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ORIGINAL ARTICLE

In vivo effects of zoledronic acid on oral mucosal epithelial cells

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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 3 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 ($P < 0.05$), whereas 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.

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Keywords: zoledronic acid; oral epithelial cells; osteonecrosis of the jaws

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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 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 with matched cancer patients with no evidence of BRONJ (Wessel *et al*, 2008).

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). Landsberg *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 pretreated 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*.

Reid *et al* (2007) 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 plays 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 re-epithelization (Ravanti and Kahari, 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 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 pathophysiology 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 2 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 2 weeks via intravenous infusion at a dose of 0.06 mg kg^{-1}, which corresponds to the 4 mg dose used in cancer patients as adjusted on a mg kg^{-1} 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 i.v. 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-min period. Control animals (CON) were untreated throughout the experimental period. All animals underwent dental extraction of the fourth right premolar on day 30 of the study. One month later, all the animals underwent extraction of the fourth 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 h, 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 min and then returned to room temperature for an additional 20 min. Endogenous peroxidase activity was then blocked by 10-min incubation in hydrogen peroxide (Thermo Fisher Scientific/Labvision, Fremont, CA, USA). The background signal was reduced by incubating the sections for 30 min with diluted blocking serum.

Table 1 Antibodies used for immunohistochemistry

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

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, USA) for 30 min at room temperature and then incubated with the ABC reagent (Vectastain Elite ABC kit). 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, USA). 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 four randomly selected fields (0.62610484 mm^2) at a magnification of 200 ×. 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, USA) 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 min. Following treatment with 3% H_2O_2 for 5 min, the slides were incubated with the terminal deoxyribonucleotidyl transferase (TdT) equilibration buffer for 20 min at room temperature. The sections were then incubated for 90 min at 37°C with TdT enzyme mixed with the TdT labeling reaction mix. After 90 min, 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 min. Tagged nucleotides were detected using streptavidin–horseradish peroxidase (HRP) in a humidifying chamber for 30 min 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 four randomly selected fields (0.62610484 mm^2) at a magnification of 200 × . Results were expressed as the number of TUNEL-positive cells/total cells×100%.

Statistical analysis

The data were reported as mean ± s.d. 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, New York, 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

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

	Mean values of immunoexpression					
	PCNA	*MMP-2*	*MMP-9*	*MMP-14*	*Caspase-3*	*Percentage of TUNEL positive cells*
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

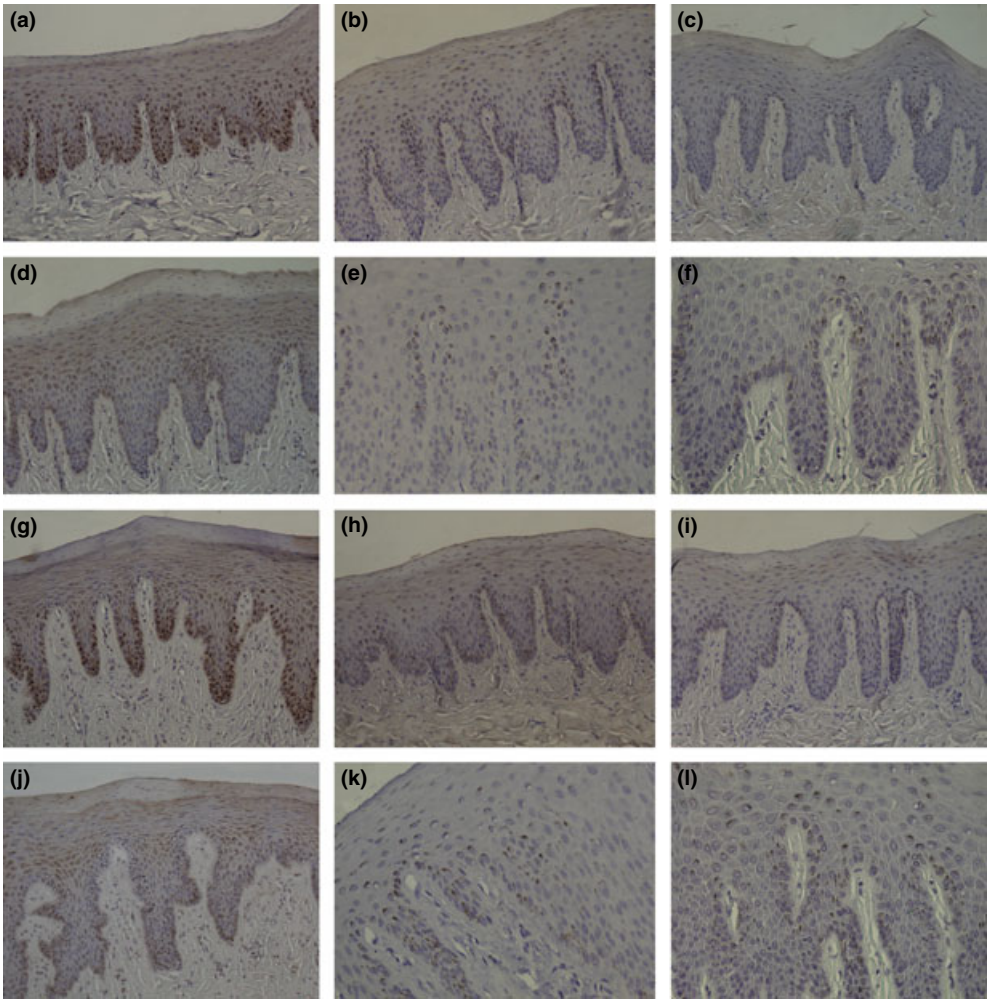

Figure 1 Immunohistochemistry analyses of untreated (**a–f**) and ZA treated (**g–l**) oral tissues stained with PCNA (**a** and **g**), MMP-2 (**b** and **h**), MMP-9 (**c** and **i**), MMP-14 (**d** and **j**), caspase-3 (**e** and **k**), and TUNEL assay (**f** and **l**). ZA decreased the number of MMP-9 positive stained oral epithelial cells and increased the number of caspase-3 positive stained oral epithelial cells. Magnification, 20 × (**a–d** and **g–j**) and 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 ± s.d

caspase-3. However for TUNEL assay, discrete staining was observed only in three cases from the CON group (50%) and four 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; Correia *et al*, 2006; Ishikawa *et al*, 2007). 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 ECM 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 ECM because of their ability to degrade the fibrillar collagens after the initial cleavage by collagenases. These enzymes are also required for the ECM remodeling after tooth extraction (Zecchin *et al*, 2005; Pardo and Selman, 2006). MMP-14 is a membrane type MMP that is involved in activating proMMP-2 and the degradation of a variety of ECM 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 specifities. 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 (Salo *et al*, 1994; Madlener, 1998). 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, which is required for wound healing, is also regulated by MMP-9 mediated cleavage, which in turn facilitates the interaction of mitogenic factors with their cell-surface receptors. MMP-9 also plays a role in the angiogenic response, as demonstrated in MMP-9-deficient mice (Vu *et al*, 1998) which have abnormal growth plate vascularization and ossification. This study suggested that the abnormality in the growth plate vascularization in the MMP-9-deficient mice was due to either a failure to release angiogenic factors from the matrix or failure to degrade the angiogenesis inhibitors that are 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 are associated with ZA treatment, whereas 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; Madlener, 1998; Pardo and Selman, 2006), 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 this 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 (Fournier *et al*, 2002; Oades *et al*, 2003; Landesberg *et al*, 2008; Hejun and Qin, 2009). However, in this study no significant difference in PCNA expression was reported between the two 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 this study, apoptosis was evaluated using two 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 this 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 with 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 (Shipman *et al*, 1997; Senaratne *et al*, 2000; Benford *et al*, 2001; 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 with 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 this 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.

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