphonates can prevent fracture-induced bone loss, inhibit serum inflammatory cytokine levels, and alleviate CRPS pain. Previously we observed that the inhibition of inflammatory cytokines or adaptive immune responses attenuated the development of pain behavior in a rat fracture model of CRPS and we hypothesized that bisphosphonates could prevent pain behavior, trabecular bone loss, post-fracture cutaneous cytokine up-regulation, and adaptive immune responses in this CRPS model.

METHODS—Rats underwent tibia fracture and cast immobilization for 4 weeks and were chronically administered either subcutaneously perfused alendronate or oral zoledronate. Behavioral measurements included hindpaw von Frey allodynia, unweighting, warmth, and edema. Bone microarchitecture was measured by uCT and bone cellular activity was evaluated by static and dynamic histomorphometry. Spinal cord Fos immunostaining was performed and skin cytokine (TNF, IL-1, IL-6) and nerve growth factor (NGF) levels were determined by EIA. Skin and sciatic nerve immunoglobulin levels were determined by EIA.

RESULTS—Tibia fracture rats developed hindpaw allodynia, unweighting, warmth, and edema, increased spinal Fos expression, trabecular bone loss in the lumbar vertebra and bilateral distal femurs as measured by uCT, increased trabecular bone resorption and osteoclast surface with decreased bone formation rates, increased cutaneous inflammatory cytokine and NGF expression and elevated immunocomplex deposition in skin and nerve. Alendronate (60 µg/kg/day s.c.) or zoledronate (3 mg/kg/day p.o.) treatment for 28 days, started at the time of fracture, completely inhibited the development of hindpaw allodynia and reduced hindpaw unweighting by $44 \pm 13\%$ and $58 \pm 5\%$, respectively. Orally administered zoledronate (3 mg/kg/day for 21 days) treatment also completely reversed established allodynia and unweighting when started at 4-weeks post-fracture. Histomorphometric and uCT analysis demonstrated that both the 3 and 60 µg/kg/day alendronate treatments reversed trabecular bone loss (a $88 \pm 25\%$ and $188 \pm 39\%$ increase in the ipsilateral distal femur BV/TV, respectively) and blocked the increase in osteoclast numbers and erosion surface observed in bilateral distal femurs and in L5 vertebra of the fracture rats. Alendronate treatment inhibited fracture-induced increases in hindpaw inflammatory mediators, reducing post-fracture levels of TNF by $43 \pm 9\%$, IL-1 by $60 \pm 9\%$, IL-6 by $56 \pm 14\%$, and NGF by $37 \pm 14\%$, but had no effect on increased spinal cord Fos expression, or skin and sciatic nerve immunocomplex deposition.

CONCLUSIONS—Collectively, these results indicate that bisphosphonate therapy inhibits pain, osteoclast activation, trabecular bone loss, and cutaneous inflammation in the rat fracture model of

CRPS, data supporting the hypothesis that bisphosphonate therapy can provide effective multimodal treatment for CRPS.

Keywords

cytokine; nerve growth factor; alendronate; zoledronate; osteopenia; fracture; pain

INTRODUCTION

Bisphosphonates adhere to calcium hydroxyapatite in bone and inhibit osteoclast differentiation, activation, and survival. They are primarily used to prevent bone loss and fractures associated with osteoporosis, bone metastases, multiple myeloma, osteogenesis deformans, and other conditions associated with increased bone fragility.^{1,2} Bisphosphonate treatment also can reduce metastatic bone pain in some patients, but the effect is not immediate and bisphosphonates are not utilized as initial or primary analgesic treatments for metastatic pain.³

Complex regional pain syndrome (CRPS) is a chronic pain syndrome that most frequently develops after limb injuries and presents with regional nociceptive sensitization, vascular changes, and periarticular bone loss that exceed the expected clinical course of the inciting injury in both magnitude and duration. CRPS symptoms gradually resolve over the first year in the majority of patients, but persistent CRPS is a serious problem resulting in chronic pain, weakness, contractures, and bone loss.⁴ The debate regarding the underlying mechanisms of CRPS has been dynamic and controversial, and despite extensive investigation, the pathophysiology of this condition remains undefined and it is uncertain whether any treatment for CRPS is effective.^{5,6} Promising data from five small randomized controlled trials suggest that bisphosphonates may be an effective CRPS treatment, especially early in the course of the disease.⁷⁻¹¹ These trials examined heterogeneous bisphosphonate preparations, used differing diagnostic criteria and outcome measures, and did not examine long-term efficacy, but the consistently positive trial results warranted further investigation and several multicenter trials are currently evaluating bisphosphonate therapy in CRPS (NIH ClinicalTrials.gov identifier NCT02504008 and EU Clinical Trial Register numbers 2014 001156-28 and 2014 001915-37).

Population based studies indicate that distal limb fracture is the most common cause of CRPS^{12,13} and we have developed a fracture model in the rat and mouse closely resembling CRPS. Distal tibia fractured rats treated with 4-weeks cast immobilization develop hindpaw allodynia, unweighting, increased spinal Fos-immunoreactivity, increased hindpaw skin temperature, edema, facilitated neuropeptide signaling, periarticular bone loss, mast cell and keratinocyte proliferation, and increased keratinocyte expression of TNF, IL-1, IL-6, and NGF inflammatory mediators in the affected skin.¹⁴⁻²² Experimental maneuvers blocking these inflammatory mediators partially inhibited the nociceptive and vascular changes that develop after fracture, but had no effect on trabecular bone loss in the injured or contralateral limbs. Adaptive immunity also contributed to post-fracture nociceptive sensitization. After tibia fracture elevated levels of IgM complexes were observed in the skin and sciatic nerve of the injured limb, and fracture mice lacking B cells and immunoglobulin had attenuated

post-fracture nociceptive sensitization.²³ Bisphosphonates can inhibit monocyte/macrophage/dendritic cell migration, proliferation, and differentiation in vitro and reduce monocyte expression of TNF, IL-1, and IL-6 cytokines.²⁴⁻²⁹ Bisphosphonate treatment also reduces serum levels of TNF, IL-1, and IL-6 in osteoporosis patients.³⁰⁻³² Because bisphosphonates can potentially reverse pain, osteoclast activation, bone loss, inflammation, and antigen presentation, they present an attractive therapeutic approach in CRPS. The aim of the current study was to determine whether bisphosphonate treatments could inhibit the post-fracture development of nociceptive sensitization, reverse established pain behaviors, preserve trabecular bone integrity, and prevent the expression of cutaneous inflammatory mediators and immunocomplex deposition in skin and nerve after fracture.

METHODS

Animals

Our institute's Animal Care and Use Committee approved these experiments. All animals were treated in accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals and followed the guidelines of the International Association for the Study of Pain (IASP). Ten-month-old male Sprague Dawley rats (Simonsen Laboratories, Gilroy CA) were used in all experiments. When they arrived in our institution, they were housed individually in the isolator cages with solid floors covered with 3 cm of soft bedding in a room maintained at 26°C with 14-h light and 10-h dark cycles. During experimental period, the animals were fed *ad libitum* Lab Diet 5012 (PMI Nutrition, LandOLakes, St Paul, MN) that contained 1.0% calcium, 0.5 % phosphorus, and 3.3 IU/g of vitamin D3 per gram. The animals were allowed free access to drinking water.

Fracture protocol

Tibia fracture was performed under 2–4% isoflurane to maintain surgical anesthesia as we have previously described.^{14,15} The right hind limb was wrapped in a stockinet (2.5 cm wide) and the distal tibia was fractured using pliers with an adjustable stop (Visegrip, Newell Rubbermaid, Atlanta, GA) that had been modified with a three-point jaw. The hind limb was wrapped in casting tape (Delta-Lite, Johnson & Johnson, New Brunswick, NJ) so the hip, knee, and ankle were flexed. The cast extended from the metatarsals of the hind paw up to a spica formed around the abdomen. The cast over the paw was applied only to the plantar surface; a window was left open over the dorsum of the paw and ankle to prevent constriction when post-fracture edema developed. To prevent the animals from chewing at their casts, the cast material was wrapped in galvanized wire mesh. The rats were given subcutaneous saline and buprenorphine immediately after procedure (0.03 mg/kg) and on the first day after fracture for postoperative hydration and analgesia. At 4 weeks the rats were anesthetized with isoflurane and the cast removed with a vibrating cast saw. All rats used in this study had union at the fracture site after 4 weeks of cast immobilization.

Drug treatment protocols

To test the hypothesis that bisphosphonate treatment can inhibit immune responses and pain behavior after tibia fracture the following treatments were evaluated: 1) no fracture controls, 2) fracture + vehicle (Fx) s.c. for 4 weeks, 3) fracture + alendronate 3 µg/kg/day s.c. for 4

weeks (Fx + ALN 3), 4) fracture + alendronate 60 µg/kg/day s.c. for 4 weeks (Fx + ALN 60), and 5) fracture + zoledronate 3mg/kg/day p.o. for 4 weeks (Fx + ZOL 3). Both alendronate and zoledronate are nitrogenous bisphosphonates that act on bone metabolism by binding and blocking the enzyme farnesyl diphosphate synthase in the HMG-CoA reductase pathway. These dosages were selected after a thorough review of the bisphosphonate CRPS literature and discussions with lead scientists at Axsome Therapeutics, taking into consideration the fact that bisphosphonate oral bioavailability is only 1% and that zoledronate is 20 times more potent than alendronate in the inhibition of farnesyl diphosphate synthase.^{33,34} Alendronate (Sigma, St. Louis, MO) was diluted in normal saline and chronically perfused by ALZET Osmotic pumps (Durect, Cupertino, CA) placed subcutaneously over the dorsum of the rat trunk immediately after fracture. Zoledronate (a generous gift from Dr Herriot Tabuteau, Axsome Therapeutics, New York, NY) was dissolved in 4 ml distilled water and starting the day after fracture the rats were gavaged daily with either zoledronate (3 mg/kg/day) or distilled water. The rats were fasted 6 hours prior to gavage treatment and returned to normal *ad lib* food and water conditions 2 hours later. Calcein (15 mg/kg body weight) (Sigma, St. Louis, MO) was injected intraperitoneally at 14 days and 4 days prior to the time of sacrifice. After cast removal at 4 weeks post-fracture the rats underwent behavioral testing for hindpaw von Frey allodynia, unweighting, edema, and warmth, then they were euthanized by CO₂ inhalation and the bilateral hindpaw skin, sciatic nerves, gastrocnemius and soleus muscles, popliteal lymph nodes, femurs and the L5 lumbar vertebra were collected.

An additional study was performed looking at the effect of zoledronate treatment on established CRPS-like symptoms in fracture rats. After cast removal at 4 weeks post-fracture the rats underwent behavioral testing for nociception, edema, and warmth, then the fracture rats were started on daily gavage treatments with either zoledronate (a 21 mg/kg loading dose the first day and 3 mg/kg/day thereafter) or distilled water for 3 weeks (weeks 4-7 post-fracture) and behavioral testing was repeated each week.

Hindpaw nociception, temperature and edema

To measure hindpaw plantar mechanical allodynia in the fracture rats an up-down von Frey testing paradigm³⁵ was used as we have previously described.^{14,15} Hind paw mechanical nociceptive thresholds were analyzed as the difference between the treatment side and the contralateral untreated side.

An incapacitance device (IITC Inc. Life Science, Woodland Hills, CA) was used to measure hindpaw unweighting. The rats were manually held in a vertical position over the apparatus with the hind paws resting on separate metal scale plates, and the entire weight of the rat was supported on the hindpaws. The duration of each measurement was 6s, and 10 consecutive measurements were taken at 20s intervals. Eight readings (excluding the highest and lowest) were averaged to calculate the bilateral hind paw weight bearing values.^{15,18} Right hindpaw weight bearing data were analyzed as a ratio between the right hindpaw weighting and the mean of right and left hindpaws values ($(2R/(R + L)) \times 100\%$).

The temperature of the hindpaw was measured using a fine wire thermocouple (Omega Engineering, Stamford, CT) applied, as previously described.^{14,15} Temperature testing was

performed over the hindpaw dorsal skin between the first and second metatarsals (medial), the second and third metatarsals (central), and the fourth and fifth metatarsals (lateral). The measurements for each hindpaw were averaged for the mean paw temperature. Hindpaw edema was determined by measuring the hindpaw dorsal-ventral thickness over the midpoint of the third metatarsal with a LIMAB laser measurement sensor (Goteborg, Sweden) while the rat was briefly anesthetized with isoflurane.^{14,15} Temperature and hindpaw thickness data were analyzed as the difference between the fracture side and the contralateral intact side.

Micro-CT

Ex vivo scanning was performed for assessment of trabecular and cortical bone structure using μ CT (Viva CT 40, Scanco Medical AG, Basserdorf, Switzerland). Specifically, trabecular bone was evaluated in the distal femur and the L5 vertebral bone and cortical bone was examined at the femur mid-shaft. CT images were reconstructed in 1024X1024-pixel matrices for vertebral, distal femur, and mid-femur samples and scored in 3-D arrays. The resulting gray scale imaging was segmented using a constrained Gaussian filter to remove noise, and a fixed threshold (25.5% of the maximal gray scale value for vertebra and distal femur and 35% for the mid-femur cortical bone) was used to extract the structure of the mineralized tissue. The μ CT parameters were set at threshold = 255, σ = 0.8, support = 1 for vertebral samples; threshold = 255, σ = 0.8, support = 1 for distal femur, threshold = 350, σ = 1.2, support = 2 for mid-femur evaluation analysis.

Each L5 vertebral body was scanned using 223 transversely oriented 21 μ m thick slices (21- μ m isotropic voxel size) encompassing a length of 4.68 mm. The trabecular bone region was manually identified and all slices containing trabecular bone between the growth plates were included for our analysis. In the distal femur, 150 transverse slices of 21 μ m thickness (21- μ m isotropic voxel size) encompassing a length of 3.15 mm were acquired, but only 100 slices encompassing 2.1 mm of the distal femur were evaluated, starting where the growth plate bridge across the middle of the metaphysic ends. The region of interest (ROI) was manually outlined on each CT slice, extending proximally from the growth plate. Relative trabecular bone volume (BV/TV, %), trabecular number (Tb.N, mm⁻¹), trabecular thickness (Tb.Th, μ m), and trabecular separation (Tb.Sp, μ m) were calculated by measuring 3D distances directly in the trabecular network and taking the mean over all voxels. The connectivity density (Conn.D, mm⁻³) based on the Euler number was also determined. By displacing the surface of the structure by infinitesimal amounts, the structure model index (SMI, 0-3) was also calculated. The SMI quantifies the plate versus rod characteristics of trabecular bone, in which a SMI of 0 represents a purely plate-like bone and SMI of 3 indicates a purely rod-like structure.

At the femur midshaft, 10 transverse CT slices were obtained, each 21 μ m thick totaling 0.21 mm in length (21 μ m isotropic voxel size) and these were used to compute the total area (T.Ar, mm²), cortical bone area (B.Ar, mm²), cortical thickness (Ct.Th, μ m) and periosteum perimeter (B.Pm, mm).

Bone histomorphometry

Rat distal femurs were embedded, without decalcification, in methylmethacrylate (Sigma, St. Louis, MO), and sectioned longitudinally with a Leica / Jung 2255 microtome (Leica Microsystems, Wetzlar, Germany) at 4- and 8- μ m-thick sections. The 4- μ m sections were stained with Toluidine blue for collection of bone mass and architecture data with the light microscope, whereas the 8- μ m sections were left unstained for measurements of fluorochrome-based indices. Static and dynamic histomorphometry were performed using an automatic image analysis system (Bioquant, Nashville, TN) linked to a microscope equipped with a transmitted and fluorescence light.

Trabecular bone in the distal femur was quantified at a magnification of $\times 200$. The area measured was defined by the cortical bone on both sides and by a line beginning 1 mm distal to the growth plate and extending further proximally to the middle femur shaft. The following parameters were measured: total bone area (T.Ar; mm^2), total trabecular bone area (B.Ar; mm^2), total trabecular bone perimeter (B.Pm; mm), single-labeled bone perimeter (sL.Pm; mm), double-labeled bone perimeter (dL.Pm; mm), and interlabeled width (Ir.L.Wi; μ m). The following parameters were calculated: trabecular bone volume (BV/TV; %), trabecular thickness (Tb.Th; μ m), trabecular number (Tb.N; mm^{-1}), trabecular separation (Tb.Sp; μ m), single-labeled surface (sLS/BS; %), double-labeled surface (dLS/BS; %), trabecular bone surface (BS), mineral apposition rate (MAR, $\mu\text{m}/\text{day}$), mineralizing surface (MS/BS; %), bone formation rate (BFR/BS, $10^{-2} \mu\text{m}^3/\mu\text{m}^2/\text{day}$), osteoclast surface (Oc.S), eroded surface (ES). All nomenclature and calculations of the histomorphometric indices are according to Parfitt et al.³⁶

Cytokine and NGF ELISA

Rats were euthanized with CO_2 and the hindpaw dorsal skin was collected and frozen immediately on dry ice. All tissues were cut into fine pieces in ice-cold phosphate buffered saline (PBS), pH 7.4, containing protease inhibitors (aprotinin (2 $\mu\text{g}/\text{ml}$), leupeptin (5 $\mu\text{g}/\text{ml}$), pepstatin (0.7 $\mu\text{g}/\text{ml}$), and PMSF (100 $\mu\text{g}/\text{ml}$, Sigma, St. Louis, MO) followed by homogenization using a rotor/stator homogenizer. Homogenates were centrifuged for 5 min at 14 000 g, 4°C. Supernatants were transferred to fresh precooled Eppendorf tubes. Triton X-100 was added at a final concentration 0.01 %. The samples were centrifuged again for 5 min at 14,000g at 4°C. The supernatants were aliquoted and stored at -80°C . The TNF, IL-1, and IL-6 levels were measured using EIA kits (R&D Systems, Minneapolis, MN). The NGF concentrations were determined by using the NGF Emax® ImmunoAssay System kit (Promega, Madison, WI) according to the manufacturer's instructions. Total protein contents in all tissue extracts were measured by the Coomassie Blue Protein Assay Kit (Pierce, Life Technologies, Waltham, MA). Each protein concentration was expressed as pg/mg total protein. The results of all assays were confirmed by repeating the experiments twice.

Fos spinal cord immunohistochemistry

Rats were euthanized with CO_2 and perfused intracardially with 200 ml 0.1 M PBS followed by 200 ml neutral 10% buffered formaldehyde. Spinal cord segments (L3–L5) were removed, post-fixed in the perfusion fixative overnight and cryoprotected in 30% sucrose at 4°C for 24 h. Serial frozen spinal cord sections, 40- μ m-thick, were cut on a

coronal plane by using a cryostat, collected in PBS, and processed as free floating sections. Fos immunostaining was performed as previously described.^{17,18} Because the sciatic nerve projects heavily to the L3-L5 segments of the spinal cord, we analyzed the numbers of Fos immunoreactive (Fos-IR) neurons at those levels.

To evaluate and compare the distribution of Fos positive neurons in the lumbar spinal cord, an image analysis system (Bioquant, Nashville, TN) attached to a Nikon Eclipse 80i microscope was used. Digital images were captured using 10X magnification. The Fos-IR neurons were identified by dense black staining of the nucleus. The Fos-IR neurons were plotted and counted with Bioquant Automated Imaging module through four arbitrary defined regions of the spinal grey matter of the L3 - L5 segments, according to the cytoarchitectonic organization of the spinal cord; the superficial laminae (laminae I - II), the nucleus proprius (laminae III - IV), and the deep laminae (laminae V - VI; neck) of dorsal horn. For each section, the Fos-IR neurons were counted for each lamina, the counts were pooled, and the average number was calculated giving a count that was the mean of all stained neurons in those three sections per each cytoarchitectonic region. The investigator responsible for plotting and counting of the Fos-IR neurons was blinded to groups.

Skin and sciatic nerve immunoglobulin ELISA

Rats were euthanized with CO₂ and hindpaw dorsal skin and sciatic nerve was collected and frozen immediately on dry ice. All tissues were cut into fine pieces in ice-cold phosphate buffered saline, pH 7.4, containing a cocktail of protease inhibitors (Roche Applied Science, Penzberg, Germany) and followed by homogenization using a Bio-Gen PRO200 homogenizer (PRO Scientific, Oxford, CT). The homogenates were centrifuged at 12,000g for 15 min at 4°C. The supernatants were aliquoted and stored at -80°C until required for ELISA performance. Total protein contents in all tissue extracts were measured by using the DC Protein Assay kit (Bio-Rad, Hercules, CA). The albumin, IgM and IgG levels were determined in duplicate by using ELISA kits (GenWay Biotech, San Diego, CA) according to the manufacturer's instructions. The results of all assays were confirmed by repeating the experiments twice.

Popliteal lymph node dissection and size measurement

The popliteal lymph node is embedded in the adipose tissue of the popliteal fossa and is spherical. Rats were euthanized with CO₂ and the bilateral popliteal lymph nodes were dissected free under a microscope. The lymph node diameters were measured using a caliper with the average diameter for each lymph node defined as the (short-axis diameter + long-axis diameter)/2.

Statistical analysis

The primary endpoint of the study was comparing vehicle treatment to bisphosphonate treatment on post-fracture hindpaw von Frey thresholds, while also confirming that fracture rats have reduced von Frey thresholds relative to nonfractured controls. Secondary outcomes of the study were; 1) comparing control (no fracture) to fracture for hindpaw unweighting, vascular changes (temperature and paw thickness), bone metabolism parameters (BV/TV, Conn.D, BFR, Oc.S/BS, ES/BS), hindpaw skin inflammatory mediator levels (TNF, IL-1,

IL-6, NGF), hindpaw skin and sciatic nerve albumin, IgM, and IgG levels, and popliteal lymph node diameters, and 2) comparing vehicle treatment to bisphosphonate treatment on post-fracture hindpaw unweighting, vascular changes (temperature and paw thickness), bone metabolism parameters (BV/TV, Conn.D, BFR, Oc.S/BS, ES/BS), hindpaw skin inflammatory mediator levels (TNF, IL-1, IL-6, NGF), hindpaw skin and sciatic nerve albumin, IgM, and IgG levels, and popliteal lymph node diameters.

Statistical analysis was performed using Prism 4.02 (GraphPad software, La Jolla, CA). Sample sizes were based on a power analysis of preliminary and published data generated from using each of the proposed assays in fracture animals. Based on this analysis we calculate that the proposed experiments would require 8 animals per cohort to provide 80% power to detect 25% differences between groups. The SD deviation of hindpaw von Frey thresholds used for sample size calculation was 4 g and the clinically important difference was set as 1 g. Animals were randomized to experimental groups using computer generated random numbers and all testing was performed in a blinded fashion when possible. No animals were excluded after enrollment into the experimental cohorts. The normal distribution of the data was confirmed using the D'Agostino-Pearson omnibus normality test. All data were evaluated using a one-way analysis of variance (ANOVA), except the time-course comparisons in Figure 2, followed by Holm-Sidak multiple comparison testing (significance level 5%) to compare between control and fracture rats that were treated with either bisphosphonate or vehicle. The time-course comparisons in Figure 2 were evaluated using a repeated measure two-way ANOVA followed by Holm-Sidak multiple comparison testing to compare between baseline and post-fracture time points and between vehicle and zoledronate treatment groups. All data are presented as the mean $\pm$ SEM.

RESULTS

Alendronate effects on fracture induced changes in body and muscle weight

All groups had similar mean body weights at the start of the experiment (control, 437.6 ± 7.9 g; fracture + vehicle (Fx + vehicle), 435.0 ± 7.8 g; fracture + alendronate 60 ug/kg/day (Fx + ALN 60), 437.6 ± 6.1 g). The fractured rats had significantly lower body weights (345.2 ± 6.8 g) than the age-matched vehicle treated control rats 4 weeks after surgery, as we had previously observed.^{14,15} After 4 weeks of alendronate treatment, when the experiment was terminated, the alendronate treated fractured rats had slightly higher observed mean body weights (Fx+ ALN 60, 351.6 ± 6.2 g) than the age-matched, vehicle treated fracture rats (Fx + vehicle, 345.2 ± 6.8 g), but the increased weights were not statistically significant. The weights of ipsilateral gastrocnemius muscles from each group were also measured. Fracture caused a significant decrease in muscle weights in bilateral hindlimbs (Fx + Vehicle: ipsilateral side, 1.20 ± 0.06 g; contralateral side, 1.87 ± 0.09 g) when compared to the control rats (control: ipsilateral side, 3.56 ± 0.07 g; contralateral side, 3.60 ± 0.08 g). Interestingly, 4 weeks of alendronate treatment (Fx + ALN 60) significantly increased the muscle weight in the fractured hindlimb (1.39 ± 0.04 g, $p < 0.05$) and in the contralateral side (2.26 ± 0.06 g, $p < 0.01$), suggesting a possible bisphosphonate anticatabolic effect in muscle after fracture.

Bisphosphonates inhibited the development of post-fracture nociceptive changes

The effects of alendronate and zoledronate treatment on fracture-induced hindpaw mechanical sensitivity, weight bearing, warmth, and edema were evaluated (Fig. 1). Fractured rats were administered vehicle (saline) or alendronate (3 µg/kg/day or 60 µg/kg/day) by subcutaneous osmotic pumps, or zoledronate (3 mg/kg/day) by oral gavage. The zoledronate controls were fracture rats gavaged daily with 4 ml distilled water. As there were no differences between the saline subcutaneous pump treated fracture rats and the distilled water gavaged fracture rats in any outcome measure, data from all the vehicle treated fracture rats were pooled in Figure 1 and in the statistical analysis.

Figure 1A illustrates that von Frey nociceptive thresholds in the ipsilateral hindpaw are reduced 4 weeks after fracture, but treatment with alendronate or zoledronate blocked the development of this mechanical allodynia. There was no significant difference between the contralateral hindpaw von Frey withdrawal threshold in the fracture cohorts and the intact controls (data not shown), indicating that the vehicle treated fracture rats did not develop mechanical allodynia in the contralateral hindpaw. In addition, there was no significant difference between the contralateral hindpaw von Frey withdrawal threshold in fracture rats treated with vehicle compared to any of the bisphosphonate treatments (data not shown), indicating that the bisphosphonate treatments had no effect on the normal mechanical nociceptive thresholds in the contralateral hindpaw. Figure 1B shows that vehicle treated fracture rats unweighted the ipsilateral hindpaw by 34% ($p < 0.0001$) and that a 4 weeks course of alendronate treatment reduced fracture induced unweighting to 26% (3 µg/kg s.c. daily, $p < 0.05$) and 18% (60 µg/kg s.c. daily, $p < 0.0001$), respectively. A 4 weeks course of zoledronate treatment (3mg/kg p.o. daily) reduced hindpaw unweighting to 14% ($p < 0.0001$).

At 4 weeks post-fracture ipsilateral hindpaw temperature (Fig. 1C) and thickness (Fig. 1D) were increased. The alendronate and zoledronate treatments had no significant effect on post-fracture temperature (Fig. 1C). Neither alendronate or zoledronate treatment had any effect on paw edema (Fig. 1D).

The possibility of persistent beneficial or curative effects after stopping zoledronate treatment was examined (Fig. 2). After the completion of a 4 weeks course of zoledronate (3mg/kg p.o. daily) the cast was removed and the rats underwent behavioral testing. Post-fracture hindpaw mechanical allodynia, unweighting, and edema was partially reversed by zoledronate treatment at 4 weeks post-fracture, but by 5 weeks post-fracture (1 week after stopping the zoledronate treatment) the inhibitory treatment effects resolved for mechanical allodynia and edema, but the inhibitory effect on hindpaw unweighting persisted (Fig. 2 A-D). At 6 weeks post-fracture (2 weeks after stopping zoledronate treatment) no differences were observed between the vehicle and treatment groups for any outcome measures, indicating that zoledronate treatment did not have a curative effect on post-fracture CRPS-like sequelae.

Oral zoledronate reversed established pain behaviors

To determine whether bisphosphonate treatment could reverse established CRPS-like symptoms, zoledronate treatment (3mg/kg p.o. daily for 3 weeks, over weeks 4-7 post-fracture) was started at 4 weeks post-fracture, immediately after the baseline behavioral testing was complete (Fig. 2 E-H). Zoledronate treatment reduced hindpaw allodynia after 2 weeks of zoledronate treatment (6 weeks post-fracture), reduced unweighting after 1-week of treatment (5 weeks post-fracture). There was no treatment effect on hindpaw warmth or edema.

Alendronate treatment reversed post-fracture bone loss in the bilateral distal femurs and L5 vertebra

The effects of tibia fracture and alendronate treatment on trabecular bone mass were examined in the bilateral distal femurs and L5 vertebra. Four weeks after fracture there was a 52% decrease in BV/TV ($p < 0.0001$), an 11% decrease in Tb.N ($p < 0.05$), a 14% decrease in Tb.Th ($p < 0.001$), a 67% decrease in Conn.D ($p < 0.0001$), a 12% increase in Tb.Sp ($p < 0.01$), and a 34% increase in SMI ($p < 0.001$) in the ipsilateral distal femur of the fractured rats (Figs. 3A,D, Table 1). Alendronate treatment significantly increased the trabecular bone mass in the ipsilateral distal femur of the fractured rats. After 4 weeks treatment, both the 3 $\mu\text{g/kg/day}$ and 60 $\mu\text{g/kg/day}$ doses of alendronate increased trabecular BV/TV in the ipsilateral femur of the fractured rats 77% ($p < 0.001$) and 158% ($p < 0.0001$), respectively, increased Tb.N by 44% ($p < 0.001$) and 58% ($p < 0.001$), respectively, increased Tb.Th by 8% ($p < 0.05$) and 15% ($p < 0.001$), respectively, increased Conn.D by 170% ($p < 0.0001$) and 414% ($p < 0.0001$), respectively, decreased Tb.Sp by 32% ($p < 0.001$) and 37% ($p < 0.001$), respectively, and decreased SMI by 13% ($p < 0.01$) and 27% ($p < 0.001$), respectively, when compared to the vehicle treated fracture rats (Fx + Vehicle, Figs. 3A,D, Table 1).

Effects of fracture without and with alendronate on trabecular bone mass in the contralateral distal femur were also investigated with μCT (Figs. 3B,E, Table. 1). Four weeks after fracture, there was a 20.2% decrease in BV/TV ($p < 0.01$), a 12.6% decrease in Tb.N ($p < 0.05$), a 7% decrease in Tb.Th ($p < 0.001$), a 17.9% decrease in Conn.D ($p > 0.05$), a 11.4% increase in Tb.Sp ($p < 0.01$), and a 17% increase in SMI ($p < 0.001$) in the contralateral distal femur of the fractured rats. After 4 weeks treatment, both the 3 $\mu\text{g/kg/day}$ and 60 $\mu\text{g/kg/day}$ doses of alendronate increased trabecular BV/TV in the contralateral femur of the fractured rats by 22% ($p < 0.05$) and 61% ($p < 0.0001$), respectively, increased Tb.N by 26% ($p < 0.001$) and 30% ($p < 0.001$), respectively, increased Tb.Th by 2% ($p > 0.05$) and 6% ($p > 0.05$), respectively, increased Conn.D by 34% ($p < 0.05$) and 118% ($p < 0.0001$), respectively, decreased Tb.Sp by 22% ($p < 0.001$) and 24% ($p < 0.001$), respectively, and decreased SMI by 5% ($p < 0.01$) and 18% ($p < 0.001$), respectively, when compared to the vehicle treated fracture rats (Fx + Vehicle, Figs. 3B,E, Table. 1).

Effects of fracture without and with alendronate on cortical bone mass were examined at the bilateral middle femurs. In this study, fracture and alendronate treatments did not affect the cortical bone parameters, cortical B.Ar/T.Ar and cortical Ct.Th (data not shown).

At 4 weeks after fracture there was a modest loss of trabecular bone in the L5 vertebral body (Fig. 3C,F, Table 1.). When compared to the intact control rats, there was a 26% decrease in BV/TV ($p < 0.05$), a 2% decrease in Tb.N ($p > 0.05$), a 10% decrease in Tb.Th ($p > 0.05$), a 26% decrease in Conn.D ($p < 0.01$), a 3% increase in Tb.Sp ($p > 0.05$), and a 46% increase in SMI ($p < 0.01$) at L5 in the fractured rats. After 4 weeks of alendronate treatment, both the 3 $\mu\text{g/kg/d}$ and 60 $\mu\text{g/kg/d}$ doses of alendronate increased the L5 trabecular BV/TV of the fractured rats by 42% ($p < 0.01$) and 80% ($p < 0.0001$), respectively, increased Tb.N by 6% ($p > 0.05$) and 13% ($p < 0.001$), respectively, increased Tb.Th by 11% ($p > 0.05$) and 22% ($p < 0.001$), respectively, increased Conn.D by 53% ($p < 0.01$) and 59% ($p < 0.0001$), respectively, decreased Tb.Sp by 6% ($p > 0.05$) and 14% ($p < 0.001$), respectively, and decreased SMI by 53% ($p < 0.05$) and 61% ($p < 0.001$), respectively, when compared to the vehicle treated fracture rats (Fx + Vehicle, Figs. 3C, F, Table. 1).

Alendronate treatment inhibited osteoclast bone resorption activity

Effects of fracture and alendronate (60 $\mu\text{g/kg/day}$) on trabecular bone mass in the ipsilateral distal femur and the L5 vertebral body were also studied by both static and dynamic histomorphometry (Fig. 4A-F). Effects of alendronate on trabecular parameters as measured by static histomorphometry were similar to our previous μCT findings (data not shown). In addition, fracture caused a decrease in BFR in both sites and an increase in bone resorption activity as indicated by OC.S/BS, and ES/BS in the ipsilateral distal femur. Four weeks of alendronate treatment did not change BFR in fracture rats, but successfully inhibited bone resorption activity by reducing the values of OC.S/BS and ES/BS (Fig. 4A-F).

Alendronate treatment inhibited post-fracture increases in hindpaw cytokine and NGF levels

Cytokine and NGF protein levels in hindpaw skin were determined by EIA (Fig. 5). At 4 weeks post-fracture there was a 338% increase in TNF ($p < 0.0001$), an 85% increase in IL-1 ($p < 0.05$), a 55% increase in IL-6 ($p > 0.05$), and a 229% increase in NGF ($p < 0.01$) protein levels in the fracture hindpaw. Four weeks of alendronate treatment (60 $\mu\text{g/kg}$ s.c. daily) inhibited post-fracture increases in cytokine and NGF protein levels in the hindpaw skin. Alendronate treatment reduced post-fracture TNF levels by 48% ($p < 0.001$), IL-1 levels by 65% ($p < 0.001$), IL-6 levels by 63% ($p < 0.001$), and NGF levels by 51% ($p < 0.01$).

Bisphosphonate treatment did not inhibit Fos expression in lumbar spinal cord after fracture

To test whether bisphosphonate treatment can inhibit post-fracture increases in immediate early gene expression in the spinal cord, the effects of alendronate treatment on spinal Fos expression were evaluated. Immunostaining for Fos was performed in the L3 – L5 dorsal horns in control rats and fracture rats treated for 4 weeks with either vehicle or alendronate 60 $\mu\text{g/kg/day}$ (Fx + ALN 60). Confirming our previous findings in the rat fracture model,^{17,18} at four weeks after the fracture Fos expression increased in the ipsilateral dorsal horn (data not shown) compared with control, but the increase was statistically significant only in laminae I-II and laminae III-IV. In the contralateral dorsal horn of control rats, there was a nonsignificant increase in Fos expression seen in lamina I through Lamina VI.

Alendronate treatment failed to reduce the Fos expression in bilateral spinal horns in the fracture rats compared with vehicle treated controls (data not shown). Contrariwise, alendronate treatment evoked an increase of Fos expression in the contralateral dorsal horn compared to the Fos immunostaining observed in unfractured controls (data not shown). Previous studies have reported occasional dissociations between opiate or NMDA antagonist induced analgesia and spinal Fos expression and this may be attributable to the fact that Fos expression is not specifically related to nociception, but is also induced by motor activity, nonnoxious sensory stimuli, stress, or even arousal.³⁷

Zoledronate treatment did not inhibit immune complex deposition in fracture limb

To determine whether bisphosphonate treatment could prevent the post-fracture deposition of immune complexes indicative of autoimmunity, IgM and IgG protein levels were measured by EIA at 4 weeks post-fracture in the skin and sciatic nerve tissues of rats treated zoledronate (3 mg/kg p.o. daily for 4 weeks) or vehicle (distilled water p.o. daily for 4 weeks). IgM levels were increased 357% in the skin and 166% in the sciatic nerve, and zoledronate treatment failed to inhibit this post-fracture increase (Fig. 6B). Similarly, IgG levels were increased 107% in the skin and 67% in the sciatic nerve, and zoledronate treatment failed to prevent this post-fracture increase in immune complex deposition (Fig. 6C). Post-fracture albumin levels only increased by 42% in the skin and by 22% in the sciatic nerve, a much lesser extent than the increases in IgM and IgG levels observed after fracture, suggesting that changes in vascular permeability did not account for the skin and nerve immunoglobulin deposition observed after fracture (Fig. 6A).

Zoledronate treatment did not inhibit post-fracture popliteal lymphadenopathy

At 4 weeks post-fracture the popliteal lymph node diameter was increased 54% in the fracture limb (Fx-ipsi + Vehicle), compared to the contralateral popliteal lymph node diameters (Fx-contra + Vehicle) or to Control nonfracture rat popliteal lymph node diameters (Fig. 7). Zoledronate treatment (3 mg/kg p.o. daily for 4 weeks) had no effect on post-fracture lymphadenopathy, compared to vehicle treatment (distilled water p.o. daily for 4 weeks).

DISCUSSION

Four weeks of daily bisphosphonate treatment with alendronate (3 or 60 ug/kg s.c./day) or zoledronate (3 mg/kg p.o./day), started at the time of fracture, prevented the development of post-fracture nociceptive sensitization, but it was not curative. When zoledronate treatment was discontinued at 4 weeks post-fracture there was a reoccurrence of hindpaw von Frey allodynia and unweighting, returning to the same levels as observed in the vehicle treated fracture rats (Figs. 1,2). Osteoclastic bone resorption is commonly observed in CRPS affected limbs and it has been postulated that the inhibitory effects of bisphosphonates on osteoclast formation and activation may mediate analgesia in CRPS patients. Bisphosphonates are not metabolized in the systemic circulation, but are instead rapidly incorporated into remodeling bone and cleared from the systemic circulation by renal elimination. Alendronate and zoledronate disappear from the circulation and soft tissues within hours or days after administration, but are very slowly released from calcific tissue

over a period of months to years.^{38,39} The reoccurrence of hindpaw allodynia and unweighting within a week of discontinuing zoledronate treatment suggests that osteoclastic inhibitory effects do not contribute to the zoledronate analgesia observed in the mouse fracture CRPS model (Fig. 2).

When zoledronate treatment (3 mg/kg, p.o. daily for 3 weeks) was started at 4 weeks post-fracture it slowly reversed established post-fracture pain behaviors (Fig. 2). The antinociceptive effects of zoledronate developed slowly over 1-2 weeks, suggesting an indirect mechanism of pain relief (Fig. 2). Zoledronate treatment had no effect on contralateral hindpaw von Frey thresholds and when the same dose of zoledronate (3 mg/kg, p.o.) was given to nonfracture control rats it had no effect on tail-flick latencies between 1 and 24 hours after administration (data not shown). Zoledronic acid is considered to be the most potent bisphosphonate and is currently approved only as an intravenous formulation.⁴⁰ This is the first report indicating that orally administered zoledronate can provide effective analgesia in a chronic pain model. Collectively, these results suggest that bisphosphonates can prevent or reverse post-fracture pain behaviors, but are not curative. Furthermore, the antihyperalgesic effects of bisphosphonates developed slowly over days or weeks, and there was no evidence that they provided effective analgesia for acute pain.

The therapeutic efficacy of bisphosphonate treatment in the rat CRPS fracture model concurs with the clinical trial results in CRPS patients demonstrating bisphosphonate therapeutic efficacy early in the course of the disease.⁷⁻¹¹ The clinical trial data is equivocal regarding the curative effects of bisphosphonate treatment for CRPS,^{10,11} but results from the current translational study (Figs. 2A,B) suggest that 4 weeks of bisphosphonate treatment would not be curative.

After distal tibia fracture there was a loss of trabecular bone volume and connectivity in the ipsilateral distal femur (Fig. 3A,D). This bone loss was attributable to a reduction in bone formation and an increase in osteoclast activation and bone resorption (Fig. 4A-C). Interestingly, there was also a post-fracture reduction in trabecular bone volume and connectivity in the contralateral distal femur and L5 vertebra (Fig. 3B,C,E,F), with an associated reduction in bone formation and increased osteoclast activity and bone resorption (Fig. 4D-F). A similar pattern of trabecular bone loss is observed in both the ipsilateral and contralateral limb and in the lumbar vertebra of lower limb fracture patients⁴¹⁻⁴³ In CRPS patients a regional patchy periarticular osteopenia is usually observed on radiographs with a loss of trabecular bone density in the involved limb and in the contralateral limb^{7,44-46} and the severity of trabecular bone loss is greater in fracture patients with CRPS signs and symptoms than in fracture patients without CRPS.^{47,48} Studies using technetium 99m labeled diphosphonates that are taken up in areas of active bone remodeling demonstrate accelerated bone resorption in the periarticular trabecular bone of the affected limb and frequently in the contralateral limb as well.^{9,45,46} Bone biopsies in CRPS affected limbs demonstrate demineralization and osteoclastic resorption,⁴⁹ similar to our histomorphometric findings in the distal femur and L5 vertebra of the fracture rats (Fig. 4).

Four weeks of alendronate treatment, started at the time of fracture, dose-dependently prevented post-fracture trabecular bone volume loss and loss of connectivity in the bilateral

distal femurs and in the L5 vertebra (Fig.3 and Table 1). Alendronate treatment had minimal effect on bone formation rates (Fig. 4A, D), but did inhibit post-fracture increases in osteoclast surface (Fig. 4B, E) and bone resorption (Fig. 4C, F). Similarly, bisphosphonate treatment inhibits the development of regional trabecular bone loss in both fracture patients and CRPS patients.^{7,50,51} Bisphosphonates also reduce urinary levels of type I collagen N-telopeptide in CRPS patients, suggesting an inhibitory effect on osteoclastic bone resorption.^{8,9}

Levels of inflammatory mediators (TNF, IL-1, IL-6, and NGF) were elevated in the hindpaw skin at 4 weeks after fracture (Fig. 5), consistent with our prior findings in fracture rats and mice. Post-fracture increases in inflammatory mediators were inhibited or completely blocked by 4 weeks of alendronate treatment (Fig. 5), in agreement with prior reports of bisphosphonate inhibitory effects on monocyte TNF, IL-1, and IL-6 expression *in vitro*²⁴⁻²⁹ and on TNF, IL-1, and IL-6 levels in osteoporotic patients.³⁰⁻³² Previously we demonstrated that epidermal keratinocytes are the primary cellular source for the expression of cutaneous inflammatory mediators in fracture rats and mice and in CRPS patient skin.^{20,52-54} Interestingly, TNF and IL-6 levels are also increased in the skin and experimental skin blister fluid of CRPS affected limbs.⁵⁴⁻⁵⁷ Treating fracture rats with TNF, IL-1, IL-6, or NGF receptor antagonists or inhibitors partially inhibits post-fracture allodynia and unweighting, but unlike alendronate, these treatments were ineffective at preventing trabecular bone loss in the CRPS fracture model.^{16-18,58}

Recently we observed that B cells contributed to the development and maintenance of CRPS – like changes in the mouse fracture model and postulated that IgM autoantibodies directed at antigens in the fracture limb skin and nerves contribute to nociceptive sensitization in CRPS.²³ Clinical support for this hypothesis include a recent study demonstrating that a third of CRPS patients exhibit strongly positive antinuclear antibody tests, a standard diagnostic test for autoimmune disease,⁵⁹ and small randomized trial of low dose IVIG demonstrated that some chronic CRPS patients had prolonged and dramatic symptom improvement after a single IVIG treatment⁶⁰. Consistent with our prior results in fracture mice, at 4 weeks post-fracture in rats there was a dramatic increase in IgM and IgG deposition in the skin and sciatic nerve of the injured limb, and 4 weeks of zoledronate treatment failed to inhibit this increase (Fig. 6B,C). These results do not support the hypothesis that bisphosphonate treatment can inhibit post-fracture autoimmunity. In addition, zoledronate treatment had no effect on post-fracture popliteal lymphadenopathy (Fig. 7). Collectively, these data suggest that bisphosphonate treatment would be ineffective in chronic CRPS patients with predominantly autoimmune mediated symptoms.

There will always be caveats in extrapolating from pharmacological studies in the rat fracture model to human CRPS. The current study examined bisphosphonate effects over the first 7 weeks after fracture, corresponding to the earliest phase of CRPS. Previously we demonstrated that early CRPS patients (less than 3 months disease duration) had cutaneous mast cell and keratinocyte proliferation and keratinocyte expression of TNF and IL-6 cytokines, but more chronic CRPS patients (greater than 3 months disease duration) failed to exhibit these cutaneous inflammatory changes.⁵⁴ Similarly, we have observed that peripheral inflammatory changes predominate at 4 weeks post-fracture in the rat CRPS

model, but that by 4 months post-fracture the peripheral inflammatory changes resolve and spinal cord inflammatory changes become crucial contributors to chronic pain behaviors.⁶¹ If the mechanisms supporting CRPS evolve over time the results of the current study may not be directly applicable to more chronic CRPS patients. Another concern with the current study is that different bisphosphonates were used for different aspects of the experimental design. Alendronate was used to examine bisphosphonate effects on post-fracture cutaneous inflammatory mediator expression, osteoclastic activity, bone loss, and resorption, while zoledronate was used to examine bisphosphonate effects on post-fracture immunocomplex deposition and popliteal lymphadenopathy. Both of these drugs are nitrogenous bisphosphonates that act on bone metabolism by binding and blocking the enzyme farnesyl diphosphate synthase in the HMG-CoA reductase pathway and both drugs provided effective analgesia in the fracture rat model, but it is a limitation in the study design that all experiments were not replicated using both drugs.

In conclusion, bisphosphonate therapy prevented the development of pain behaviors, osteoclast activation, trabecular bone loss, and cutaneous inflammation in the rat CRPS fracture model. Furthermore, oral zoledronate reversed established pain behaviors in the fracture model. These results support the hypothesis that bisphosphonates can provide effective multimodal treatment in the early stages of post-fracture CRPS.

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Figure 1.

The antinociceptive effects of bisphosphonate treatment in fracture rats. After baseline testing, the right distal tibia was fractured and the hindlimb casted for 4 weeks. Low dose alendronate (3 ug/kg s.c. daily), high dose alendronate (60 ug/kg s.c. daily), zoledronate (3 mg/kg p.o. daily), or vehicle were administered for 4 weeks, starting immediately after fracture. At 4 weeks after fracture, hindpaw mechanical allodynia (A), unweighting (B), warmth (C), and edema (D) were determined. Measurements for (A), (C), and (D) represent the difference between the fracture side and the contralateral paw, thus a negative value represents a decrease in mechanical nociceptive thresholds (A) on the fracture side, while a positive value represents an increase in hindpaw temperature (C) or thickness (D) on the affected side. Measurements for (B) represent weight bearing on the fracture hindlimb as a ratio to 50% of bilateral hindlimb loading, thus a percentage lower than 100% represents hindpaw unweighting. Control; nonfracture control rats (n=10), Fx + Vehicle; vehicle treated fracture rats (n=10), Fx + ALN 3; low dose alendronate (3 ug/kg s.c. daily) treated fracture rats (n=6), Fx + ALN 60; high dose alendronate (60 ug/kg s.c.) treated fracture rats (n=10), Fx + ZOL3; zoledronate (3 mg/kg p.o.) treated fracture rats (n = 8). All data were evaluated using a one-way ANOVA, followed by Holm-Sidak multiple comparison testing to compare between control and fracture rats that were treated with either bisphosphonate or vehicle. **** $P < 0.0001$, *** $P < 0.001$, * $P < 0.05$ vs. Control; and #### $P < 0.0001$, ### $P < 0.001$, # $P < 0.05$ vs. Fx + Vehicle.

Figure 2.

Time course of bisphosphonate antinociceptive effects after fracture. After baseline testing, the right distal tibia was fractured and the hindlimb casted for 4 weeks. Zoledronate (3 mg/kg p.o. daily), or vehicle were administered for 4 weeks, starting immediately after fracture (A-D) or for 3 weeks starting at 4 weeks after fracture (E-H). The time period of drug administration is denoted by the black line above the x-axis of each graph that illustrates the time in weeks after fracture. At 4, 5, 6, and 7 weeks after fracture, hindpaw edema (A), warmth (B), and mechanical allodynia (C) and the hindlimb unweighting (D) were determined. Measurements for (A), (B), and (C) represent the difference between the fracture side and the contralateral paw, thus a positive value represents an increase in thickness or temperature on the fracture side, a negative value represents a decrease in mechanical nociceptive thresholds on the affected side. Measurements for (D) represent weight bearing on the fracture hindlimb as a ratio to 50% of bilateral hindlimb loading, thus a percentage lower than 100% represents hindpaw unweighting. Fx + Vehicle; vehicle treated fracture rats (n=8), Fx + ZOL3; zoledronate (3 mg/kg p.o.) treated fracture rats (n=8). All data were evaluated using a repeated measures two-way ANOVA, followed by Holm-Sidak multiple comparison testing to compare between baseline and post-fracture time point and between vehicle and zoledronate treatment groups at each time point. #### $P < 0.0001$, ### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$ vs. Fx + Vehicle.

Figure 3.

Fracture induced trabecular bone loss was prevented by bisphosphonate treatment. After the right tibia was fractured and the hindlimb casted for 4 weeks the rats were sacrificed and the bilateral distal femurs and L5 vertebrae were collected for ex-vivo uCT scanning. There was extensive trabecular bone loss in the ipsilateral (A) and contralateral (B) distal femurs, as well as in the L5 vertebral body (C) compared to nonfractured controls. Similarly, a post-fracture loss of trabecular bone connectivity was observed in the ipsilateral distal femur (D) and L5 vertebral body (F), compared to controls, but there was no significant post-fracture change in the contralateral distal femur (E). Four weeks of daily alendronate treatment, starting at the time of fracture, prevented post-fracture trabecular bone loss and reduction in connectivity density in the distal femurs and L5 vertebra. BV/TV%; trabecular bone volume, Conn.D; connectivity density, Control; nonfracture control rats (n=10), Fx + Vehicle; vehicle treated fracture rats (n=10), Fx + ALN 3; low dose alendronate (3 ug/kg s.c. daily) treated fracture rats (n=6), Fx + ALN 60; high dose alendronate (60 ug/kg s.c.) treated fracture rats (n=10). All data were evaluated using a one-way ANOVA, followed by Holm-Sidak multiple comparison testing to compare between control and fracture rats that were treated with either bisphosphonate or vehicle. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ vs. Control, and #### $P < 0.0001$, ### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$ vs. Fx + Vehicle.

Figure 4.

Fracture induced osteoclast activation and bone resorption was inhibited by bisphosphonate treatment. After the right tibia was fractured and the hindlimb casted for 4 weeks the rats were sacrificed and the ipsilateral distal femurs and L5 vertebrae were collected for dynamic histomorphometry. There was a post-fracture decrease in trabecular bone formation (BFR) with an increase in osteoclast surface (Oc.S/BS), and bone resorption (ES/BS) in the distal femur (A-C) and in the L5 vertebral body (D-F) compared to nonfractured controls. Four weeks of high dose alendronate (60 ug/kg s.c. daily) treatment, started at the time of fracture, had minimal effect on bone formation rates (A, D), but did inhibit post-fracture increases in osteoclast surface (B, E) and bone resorption (C, F), relative to vehicle treatment. BFR; bone formation rate, Oc.S; osteoclast surface, ES; eroded surface, BS; trabecular bone surface, Control; nonfractured control rats (n = 9), Fx + Vehicle; vehicle treated fracture rats (n = 8), Fx + ALN 60; high dose alendronate (60 ug/kg s.c. daily) treated fracture rats (n = 8). All data were evaluated using a one-way ANOVA, followed by Holm-Sidak multiple comparison testing to compare between control and fracture rats that were treated with either bisphosphonate or vehicle. ** $P < 0.01$, * $P < 0.05$ vs. Control, and ## $P < 0.01$, # $P < 0.05$ vs. Fx + Vehicle.

Figure 5.

Increases in cutaneous inflammatory mediator expression after fracture were inhibited by bisphosphonate treatment. After the right tibia was fractured and the hindlimb casted for 4 weeks the rats were sacrificed and the ipsilateral hindpaw skin was collected for ELISA. Hindpaw skin TNF α (A), IL-1 β (B), IL-6 (C), and NGF (D) levels dramatically increased after fracture. Four weeks of high dose alendronate (60 μ g/kg s.c. daily) treatment, started at the time of fracture, inhibited the post-fracture increases of inflammatory mediators. TNF α ; tumor necrosis factor-alpha, IL-1 β ; interleukin - 1-beta, IL-6; interleukin 6, NGF; nerve growth factor, Control; nonfracture control rats (n=10), Fx + Vehicle (n=10); vehicle treated fracture rats (n=10); Fx + ALN 60, the high dose alendronate (60 μ g/kg s.c. daily) treated fracture rats (n=10). All data were evaluated using a one-way ANOVA, followed by Holm-Sidak multiple comparison testing to compare between control and fracture rats that were treated with either bisphosphonate or vehicle. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ vs. Control, and ### $P < 0.001$, ## $P < 0.01$, vs. Fx + Vehicle.

Figure 6.

Bisphosphonate treatment had no effect on post-fracture immunocomplex deposition in hindlimb skin or sciatic nerve. After the right tibia was fractured and the hindlimb casted for 4 weeks the rats were sacrificed and the ipsilateral hindpaw skin and sciatic nerve were collected for ELISA. Hindpaw skin and sciatic nerve IgM (B), and IgG (C) levels dramatically increased after fracture, but there was no significant fracture effect on albumin (A) levels. Four weeks of zoledronate (3 mg/kg p.o. daily) treatment, started at the time of fracture, had no effect on post-fracture immunocomplex deposition in skin and sciatic nerve. Control; nonfracture control rats (n=6), Fx + Vehicle; vehicle treated fracture rats (n=6), Fx + ZOL 3; zoledronate (3 mg/kg p.o. daily) treated fracture rats (n=6). All data were evaluated using a one-way ANOVA, followed by Holm-Sidak multiple comparison testing to compare between control and fracture rats that were treated with either bisphosphonate or vehicle. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$ vs. Control, and ##### $P < 0.0001$, ## $P < 0.01$ vs. Fx + Vehicle.

Figure 7.

Bisphosphonate treatment had no effect on post-fracture popliteal lymphadenopathy. After the right tibia was fractured and the hindlimb casted for 4 weeks the rats were sacrificed and both ipsilateral (-ipsi) and contralateral (-contra) popliteal lymph nodes were collected and their diameters measured. Four weeks of zoledronate (3 mg/kg p.o. daily) treatment, started at the time of fracture, had no effect on post-fracture lymphadenopathy. Control; nonfracture control rats (n=15), Fx + Vehicle; vehicle treated fracture rats (n=13), Fx + ZOL 3; zoledronate (3 mg/kg p.o. daily) treated fracture rats (n=5). All data were evaluated using a one-way ANOVA, followed by Holm-Sidak multiple comparison testing to compare between control and fracture rats that were treated with either bisphosphonate or vehicle.

**** $P < 0.0001$, *** $P < 0.001$ vs. Control, and #### $P < 0.0001$ vs. Fx + Vehicle.

Table 1

Distal femur and the L5 vertebral cancellous bone evaluated by μ CT

Parameters	n	Tb.N (1/mm)	Tb.Th (μ m)	Tb.Sp (μ m)	Conn.D (1/mm)	SMI (0-3)
<i>Ipsilateral DF</i>						
Control	10	2.2 $\pm$ 0.08	68.5 $\pm$ 1.0	465.2 $\pm$ 15.1	30.0 $\pm$ 2.1	2.2 $\pm$ 0.05
Fx + Vehicle	9	2.0 $\pm$ 0.08 ^c	58.7 $\pm$ 1.6 ^a	521.4 $\pm$ 19.6 ^b	9.9 $\pm$ 2.4 ^a	2.9 $\pm$ 0.10 ^a
Fx + ALN 3	6	2.9 $\pm$ 0.11 ^{a,d}	63.4 $\pm$ 1.6 ^f	356.8 $\pm$ 13.3 ^{a,d}	26.8 $\pm$ 1.2 ^d	2.6 $\pm$ 0.05 ^{b,e}
Fx + ALN 60	10	3.1 $\pm$ 0.08 ^{a,d,i}	67.2 $\pm$ 1.3 ^d	329.4 $\pm$ 9.1 ^{a,d}	51.0 $\pm$ 2.5 ^{a,d,g}	2.2 $\pm$ 0.07 ^{d,h}
<i>Contralateral DF</i>						
Control	10	2.2 $\pm$ 0.06	68.3 $\pm$ 0.8	474.1 $\pm$ 13.0	28.3 $\pm$ 1.6	2.2 $\pm$ 0.04
Fx + Vehicle	9	2.5 $\pm$ 0.08 ^b	63.5 $\pm$ 1.0 ^c	420.2 $\pm$ 14.3 ^b	23.2 $\pm$ 2.2	2.6 $\pm$ 0.06 ^a
Fx + ALN 3	6	3.2 $\pm$ 0.05 ^{a,d}	67.1 $\pm$ 1.3	320.6 $\pm$ 5.5 ^{a,d}	31.2 $\pm$ 2.2 ^f	2.5 $\pm$ 0.06 ^b
Fx + ALN 60	10	3.1 $\pm$ 0.10 ^{a,d}	65.0 $\pm$ 1.7	326.5 $\pm$ 12.8 ^{a,d}	50.8 $\pm$ 1.7 ^{a,d,g}	2.1 $\pm$ 0.07 ^{d,h}
<i>L5 vertebral body</i>						
Control	10	3.9 $\pm$ 0.06	73.8 $\pm$ 1.0	257.3 $\pm$ 4.6	77.6 $\pm$ 4.1	1.5 $\pm$ 0.10
Fx + Vehicle	9	3.8 $\pm$ 0.07	66.8 $\pm$ 2.8	263.8 $\pm$ 4.7	57.7 $\pm$ 6.2 ^b	2.2 $\pm$ 0.16 ^b
Fx + ALN 3	6	4.0 $\pm$ 0.19	73.8 $\pm$ 1.2	246.9 $\pm$ 13.8	88.1 $\pm$ 7.9 ^e	1.4 $\pm$ 0.08 ^f
Fx + ALN 60	10	4.3 $\pm$ 0.04 ^{b,d}	81.7 $\pm$ 2.3 ^d	227.0 $\pm$ 3.0 ^{b,d,i}	91.5 $\pm$ 3.1 ^d	0.9 $\pm$ 0.22 ^{c,d,i}

DF, distal femur; ALN, alendronate; Fx + Vehicle, fracture rats treated with vehicle for 4 weeks; Fx + ALN 3; fracture rats treated with low dose alendronate (3 μ g/kg/day) for 4 weeks; Fx + ALN 60, the fracture rats treated with high dose alendronate (60 μ g/kg/day)for 4 weeks. All data presented as mean $\pm$ SEM.

^a p<0.001

^b p<0.01

^c p<0.05 vs control

^d p<0.001

^e p<0.01

^f p<0.05 vs Fx + Vehicle

$$3 \text{ LTV} + x \text{Fs}1 \text{ 50} > d_j$$

$$10^0 > d_{ij}$$

$$1000 > d_g$$

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