

Published in final edited form as:

Oral Dis. 2011 April ; 17(3): 291–297. doi:10.1111/j.1601-0825.2010.01739.x.

In vivo effects of zoledronic acid on oral mucosal epithelial cells

Eman Allam, B.D.S., M.S., Ph.D.[‡], Matthew R. Allen, Ph.D.[†], Tien-Min Chu, D.D.S., Ph.D.[¶], Ahmed Ghoneima, B.D.S., M.S., Ph.D.[£], and L. Jack Windsor, Ph.D.^{‡,*}

[‡] Department of Oral Biology, Indiana University School of Dentistry, Indianapolis, IN

[†] Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN

[¶] Department of Restorative Dentistry, Indiana University School of Dentistry, Indianapolis, IN

[£] Department of Orthodontics & Oral Facial Genetics, Indiana University School of Dentistry, Indianapolis, IN, USA

Abstract

Objective—Osteonecrosis of the jaw is a serious complication of bisphosphonate treatment for which the pathophysiology is unknown. The purpose of this study was to investigate whether in vivo zoledronic acid (ZA) induces alterations in cell proliferation, apoptosis, and matrix metalloproteinases (MMPs) expression in oral mucosal epithelial cells.

Methods—One year old dogs were either untreated (control group) or given high doses of intravenous ZA (ZA group) for three months. The doses of ZA were equivalent to those given to cancer patients, yet were administered two times more frequently (every 2 weeks). Mucosal tissues were assessed immunohistochemically for cell proliferation (proliferating cell nuclear antigen, PCNA), matrix metalloproteinase (MMP) expression, and apoptosis (caspase 3 and TUNEL).

Results—There were no significant differences between the groups with respect to PCNA, MMP-2, MMP-14, and TUNEL positive cells. However, the expression of MMP-9 was significantly higher in the control group than in the ZA group ($p < 0.05$), while the expression of caspase 3 was significantly lower in the control group than in the ZA group ($p < 0.05$).

Conclusion—These results suggest that high doses of ZA resulted in higher levels of apoptosis and lower levels of MMP-9 in the oral epithelial cells supporting the idea of bisphosphonate treatment affects the oral mucosa.

Keywords

zoledronic acid; oral epithelial cells; osteonecrosis of the jaws

Introduction

Reports of bisphosphonate-related osteonecrosis of the jaw (BRONJ) have raised great concerns among patients and health care professionals. Clinically, BRONJ is an area of exposed bone in the maxilla or mandible that did not heal within 8 weeks after identification by a health care provider in a person who is currently or previously treated with bisphosphonates but not radiation to the head/neck region (Ruggiero, 2007). Most of the

*Corresponding author/contact author L. Jack Windsor, Ph.D. Department of Oral Biology, Indiana University School of Dentistry, 1121 West Michigan Street, DS 271, Indianapolis, IN 46202, Phone: 317-274-1448, Fax: 317-278-1411, ljwindso@iupui.edu.

reported cases have occurred in cancer patients who received intravenous nitrogen-containing bisphosphonates. Sixty percent of the cases occurred after recent dental extraction, trauma, or oral surgery (Woo *et al*, 2006). However, there are also cases in which there was no history of a dental procedure.

Zoledronic acid, a nitrogen-containing bisphosphonate, is a highly potent anti-osteolytic drug with a unique structure. Specifically, there is a second nitrogen atom that may account for its substantially increased *in vitro* and *in vivo* potency compared with other bisphosphonates (Green and Rogers, 2002). In a case-control study, cancer patients who had received zoledronate exhibited a significant 30-fold increase in their risk of developing BRONJ compared to matched cancer patients with no evidence of BRONJ (Wessel *et al*, 2008).⁴

The mechanisms involved in the pathogenesis of BRONJ are not yet understood. Several possible pathogenic mechanisms have been suggested in the literature. These include ischemia, suppression of bone turnover, toxicity to the bone, and infection (Reid, 2009). Most of these proposed mechanisms focus on the origin of the disease from the bone. Although the lesion is almost always associated with loss of the soft tissue covering the maxillary or mandibular bone, the effects of these drugs on oral mucosal cells have not been fully elucidated.

Several *in vitro* studies have previously examined the effects of bisphosphonates on cells of the oral cavity (Landesberg *et al*, 2008; Cornish *et al*, 2009; Scheper *et al*, 2009; Allen *et al*, 2010). Landesberg *et al* (2008) showed that pamidronate inhibits mucosal cell proliferation and that this was not an effect of increased apoptosis of the keratinocytes. Cornish *et al* (2009) used cultures of a human epithelial cell line to address cell growth over normal bone and bone that had been pre-treated with bisphosphonates. They suggested that the presence of bisphosphonates in bone does inhibit epithelial cell growth *in vitro* (Cornish *et al*, 2009). One concern with translating these *in vitro* results to *in vivo* conditions is that it remains unclear whether the concentrations that were used in culture to produce these effects are ever achieved *in vivo*.

Reid⁵ proposed that bisphosphonates in patients receiving long-term high-dose intravenous treatment is accumulated in the bone in concentrations sufficient to be directly toxic to the oral epithelium and this could be a possible explanation of the impaired wound healing and necrosis of the underlying bone. Scheper *et al* (2009) demonstrated that low concentrations of zoledronic acid (ZA) rapidly and directly affected the oral mucosal tissues through induction of a gene-regulated apoptotic process. In their *in vitro* study, they exposed human gingival fibroblast and keratinocyte cell lines to different concentrations of ZA and reported a dose response effect on apoptosis and cell proliferation. After gene expression analysis, they demonstrated the differential expression of multiple genes involved in apoptosis including: *TNF*, *BCL-2*, *CASPASE*, *IAP*, *TRAF*, and *DEATH DOMAIN* families. They stated that these findings support the possibility that soft tissue injury is an event that play a role in the development of osteonecrosis.

Matrix metalloproteinases (MMPs) are proteolytic enzymes that are involved in both injury and repair mechanisms in the oral wounds. These enzymes are also required for the extracellular matrix (ECM) remodeling after tooth extraction (Zecchin *et al*, 2005). During wound healing, degradation of ECM components by the MMPs is required to remove and reorganize provisional matrices and to allow cell migration and reepithelization (Ravanti *et al*, 2000). Human keratinocytes synthesize and secrete MMPs and this expression is required to regenerate the injured tissue (Santoro and Gaudino, 2005). Experimental studies have described the effects of bisphosphonates on MMPs expression in cancer cells (Teronen *et al*,

1999; Heikkil *et al*, 2002; Senaratne and Colston, 2002; Giraudo *et al*, 2004). It was proposed that the beneficial effects of the bisphosphonates on the metastatic process of cancerous lesions is related to the inhibition and down regulation of various MMPs. However, the effects of these drugs on the MMPs from the normal oral mucosal cells have not yet been studied. In addition, since the etiopathogenesis of BRONJ is poorly understood and the soft tissue toxicity is one of the proposed theories, studying the effect of these drugs on proliferation and apoptosis of the oral mucosal cells will provide useful insights into the disease.

Based on these previous studies, it was hypothesized in this study that the oral mucosal epithelial cells would be affected by ZA treatment. Therefore, the aim of this study was to examine the effects of high dose zoledronic acid treatment on the oral mucosal epithelial cells using an *in vivo* experimental animal model. Cellular proliferation, apoptosis, and matrix metalloproteinases expression in the hard palate mucosa were studied using immunohistochemistry.

Materials and methods

Twelve skeletally mature female beagles (~ 1 year old) were housed for the duration of the experiment in environmentally controlled rooms at Indiana University School of Medicine's Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility. All animal procedures were approved prior to the study by the Indiana University School of Medicine Animal Care and Use Committee.

Following two weeks of acclimatization, the animals were assigned to untreated control (CON; n=6) or zoledronic acid (ZA; n=6) treatment groups. ZA (Zometa®) was administered every two weeks via intravenous infusion at a dose of 0.06 mg/kg, which corresponds to the 4 mg dose used in cancer patients as adjusted on a mg/kg basis. Although the infusion frequency was twice that used clinically, it was chosen to maximize drug exposure during the 3 month study duration (Allen *et al*, 2010). For IV administration, animals were sedated and an over-the-needle catheter was inserted into the leg vein (rotated for each infusion between the cephalic and saphenous) with the drug administration in a 40 mL total volume over a 15 minute period. Control animals (CON) were untreated throughout the experimental period. All animals underwent dental extraction of the 4th right premolar on day 30 of the study. One month later, all the animals underwent extraction of the 4th left premolar. Details of the extraction protocol have been published previously (Allen *et al*, 2010). Animals were euthanized by intravenous administration of sodium pentobarbital 3 months after the first treatment and the oral mucosa was dissected from the hard palate and fixed in 10% neutral buffered formalin.

Tissue Preparation and immunohistochemistry

After the oral mucosal tissues were fixed in 10% buffered formalin for 24 hours, they were transferred to 70% ethanol and embedded in paraffin wax. Semi-thin sections (6 µm) were cut and processed for routine histological and subsequent immunohistochemical examinations. Immunohistochemistry was performed by a standard avidin-biotin peroxidase procedure. The sections were deparaffinized with xylene and rehydrated through a series of decreasing percentages of ethanol. Antigen retrieval was performed by treating the sections with 10 mM citrate buffer (pH 6.0) at 95° C for 20 minutes and then returned to room temperature for an additional 20 minutes. Endogenous peroxidase activity was then blocked by 10 minute incubation in hydrogen peroxide (Thermo Fisher Scientific/Labvision, Fremont, CA). The background signal was reduced by incubating the sections for 30 min with diluted blocking serum. Sections were incubated with primary antibody (anti-PCNA,

anti-caspase 3, anti-MMP-2, anti-MMP-9, and anti-MMP-14, table 1) in a humidifying chamber. Sections were then incubated with biotinylated universal secondary antibody (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature and then incubated with the ABC reagent (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB) staining was performed to visualize the antigens in the tissue sections. Finally, the sections were dehydrated with increasing percentages of ethanol and then cleared with xylene. The slides were mounted with Permount (Fisher Scientific, Hanover Park, IL). Tissues from an oral squamous cell carcinoma were utilized as positive controls and sections where the primary antibodies were omitted served as the negative controls. The immunoreactivity of the samples was graded on the basis of the number of positively stained cells. PCNA, caspase-3, MMP-2, MMP-9, and MMP-14 positive cells were counted in 4 randomly selected fields (0.62610484 mm^2) at a magnification of $200\times$. The results of the cell counts were given as means of percentages of positive cells of all the cells counted in a defined field.

TUNEL Assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling TUNEL assay was performed with the commercially available FregEL DNA Fragmentation Detection Kit (Calbiochem, Gibbstown, NJ) according to the manufacturer's instructions. In brief, the paraffin sections were deparaffinized with xylene and then rehydrated through a series of decreasing concentrations of ethanol. Next, the slides were partially digested with proteinase K at room temperature for 20 minutes. Following treatment with 3% H_2O_2 for 5 minutes, the slides were incubated with the terminal deoxyribonucleotidyl transferase (TdT) equilibration buffer for 20 minutes at room temperature. The sections were then incubated for 90 minutes at 37°C with TdT enzyme mixed with the TdT labeling reaction mix. After 90 minutes, the slides were washed with Phosphate-Buffered Saline/Tween (PBS-T) and the reaction was stopped by adding the stop buffer provided by the manufacturer. Non-specific binding was blocked with the blocking reagent provided by the manufacturer at room temperature for 10 minutes. Tagged nucleotides were detected using streptavidin-horseradish peroxidase (HRP) in a humidifying chamber for 30 minutes at room temperature. Sections were then stained with diaminobenzadine (DAB)/ H_2O_2 solution and counterstained with methyl green, dehydrated, mounted, and examined under light microscope. Control slides were incubated under the same conditions by substituting dH_2O for the TdT in the reaction mixture. To determine the percentage of apoptotic cells, TUNEL positive and TUNEL negative cells were counted in 4 randomly selected fields (0.62610484 mm^2) at a magnification of $200\times$. Results were expressed as the number of TUNEL-positive cells/total cells $\times 100\%$.

Statistical Analysis

The data was reported as mean \pm SD. The number of positive cells and the total cells from the four selected fields were added and calculated as % for each animal. Mean values from both groups were compared by Mann-Whitney U test and p values of < 0.05 were considered to be significant. All statistical calculations were done using computer programs Microsoft Excel 2003 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

Results

Qualitative observation showed that all of the specimens consisted of keratinized epithelium with elongated rete pegs overlying the connective tissue layer that showed no signs of inflammatory changes. Immunohistochemical findings and percentage of TUNEL positive

cells for both groups are presented in table 2 and figure 1. All the specimens analyzed were immunopositive for PCNA, MMP-2, MMP-9, MMP-14 and caspase-3. However for TUNEL assay, discrete staining was observed only in 3 cases from the CON group (50%) and 4 cases from the ZA group (67%). All the specimens showed homogenous immunoreactivity for PCNA, MMP-2, MMP-9, and MMP-14 either in the entire thickness of the epithelium or in the basal and suprabasal layers with a tendency to diminish towards the surface. With respect to caspase-3, discrete staining was observed in all specimens (figure 1).

There were no significant differences between the groups with respect to PCNA, MMP-2, MMP-14, and TUNEL positive cells ($p < 0.05$). However, the expression of MMP-9 was significantly higher in the CON group than in the ZA group ($p < 0.05$), while the expression of caspase-3 was significantly lower in CON group than in the ZA group ($p < 0.05$) (figure 1).

Discussion

BRONJ is now considered a major enigma in the dental community and in the field of skeletal biology. Confusion surrounding BRONJ exists for several reasons, including insufficient data about how and why the condition develops (Allen and Burr, 2009). It has been hypothesized that delayed wound healing after tooth extraction and remodeling suppression due to soft tissue toxicity may be one of the suggested underlying pathogenic mechanisms (Reid *et al*, 2007). *In vitro* studies had demonstrated that exposure to different concentrations of bisphosphonates induces toxic effects in many cell types (Hewitt *et al*, 2005; Ishikawa *et al*, 2007; Correia *et al*, 2006). However, the effects of these drugs on oral mucosal epithelial cells *in vivo* have not been previously investigated. In this study, the effect of ZA treatment on several important proteins that are critical for normal oral soft tissues homeostasis was investigated in an attempt to elucidate the role that oral mucosa might play in BRONJ pathogenesis.

Following the process of tooth extraction and during wound healing, epithelial cells detach from the basement membrane and migrate to cover the exposed connective tissue. Subsequently, the wound clot is degraded gradually and replaced by the epithelial cells and the granulation tissue. Both of these processes are likely to be affected by matrix-modifying enzymes such as the MMPs (Salo *et al*, 1994). MMPs form an enzyme family capable of degrading almost all of the constituents of the extracellular matrix and the basement membrane. They regulate multiple cellular functions including cellular growth, apoptosis, angiogenesis, inflammation, and immune responses (Birkedal-Hansen, 1993). MMP-2 and MMP-9 are members of the MMP family known to play an important role in the remodeling of wound extracellular matrix because of their ability to degrade the fibrillar collagens after the initial cleavage by collagenases. These enzymes are also required for the extracellular matrix remodeling after tooth extraction (Pardo and Selman, 2006; Zecchin *et al*, 2005). MMP-14 is a membrane type MMP that is involved in activating proMMP-2 and the degradation of a variety of extracellular matrix molecules (Misung *et al*, 2004). MMP-2 and MMP-9 belong to the gelatinase subfamily of the MMPs. Differences exist in their expression, proenzyme activation and substrate specificities. Despite their largely overlapping functions, they may have some opposing biological activity as illustrated by the finding that MMP-2 promotes platelet aggregation, while MMP-9 inhibits the same process (Fernandez-Patron *et al*, 1999).

MMP-9 is a gelatinase that is normally found in migrating keratinocytes during wound healing (Madlener *et al*, 1998; Lund *et al*, 1999). This enzyme is thought to have multiple roles in wound healing such as contributing to the detachment of keratinocytes from the BM,

promoting migration of the cells over the matrix, and remodeling of the granulation tissue (Salo *et al*, 1994). In addition, MMP-9 can activate inactive TGF- β ligands thus generating a positive loop that sustains keratinocytes migration (Yu and Stamenkovic, 2000). Cellular proliferation required for wound healing was also found to be regulated by MMP-9 mediated cleavages that facilitates the interaction of the mitogenic factors with their cell-surface receptors. MMP-9 also plays a role in the angiogenic response, which was demonstrated in MMP-9 deficient mice (Vu *et al*, 1998). These mice exhibited abnormal growth plate vascularization and ossification. This study demonstrated that the abnormality in the growth plate vascularization in the MMP-9-deficient mice was due to failure to release angiogenic factors from the matrix or alternatively, failure to degrade the angiogenesis inhibitors that is present in the matrix. Relevant to angiogenesis, MMP-9 also cleaves the pro-inflammatory, proangiogenic cytokine IL-8, as well as degrade and inactivate the angiogenesis inhibitor platelet factor-4 (Opdenakker *et al*, 2001).

The data from the present study demonstrated that significant reduction in MMP-9 expression is associated with ZA treatment, while MMP-2 and MMP-14 were non-significantly changed. Given that MMP-9 is required for facilitating the migration of epithelial cells (re-epithelialization) and the subsequent wound closure (Salo *et al*, 1994; Pardo and Selman, 2006; Madlener, 1998), these data suggest that ZA inhibitory effect on MMP-9 expression could play an important part in explaining the failure of wound closure following tooth extraction in patients treated with ZA.

In the present study, the rate of cellular proliferation was determined by detecting PCNA expression. Immunohistochemical localization of PCNA is used as a reliable marker of cells undergoing active proliferation (Paunesku *et al*, 2001). Previous studies demonstrated that bisphosphonates can inhibit epithelial cell proliferation (Landesberg *et al*, 2008; Hejun and Qin, 2009; Oades *et al*, 2003; Fournier *et al*, 2002). However, in the present study no significant difference in PCNA expression was reported between the 2 groups which could possibly be explained by the use of different concentrations of bisphosphonates.

Apoptosis, or programmed cell death, plays a critical role in the regulation of inflammation, wound healing, and the immune response. During this process, a series of coordinated morphological and biochemical events is induced in the affected cells resulting in cell death and subsequent removal by scavenger phagocytes (Cohen, 1991). In the present study, apoptosis was evaluated using 2 different techniques: caspase-3 detection and TUNEL assay. Although all of the specimens showed positive caspase-3 immunoreactivity, some specimens were TUNEL negative which might be explained by the difference in sensitivity between the two methods. The presence of caspase-3 positive cells is considered a hallmark of apoptosis activation (Krajewska *et al*, 1997; Bulut *et al*, 2006). A study compared the sensitivity and reliability of TUNEL method with immunohistochemistry for caspase-3 indicated that caspase-3 immunohistochemistry was more reliable for the early identification and quantification of apoptosis at early stages even before the morphological features of apoptosis occur (Duan *et al*, 2003).

In the present study, a significant percent of the specimens from both groups were TUNEL negative. This finding was consistent with a recent report that in an animal model of Sprague-Dawley rats in which clinical and radiographic features of BRONJ were replicated, while apoptotic cells were identified in bone and marrow cells, none were seen in the epithelium (Sonis *et al*, 2009).

Data from the present study showed a significant increase in caspase-3 expression in the mucosal epithelial cells of ZA treated animals compared to the control animals. These results are consistent with an earlier report of an *in vitro* model using human gingival

fibroblasts and oral mucosal cell lines that demonstrated that ZA directly affected the oral mucosal cells through the induction of a gene-regulated apoptotic process (Scheper *et al*, 2009).

One mechanism by which bisphosphonates induce apoptosis is through the production of ATP analogues (either as direct metabolites or as a result of inhibition of the mevalonate pathway), which can disrupt mitochondrial ATP/ADP translocase (Green, 2004). ZA was reported to induce apoptosis of osteoclasts and tumor cells by activation of caspases (Benford *et al*, 2001, Senaratne *et al*, 2000; Shipman *et al*, 1997; Tassone *et al*, 2003,). A recent study investigating the mechanism by which ZA acid induced apoptosis in human breast cancer cell lines (MDA-MB-231 and MCF-7) indicated that this response was associated with cytochrome c release from the mitochondria and subsequent caspase-3 activation, and that ZA may induce cytochrome c release by modulating expression of Bcl-2, a key antiapoptotic regulatory protein (Senaratne *et al*, 2002).

All animals in this study underwent dental extractions after which, all untreated animals and five of the six ZA-treated animals had uneventful soft tissue healing. One ZA-treated animal did have delayed soft-tissue healing to the point that exposed bone existed for over 3 weeks. A sequestrum eventually formed at this site and thereafter the site appeared normal (Allen *et al*, 2010). Analyses of the socket osseous healing revealed significant delays in the healing of ZA-treated animals compared to untreated animals and this delay was associated with dramatic suppression of bone remodeling (Allen *et al*, 2010). Whether or not the changes in mucosa of the hard palate are similar to those that occurred near the extraction site is unclear.

From the findings of the present study, it was concluded that the short-term high doses of ZA used altered the normal epithelium. It is possible to speculate that increased apoptosis and reduced MMP-9 expression from the oral epithelial cells as a result of ZA treatment may play a role in the delayed soft tissue healing, bone exposure and subsequently development of BRONJ. Further studies are needed to provide more data and further explore the association between the effects of these drugs on the oral soft tissues and the pathogenesis of the bone lesion.

Acknowledgments

This work was supported by grants from the Showalter Foundation and the NIH (R21-DE019686 and S10-RR023710) to M.R. Allen. This investigation utilized an animal facility constructed with support from Research Facilities Improvement Program Grant Number C06RR10601 from the NIH National Center for Research Resources

References

- Allen MR, Burr DB. The Pathogenesis of Bisphosphonate- Related Osteonecrosis of the Jaw: So Many Hypotheses, So Few Data. *J Oral Maxillofac Surg* 2009;67:61–70. [PubMed: 19371816]
- Allen MR, Kubek DJ, Burr DB, Ruggiero SL, Chu TG. Compromised Osseous Healing of Dental Extraction Sites in Zoledronic Acid- Treated Dogs. *Osteoporosis International*. 2010 in press.
- Benford HL, McGowan NWA, Helfrich MH. Visualization of bisphosphonate-induced caspase-3 activity in apoptotic osteoclasts in vitro. *Bone* 2001;28:465–473. [PubMed: 11344045]
- Birkedal-Hansen EL. Role of matrix metalloproteinases in human periodontal diseases. *J Periodontol* 1993;64:474–484. [PubMed: 8315570]
- Bulut S, Uslu1 H, Özdemir B, Bulut O. Expression of caspase-3, p53 and Bcl-2 in generalized aggressive periodontitis. *Head & Face Medicine* 2006;2:17. [PubMed: 16787530]
- Cohen JJ. Programmed cell death in the immune system. *Adv Immunol* 1991;50:55–58. [PubMed: 1950799]

- Cornish J, Bava U, Reid I. Bisphosphonate-treated bone is toxic to epithelial cells-mechanism of ONJ? *Bone* 2009;44:S283–S283.
- Correia VDP, Caldeira CL, Marques MM. Cytotoxicity evaluation of sodium alendronate on cultured human periodontal ligament fibroblasts. *Dental Traumatol* 2006;22:312–7.
- Duan WR, Garner DS, Williams SD, Funckes-Shippy CL, Spath IS, Blomme EA. Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. *J Pathol* 2003;199:221–8. [PubMed: 12533835]
- Fernandez-Patron C, Martinez-Cuesta MA, Salas E, Sawicki G, Wozniak M, Radomski MW, Davidge ST. Differential regulation of platelet aggregation by matrix metalloproteinases-9 and -2. *Thromb Haemost* 1999;82:1730–5. [PubMed: 10613662]
- Fournier P, Boissier S, Filleur S, Guglielmi J, Cabon F, Colombel M, Clézardin P. Bisphosphonates inhibit angiogenesis in vitro and testosterone-stimulated vascular regrowth in the ventral prostate in castrated rats. *Cancer Res* 2002;62:6538–44. [PubMed: 12438248]
- Green JR. Bisphosphonates: Preclinical review. *The Oncologist* 2004;9:3–13. [PubMed: 15459425]
- Green JR, Rogers MJ. Pharmacologic profile of zoledronic acid: a highly potent inhibitor of bone resorption. *Drug Dev Res* 2002;55:210–224.
- Yu, Hejun; Qin, An. Could local delivery of bisphosphonates be a new therapeutic choice for hemangiomas? *Medical Hypothesis* 2009;73:495–497.
- Hewitt RE, Lissina A, Green AE, Slay ES, Price DA, Sewell AK. The bisphosphonate acute phase response: rapid and copious production of proinflammatory cytokines by peripheral blood gamma delta T cells in response to aminobisphosphonates is inhibited by statins. *Clin Exp Immunol* 2005;139:101–11. [PubMed: 15606619]
- Ishikawa C, Matsuda T, Okudaira T, Tomita M, Kawakami H, Tanaka Y, et al. Bisphosphonate incadronate inhibits growth of human T-cell leukaemia virus type I-infected T-cell lines and primary adult T-cell leukaemia cells by interfering with the mevalonate pathway. *Brit J Haematol* 2007;136:424–32. [PubMed: 17233845]
- Krajewska M, Wang HG, Krajewski S, Zapata JM, Shabaik A, Gascoyne R, Reed JC. Immunohistochemical analysis of in vivo patterns of expression of CPP32 (caspase-3), a cell death protease. *Cancer Res* 1997;57:1605–1613. [PubMed: 9108467]
- Landesberg R, Cozin M, Cremers S, Woo V, Kousteni S, Sinha S, Garrett-Sinha L, Raghavan S. Inhibition of Oral Mucosal Cell Wound Healing by Bisphosphonates. *J Oral Maxillofac Surg* 2008;66:839–847. [PubMed: 18423269]
- Madlener M. Differential expression of matrix metalloproteinases and their physiological inhibitors in acute murine skin wounds. *Arch Dermatol Res* 1998;290:S24–29. [PubMed: 9710380]
- Misung, Jo; Thomas, Lauren E.; Wheeler, Sarah E.; Curry, Thomas E, Jr. Membrane type 1-matrix metalloproteinase (MMP)-associated MMP-2 Activation Increases in the Rat Ovary in Response to an ovulatory dose of human chorionic gonadotropin. *Biology of Reproduction* 2004;70:1024–1032. [PubMed: 14668206]
- Oades GM, Senaratne SG, Clarke IA, Kirby RS, Colston KW. Nitrogen containing bisphosphonates induce apoptosis and inhibit the mevalonate pathway, impairing Ras membrane localization in prostate cancer cells. *J Urol* 2003;170:246–52. [PubMed: 12796698]
- Pardo A, Selman M. Matrix Metalloproteinases in aberrant fibrotic tissue remodeling. *Proc Am Thorac Soc* 2006;3:383–388. [PubMed: 16738205]
- Paunesku T, Mittal S, Protic M, Oryhon J, Korolev SV, Joachimiak A, Woloschak GE. Proliferating cell nuclear antigen (PCNA): ringmaster of the genome. *Int J Radiat Biol* 2001;77:1007–1021. [PubMed: 11682006]
- Reid IR. Osteonecrosis of the jaw - Who gets it, and why? *Bone* 2009;44:4–10. [PubMed: 18948230]
- Reid IR, Bolland MJ, Grey AB. Is bisphosphonate-associated osteonecrosis of the jaw caused by soft tissue toxicity? *Bone* 2007;41:318–20. [PubMed: 17572168]
- Ruggiero SL. Guidelines for the diagnosis of bisphosphonate-related osteonecrosis of the jaw (BRONJ). *Clin Cases Miner Bone Metab* 2007;4:37–42.

- Salo T, Mäkelä M, Kylmäniemi M, Autio-Harminen H, Larjava H. Expression of matrix metalloproteinase-2 and -9 during early human wound healing. *Lab Invest* 1994;70:176–82. [PubMed: 8139259]
- Scheper MA, Badros A, Chaisuparat R, Cullen KJ, Meiller TF. Effect of zoledronic acid on oral fibroblasts and epithelial cells: a potential mechanism of bisphosphonate-associated osteonecrosis. *Br J Haematol* 2009;144:667–76. [PubMed: 19036117]
- Senaratne SG, Mansi JL, Colston KW. The bisphosphonate zoledronic acid impairs Ras membrane localisation and induces cytochrome c release in breast cancer cells. *Br J Cancer* 2002;86:1479–1486. [PubMed: 11986784]
- Senaratne SG, Pirianov G, Mansi JL. Bisphosphonates induce apoptosis in human breast cancer cell lines. *Br J Cancer* 2000;82:1459–1468. [PubMed: 10780527]
- Shipman CM, Rogers MJ, Apperley JF. Bisphosphonates induce apoptosis in human myeloma cell lines: a novel antitumour activity. *Br J Haematol* 1997;98:665–672. [PubMed: 9332325]
- Sonis ST, Watkins BA, Lyng GD, Lerman MA, Anderson KC. Bony changes in the jaws of rats treated with zoledronic acid and dexamethasone before dental extractions mimic bisphosphonate-related osteonecrosis in cancer patients. *Oral Oncol* 2009;45:164–72. [PubMed: 18715819]
- Tassone P, Tagliaferri P, Viscomi C. Zoledronic acid induces antiproliferative and apoptotic effects in human pancreatic cancer cells in vitro. *Br J Cancer* 2003;88:1971–1978. [PubMed: 12799645]
- Wessel JH, Dodson TB, Zavras AI. Zoledronate, Smoking, and Obesity Are Strong Risk Factors for Osteonecrosis of the Jaw: A Case-Control Study. *J Oral Maxillofac Surg* 2008;66:625–631. [PubMed: 18355585]
- Woo SB, Hellstein JW, Kalmar JR. Systematic Review: Bisphosphonates and Osteonecrosis of the Jaws. *Ann Intern Med* 2006;144:753–761. [PubMed: 16702591]
- Zecchin KG, Pereira MC, Coletta RD, Graner E, Jorge J. Ovariectomy reduces the gelatinolytic activity and expression of matrix metalloproteinases and collagen in rat molar extraction wounds. *Calcif Tissue Int* 2005;76:136–145. [PubMed: 15549640]

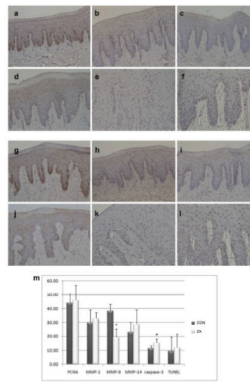


Figure 1.

Immunohistochemistry analyses of untreated (a–f) and ZA treated (g–l) oral tissues stained with PCNA (a and g), MMP-2 (b and h), MMP-9 (c and i), MMP-14 (d and j), caspase-3 (e and k) and TUNEL assay (f and l). ZA decreased the number of MMP-9 positive stained oral epithelial cells and increased the number of caspase-3 positive stained oral epithelial cells. Magnification, $\times 20$ (a–d and g–j) and $\times 40$ (e–f and k–l). m): Comparison of PCNA, MMP-2, MMP-9, MMP-14, caspase-3, and TUNEL labeling in untreated (dark bars), and ZA treated (white bars) specimens. Significant differences in the percent of MMP-9 and caspase-3 stained cells were detected ($p < 0.05$). Data are mean percent of positive cells \pm SD.

Table 1

Antibodies Used for Immunohistochemistry

Antibody (Clone)	Source	Dilution/Incubation time
PCNA (PC 10)	Sigma Chemical Co, St Louis, MO	1:3000 overnight at 4°C
Caspase-3 (E87)	Abcam, Cambridge, MA	1:50 overnight at 4°C
MMP-2 (Ab-4)	NeoMarkers, Fremont, CA	5µg/ml 2hr at 23°C
MMP-9 (Ab-5)	NeoMarkers, Fremont, CA	1µg/ml overnight at 4°C
MMP-14 (AB8345)	Millipore, Billerica, MA	5µg/ml 1hr at 23°C

Table 2

Expression of PCNA, MMP-2, MMP-9, MMP-14, caspase-3, and percentage of TUNEL positive cells

	PCNA	Mean values of immunoeexpression				Percentage of TUNEL positive cells
		MMP-2	MMP-9	MMP-14	Caspase-3	
CON	44.00 ± 7.18	30.00 ± 9.03	38.17 ± 4.71	23.00 ± 7.27	11.33 ± 1.86	9.33 ± 10.27
ZA	46.17 ± 10.65	33.17 ± 4.17	19.33 ± 5.79	28.83 ± 10.26	15.33 ± 2.94	11.83 ± 9.70
P value	0.81	0.68	0.004*	0.262	0.024*	0.803