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Resolution of pathological inflammatory condition before tooth extraction ameliorates ONJ development in mice

A thesis submitted in partial satisfaction of the requirement for the degree Master of Science in Oral Biology

by

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ABSTRACT OF THE THESIS

Resolution of pathological inflammatory condition before tooth extraction ameliorates ONJ development

by

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Master of Science in Oral Biology
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Dental-related trauma such as tooth extraction is one of the major risk factors significantly associated with development of medication-related osteonecrosis of the jaw (ONJ). Tooth extraction is usually performed due to underlying pre-existing pathological inflammatory conditions such as periodontitis but it remains largely unknown whether pathological inflammatory conditions exacerbate ONJ development following tooth extraction, or resolving pathological inflammatory condition before tooth extraction ameliorates ONJ development. The aim of the study is to determine the role of pathological inflammatory conditions in ONJ development induced by tooth extraction in mice. ZOL (Zometa, 125 μg/kg; Novartis Oncology, East Hanover, NJ, USA) or vehicle (veh) solution (0.9% NaCl saline) was i.v. administered bi-weekly throughout the experiment (n = 5-10 per group). One week after ZOL-administration, pathological inflammatory conditions were induced by placing ligature (6-0) on the second maxillary molar for 3 weeks. To resolve ligature-induced inflammation, the ligatures were removed after 3 weeks and allowed to heal for 2 weeks following tooth extraction. After 3
weeks, maxillae were harvested to analyze for the microCT and bone loss as measured from the cement-enamel junction to alveolar bone ridge. Bone necrosis was measured from H&E stained slides by measuring empty lacunae and necrotic bone. Palatal tissues were subjected to qRT-PCR for expression of IL-1B, IL-6, and IL-17. The sectioned slides were stained for tartrate-resistant acid phosphatase (TRAP)+ osteoclasts. Extraction of the tooth with ligature caused increased numbers of empty lacunae and percentages of necrotic bone when compared to that of without ligature. When ligature was removed and allowed to heal before tooth extraction, bone exposure, numbers of empty lacunae, and necrotic bone percentages were all reduced when compared to the unremoved group. These results signify that pathological inflammatory conditions exacerbate ONJ lesions following tooth extraction, and resolution of these conditions before extraction ameliorates ONJ development.
The thesis of Terresa Kim is approved.

Mo Kang
Yeumin Hong
Reuben Han-Kyu Kim, Committee Chair

University of California, Los Angeles
2016
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1. Introduction

Medication-related osteonecrosis of the jaw (MRONJ) is an oral malady first reported in 2003 (Marx, 2003) and since has increased in occurrence. MRONJ is characterized in patients by presence of exposed bone in the maxillofacial region which does not heal within 8 weeks after identification by a health care provider, exposure to an antiresorptive agent such as nitrogen containing bisphosphonates (BP), or receptor activator of nuclear factor kappa-B ligand (RANKL) specific antibody denosumab, and no history of radiation therapy to the craniofacial region (Khan, 2015). Most notable risk factors involved are traumatic dental surgery such as tooth extraction and pre-existing inflammatory dental diseases, including periodontal diseases and periapical diseases (Thumbiegere-Math, 2014). MRONJ occurs roughly 6-10% in cancer patients receiving high doses of intravenous BP treatment for multiple myeloma or breast cancer, especially with the use of zoledronic acid (ZOL) or pamidronate in high doses (Aguirre, 2012; Khan, 2015).

Bisphophonates prevent the loss of bone mass and is frequently used in cancer and osteoporosis treatments. Of the two types of BP, nitrogen contain BP (e.g zoledronic acid, pamidronate, alendronate) is taken up by osteoclasts from the bone surface and blocks the enzyme farnesyl disphosphoate synthase (FPPS) in the mevalonate pathway (Drake, 2008). The binding of BP at the FPPS level inhibits the post-translational modification of number of proteins that regulate osteoclast cellular activity, which ultimately leads to osteoclast apoptosis (Drake, 2008). Due to its pyrophosphate-like structure, 80% of intravenously administered BP will deposit to the bone with high affinity. Along with its strong adhesion to bone and poor metabolism by many enzymes, BP is also known to have half-life up to 10 years in the enriched bone (Ikebe, 2013). Denosumab, on the other hand, is a fully human monoclonal antibody
specific for RANKL that inhibits the formation, activation, and survival of the osteoclasts (Lipton, 2011). In many patients, denosumab has shown to be superior than zoledronic acid in preventing skeletal-related events but its incorporation and long-term effects on bone remodeling as well as its half-life has not been fully explored (Lipton, 2012; Lewiecki, 2009). Despite its promising efficacy, it has been shown that denosumab also contributes to development of ONJ (Aghaloo, 2010).

Bone remodeling is a tightly orchestrated healing of old or damaged bone by osteocytes, osteoclast and osteoblast. It is suggested that when the osteocytes sense damaged bone, osteocytes recruit osteoclast progenitors to the site of damage and with exposure to M-CSF and RANKL mature into multinucleated osteoclasts. The osteoclasts performing bone resorption send out signals to recruit osteoblast progenitors for the synthesis of new bone (Feng, 2011). With the use of BP or denosumab, bone remodeling procedure is greatly inhibited, leading to difficulty following traumatic surgeries in the jaw.

Tooth extraction is a major risk of ONJ development. Previous clinical data shows that high occurrence of ONJ was followed by tooth extraction in patients taking BP (Marx, 2003; Ficarro, 2005). The jaws are unique amongst the skeletal structure in that they are the only bones exposed to an external environment via the teeth, and frequently exposed to inflammation which require increased demand for bone turnover (Marx, 2003). While the etiological role of BP still remains to be elucidated, the alteration in bone metabolism paired with traumatic surgical procedures such as tooth extraction seems to play a key role in development of ONJ (Ficarra, 2005).

Previously, we have established the mouse model for MRONJ and showed that tooth extraction alone can induces BRONJ and also DRONJ. Higher occurrence of ONJ in both BP
and denosumab injected mice paired with tooth extraction was shown to be not only on the inhibition of osteoclasts’ bone resorptive function, but also related to the disrupted orchestration of appositional bone forming and *de novo* bone formation (Williams, 2014). It is speculated that anti-resorptive agents interrupt appositional bone formation and instead leave bone remodeling solely on woven bone formation. It was also suggested from this study that the inability to remove bacteria-infected bone by osteoclasts may have also lead to necrosis, leading to the absence of woven bone, and ultimately, MRONJ (Williams, 2014).

Clinically, pre-existing inflammatory lesions are always present in patients preceeding tooth extractions and related MRONJ. Case studies have shown that high number of oncological patients taking BP with active periodontal conditions, followed by extraction of the unrestorable teeth, frequently developed BRONJ (Thunbigere-Math, 2013). Tooth extractions is generally performed when the deteriorating condition brought on by pre-existing inflammation can no longer sustain the integrity of the tooth and bone structure. However, clinical studies have shown that when the pre-existing inflammatory lesions were treated prior to the start of BP regiment, the incidents of ONJ decreased (Dimopoulos, 2009). Nevertheless, undiagnosed inflammatory conditions can surface following BP administration rather that prior, leading to more debilitating development of ONJ.

Currently there is no animal model that recapitulate such clinical observations. Previous studies have established a pathological periodontitis mouse model with ligature placement on mice molars (Abe, 2013). In addition, MRONJ mice model has also been established previously showing high doses of BP or denosumab paired with tooth extraction alone can cause ONJ like lesions (Williams, 2014). Here, we developed a mouse model that connect ligature-induced
periodontitis model and extraction model to test a hypothesis that pre-existing inflammatory lesions exacerbates ONJ lesions.

2. Materials and Methods

2.1. Animals

6 week old female C57BL/6J mice were purchased from Jackson Laboratories, and housed in the vivarium at University of California, Los Angeles, Division of Laboratory Animal Medicine. All experimental protocols were approved by institutional guidelines from the Chancellor’s Animal Research Committee (#2011-062).

2.2. Ligature induced periodontitis model and tooth extraction model in mice

In the ligature induced periodontitis and extraction model, twenty 6 week old female mice were injected 0.9% NaCl saline as control (Veh; n=10) or zoledronic acid (ZOL, Zometa, 125 μg/kg; Novartis Oncology, East Hanover, NJ, USA) (n=10) intravenously through the tail vein one week prior to placement of suture on the second maxillary molar. The ligatured molars were extracted on the 4th week under anesthetics ketamine/xylazine. The mice were injected bi-weekly with saline or zoledronic acid every 3.5 days through the duration of 7 weeks at which the mice were euthanized and maxillae were harvested for analyses.

In the inflammation resolution model, twenty 6 week old female C57BL/6J were injected with saline (Veh; n=10) or ZOL (n=10) on week 0, and ligature placed in the following week. Ligatures from five ZOL group mice and five mice from the Veh group were removed after 3 weeks. The mice were continuously injected every 3.5 days for the duration of 6 weeks. The mice were euthanized and maxillae were harvested on the 6th week.
In the combined inflammation resolution and tooth extraction model, forty 6 week old female C57BL/6J mice were injected with saline (Veh; n=20) or ZOL (n=20) through the tail vein starting on week 0 every 3.5 days. The ligatures were placed on second maxillary molars of all mice at week 1. The ligatures were removed from half of each group (Veh, n=10; ZOL, n=10) and allowed healing for 2 weeks. Ligatured and ligature-removed molars from the maxillae were removed at week 6, and mice were allowed 3 weeks to heal or to develop ONJ-like lesions. All mice were euthanized and maxillae were harvested on week 9.

2.3. Tissue Harvest and Embedding

Maxillary tissues were harvested and fixed in 4% para-formaldehyde/1x PBS solution overnight. The tissues were washed in 1x PBS and stored in 70% ethanol the following day. With completion of the μCT scan, the maxillae were decalcified in 5% EDTA/4% sucrose solution changed daily for two weeks. The tissues were trimmed, then embedded in paraffin through the UCLA Translational Procurement Core Laboratory (TPCL). The paraffin blocks were cut at 4μm thickness, with 20 consecutive slices each for each block.

2.4. MicroCT Scan

Prior to embedding, the fixed maxillae were scanned in Scanco uCT 40 machine at voxel size of 20 μm³ and a 0.5 mm Aluminum filter at 55 kVp and 145 μA with an integration time of 200 ms using a cylindrical tube (FOV/Diameter: 20.48 mm). Maxillary tissues were reconstructed and analyzed via CTan atnd CTvol program to generate 3-D images and cross-section images. Bone loss was quantified by measuring the distance between cementoenamal junction (CEJ) to the alveolar ridge on palatal and buccal roots of the first molar and second molars in extraction model and non-extraction periodontitis model, respectively, on Dataviewer.
2.5. **H&E Staining**

The embedded tissues were sectioned at mesiodistal plane at 4 micron thickness, with 20 sections from each tissue sample. The sectioned slides numbered 1, 6, 11, and 16 were de-paraffinized in 60°C, then rehydrated in ethanol with increasing concentration of water. The rehydrated tissue slides were stained with hematoxylin for 2.5 minutes, washed with water and 95% ethanol, then stained with eosin for 1 minute. The stained slides were dehydrated in 70%, 95%, and 100% ethanol, followed by xylene. The slides were mounted using mounting medium (Permount, Fisher Scientific). Bone area quantification was measured using ImageJ software version 1.48 (NIH) on digital pictures taken through Olympus microscope (model DP72; Olympus) at 100x magnification.

2.6. **Tartrate – Resistant Acid Phosphatase (TRAP Stain)**

Osteoclast population was quantified using Acid Phosphatase, Leukocyte (TRAP) kit (Sigma-Aldrich). The tissue section slides numbered 2, 7, and 12 were incubated with TRAP solution in a humidification chamber at 37°C in the dark for 1 hour. The slides were washed in water and counterstained in hematoxylin then mounted with ImmunoHistoMount (Sigma-Aldrich). Osteoclasts were identified by staining and presence of multiple nuclei (n>5). Osteoclasts number quantification and surface area were measured using ImageJ software version 1.48 (NIH) on digital pictures taken through Olympus microscope (model DP72; Olympus) at 100x magnification.

2.7. **Statistical Analysis**

One-way analysis of variance (ANOVA) and Tukey’s post hoc test were used to compare the number of osteoclast, empty lacunae, and necrotic bone (%) among four groups. All of the
statistical analyses were performed with SPSS version 19.0 software (IBM Corp) with a significance level of 0.05.

2.8. **Real Time Quantitative PCR (PR-qPCR)**

Palatal tissues for mRNA extraction was harvested from the immediate areas near the ligature site on the second maxillary molars. Tissues were immediately prepared for messenger RNA (mRNA) isolation. The harvested tissues were treated with 1ml of TRIzol® Reagent (ThermoFisher Scientific) according to manufacturer’s protocol. The quality of isolated mRNA was assessed using NanoDrop Spectrophotometer (ThermoFisher Scientific). Complementary DNA (cDNA) was synthesized from 2.5 μg of total RNA extracted using SuperScript First-Strand Synthesis system (Invitrogen) and Random Primer (Invitrogen). 2.5 μl cDNA was amplified using SYBR Green I Master Mix (Roche Applied Sciences) with the LightCycler 480 II real-time PCR system with primers for IL-1β, IL-6, and IL-17, and GAPDH was used as internal control. The cDNA were loaded in triplicates in LightCycler 96 well plates (Roche). Second derivatives Cq values of the genes and GAPDH were compared to assess the fold-differences of amplification following the manufacturer's instruction (Roche).

3. **Results**

3.1. **Pre-existing inflammation exacerbate BRONJ lesions following tooth extraction in mice**

Previous studies have shown that with a placement of ligatures around the second molars of the maxilla was sufficient enough to induce periodontitis in mice. With this knowledge, we investigated whether tooth extracted under periodontal inflammatory condition could induce ONJ, and if the development of ONJ would be exacerbated in BP treated mice. To stimulate
periodontitis in mice, ligatures were placed around the second molar on the first week, and was followed by extraction in week 4 (Fig. 1A). When teeth were extracted after ligature placement in control group mice injected with saline (Veh), there was significantly higher bone loss at the site of the extraction compared to those without initial ligature placement (Fig. 1B). The severity of the bone loss was measured from the CEJ of the distal root of the first molar to the solid mass of the alveolar ridge immediate to the root apex. In the ZOL group, the severity of the bone loss in both the ligature and the unligated mice was noticeably less than that of the Veh group, which indicated the effects of zoledronic acid on the inhibition of osteoclast function (Fig 1C).

However, the amount of bone loss in the ZOL mice was nevertheless much higher in those with ligature placement.

The maxillae were sectioned at mesiodistal plane to observe the bone loss at the histological level. As presence of large number of empty lacunae localized in bone signifies as necrotic bone, the bone area and empty lacunae were quantified on the H&E stained slides 1, 6, 11 from total of 20 consecutive cuts. The number of empty lacunae extremely low in Veh mice maxillae and sporadically located around the site of extraction for both ligated and unligated (Fig. 1D, top row). However the presence of empty lacunae in the extraction site for ZOL mice was much higher, compared to the control Veh mice. The number of empty lacunae was significantly higher in the ZOL mice with ligatured tooth compared to that of the Veh mice (Fig. 1D – E). The area where high number of empty lacunae were present were determined as necrotic area and the percentage against the total area was calculated (Fig. 1F). The percent of necrotic area for the ZOL maxillae was notably higher compared to the Veh maxillae. Of the ZOL mice, the percentage of necrotic area on ligatured maxillae were also significantly higher compared to the unligatured maxillae, indicating development of BRONJ in the ZOL mice following dental
trauma of tooth extraction. With the increased severity of the symptoms of BRONJ in ZOL group with ligature induced inflammation, we concluded that pre-existing inflammation induced by ligature placement exacerbates the development of ONJ in mice following tooth extraction.

3.2. Removal of ligature ameliorate inflammation and bone loss

Since it was shown that chronic inflammation aggravated ONJ development, we wanted to investigate if the condition can be ameliorated. However, we first examined if ligature – induced periodontitis could be reduced if the source of inflammatory condition is removed. To simulate the amelioration of the inflammatory conditions, we removed the ligature from the mice (Veh; n= 5, ZOL; n=5) 3 weeks following its placement (Fig. 2A). When the maxillary tissues were removed from the mice, the typical signs of inflammation such as soft tissue edema was readily observed from the mice when ligature was left in compared to the mice with the ligature taken out in both Veh and Zol group (Fig. 2B). Inflammation induced bone loss was measured from the CEJ of the second molar from the distal buccal root to the alveolar ridge, and also from the proximal root in the μCT scans of the maxillae (Fig. 2C – E, Left). Veh maxillae showed significant bone loss when the ligature was left placed on the second molar, but had reduction of bone loss when the ligature was removed (Fig. 1D and E). ZOL mice showed significantly less bone loss in the alveolar ridge overall, in observance of inhibition of osteoclastic activity by ZOL. However, there was notable decrease in bone loss in the ZOL group when the ligature was taken out (Fig. 2C-E). At the histological level the extraction site showed reduced bone loss in both Veh and ZOL ligatured removed maxillae, with ZOL group showing noticeable decrease in bone resorption (Fig. 2F).

To further confirm that the bone loss was due to inflammation, presence of osteoclasts and level of inflammatory cytokines were measured. Maxillae sections 2, 7, and 12 were stained
for osteoclasts using TRAP staining. Despite the lack of bone resorption in the ZOL mice maxillae, there were higher number of osteoclasts present in both the ZOL ligature and unligatured mice (Fig. 3A). Though the relationship is unknown, high count of osteoclast numbers has shown to be in correlation to inflamed tissues. To supplement the osteoclast count, isolated mRNA levels for inflammatory cytokines IL-1β, IL-6, and IL-17 quantified through RT-qPCR. The palatal tissues from both the Veh and ZOL mice were harvested and mRNA was extracted. The level of IL-1β, IL-6, and IL-17 were greater for the ligatured mice maxillae for both Veh and ZOL group compared to the ligature removed mice, but the level of these cytokines were significantly higher in the ZOL ligatured group compared to the other mice (Fig. 3C – D). This indicated that ligature placement combined with ZOL injection greatly increases inflammation in mice, and when removed, ameliorates the inflammatory conditions in both Veh and ZOL injected mice.

3.3. **Removal of ligature ameliorates ligature – and extraction-induced BRONJ lesions in mice**

Since the preceding experiments showed that removal of ligature can mollify inflammatory condition, and pre-existing inflammatory condition indeed exacerbated ONJ lesions in mice following extraction, we investigated whether removal of pre-inflammatory condition could resolve the development of ONJ in mice following extraction. The schematics for drug administration and surgical procedures were modified to include both the ligature placement and removal as well as tooth extraction to combine the resolution of the inflammatory conditions (Fig. 4A). The ligatures were removed from half of the mice population in each group (Veh, n=10, ZOL, n=10) and mice were allowed to heal from inflammatory conditions for two weeks before the tooth were extracted. The maxillae were harvested from all mice at week 9. The
isolated maxillae displayed ONJ-like lesions in its soft tissues around the extraction site with exposed bone in the ZOL groups (Fig. 4B). ZOL ligatured mice (Lig-in) had upwards to 40% of bone exposure compared to 14% of the ZOL ligature removed (Lig-out) group, indicating decrease in occurrence of ONJ lesions (Fig. 4C). μCT scan of the maxillae revealed that the structure of the alveolar ridges of the Veh lig-in mice had severe bone loss in comparison to the Veh lig-out and both ZOL mice groups (Fig. 4D). The visual comparison of the ZOL lig-in and ZOL lig-out μCT scans showed noticeable decrease in bone structure damage in the ZOL lig-out maxillae (Fig. 4D, right column). To quantitate the severity of bone loss, the distance between the CEJ of the first maxillary molars to the alveolar ridge surrounding the distal buccal (DB) and distal palatal (DP) root apices were measured. Veh lig-in showed greater amount of bone loss measurement between the CEJ and the alveolar ridge on both DB and DP root which (Fig. 4 E and F), which decreased in the ligature removed Veh mice. The same pattern was observed in the ZOL mice maxillae, though the amount of bone loss itself was less than that of the Veh group.

Histological analysis on the maxillae show that necrosis was only present in the ZOL mice. The necrotic area was again identified by the presence of empty lacunae (Fig. 5A), and large areas of ZOL maxillae bone showed increased number of empty lacunae, which decreased significantly in the lig-out mice (Fig. 5B). When quantified, the areas of necrotic bone were noticeably high in ZOL lig-in mice, which then decreased drastically in the ZOL lig-out mice maxillae (Fig. 5C). Taken together, these data strongly suggests that removal of ligature ameliorates ligature and extraction-induced BRONJ lesions in mice.

4. Discussion

In this study, it was reinstated that pre-existing pathological inflammation was highly likely to lead to BRONJ development in mice following extractions. Furthermore, ligature
induced periodontal inflammation alone was sufficient in inducing bone loss. However, when the source of the inflammation was ablated, bone loss was significantly placated.

Periodontitis is described as the presence of gingival inflammation at sites where there has been apical migration of the epithelial attachment onto the root surfaces accompanied by loss of connective tissue and alveolar bone (Armitage, 1995), frequently with decreased host resistance to infections or perturbations in gingival connective tissue that increases its susceptibility to inflammation-induced degradation (Armitage, 2004). Inflammation is a biological reaction to a disrupted homeostasis, during which the impacted tissues are destroyed while blood-derived products such as plasma proteins, fluids, and leukocytes are recruited to destroy the underlying sources in order to restore the homeostasis (Medzhitov, 2008). Acute phase of inflammation is triggered immediately following infection or tissue injury with the localization of plasma proteins and leukocytes, especially neutrophils, to the site of infection or injury (Medzhitov, 2008). The normally blood vessel neutrophils are allowed entry to the inflammation site through extravasation orchestrated by inflammatory cytokines, and attempt to eliminate the source of infectious agents. Once the source has been removed, acute inflammatory response ends with the healing of the damaged tissues. However, during chronic inflammation, neutrophils are replaced with macrophages and T cells. When the combined effects of these cells fail, they are replaced with granulomas and tertiary lymphoid tissues. With slow decline of pathogens and increase of self-antigens at the site of chronic inflammation, tissue damage is drastically increased (Medzhitov, 2008).

Previous animal studies suggested that periodontitis alone can cause ONJ (Aghaloo et al., 2011). Here, we found that when tooth extraction follows pre-existing inflammatory conditions, ONJ development is exacerbated. This suggested that structural defects in the presence of long-
term inflammatory lesions cause ONJ. Tooth extraction is often the last resort procedure to quickly resolve pre-existing pathological inflammation, and leads to structure defect in the alveolar bone and bony sockets which otherwise heal without difficulty in uncompromised patients (Kang et al, 2013). However, in patients taking BP, abnormal bone healing and interference of woven bone formation has been observed (Williams et al, 2014), especially within the site of heavy inflammation (Ruggiero, 2004; Bilezikian, 2006; Ficarra et al, 2005). It has been suggested that the presence of bacteria play a role in necrosis of bones leading to poor healing, and providing a link between pathological inflammatory conditions and ONJ development in both in clinical level and in animal models (Mawardi, 2011).

Previous studies have suggested that woven bone formation may play important role in osteomucosal healing by bridging soft and hard tissue healing processes (Williams, 2014). BP has shown to inhibit the bone resorption by osteoclasts, and subsequently the woven bone formation, which may lead to its inability to remove bacteria infected bone leading to necrosis (Williams, 2014). With pre-existing inflammation the ensuing necrosis of the bone can decrease the signal for bone resorption, coupled with inhibition of osteoclast activities can further disrupt the oralmucosal healing process. However, removal of inflammatory signals reduced ONJ development, suggesting that inflammation plays a key role in ONJ.

IL-1β, IL-6, and IL-17 are pro-inflammatory cytokines that are released during periodontitis by leukocytes (Graves, 2011). IL-1 especially has been shown to play a significant role in bone resorption since inhibition of IL-1 significantly reduces the progression of inflammation toward alveolar bone and the destructive periodontal pathogens (Delima, 2002). When inflammatory factors were examined in the ligature-induced inflammation animal model, it was seen that the level of IL-1β, IL-6, and IL-17 mRNA levels were extremely high in mice.
with intact ligatures and injected with BP. However, when the source or inflammation was removed, the level of these cytokines dramatically decrease. The changing level of pro-inflammatory cytokine not only signaled the presence of inflammation induced by ligature placement, but also suggested that these cytokines play a significant role in BRONJ.

These results suggest important clinical implications; inflammatory lesions can cause devastating results in patients under BP therapy. To some, preventative measures can be taken prior to the BP regiment to lessen the occurrence of ONJ. However, for many inflammatory condition may develop during, or as a result of anti-resorptive regimen. Given the improved prognosis for MRONJ following removal of ligature induced inflammation in mice it can be inferred that in lieu of extraction, removal or placating inflammatory condition prior to extraction may prevent the development of MRONJ in patients taking BP or denosumab.
5. Figure Legends and Figures

5.1. Figure 1. Pre-existing periodontal disease exacerbates BRONJ lesions following tooth extraction in mice

A) Schematic showing the injection, ligature placement, and extraction schedule of the experiment. B) μCT cross-section of the maxillae following the experiment showing the bone loss. C) Quantification of bone loss on the maxillae. D) H&E stain was performed on the site of extraction of second molar. Empty lacunae are indicated by arrows. E) Quantification of number of empty lacunae. F) Quantification of the necrotic bone area.
5.2. Figure 2. Removal of ligature prevents bone loss

A) Schematic showing the injection and ligature placement schedule of the experiment. B) Harvested maxillae tissues with signs of inflammation and presence of ligatures C) μCT scan of the maxillae following the experiment showing the bone loss on alveolar ridge. D) Quantification of bone loss on the maxillae measured by distance from CEJ from distal buccal root of first molar. E) Quantification of bone loss on the maxillae measured by distance from CEJ from palatal root of first molar. F) H&E stain of the site of ligature placement (second maxillary molar).
5.3. Figure 3. Removal of ligature ameliorates inflammation and bone resorption

A) TRAP staining at the site of ligature placement. Arrows show TRAP+ osteoclasts. B) Quantification of TRAP+ osteoclast. C) Quantitative Real-Time PCR measuring relative mRNA level of IL-1β. D) qRT-PCR measuring relative mRNA level of IL-6. E) qRT-PCR measuring relative mRNA level of IL-17.
5.4. Figure 4. Removal of ligature ameliorates ligature- and extraction-induced BRONJ in mice

A) Schematic showing the injection, ligature placement and removal, and extraction schedule of the experiment. B) Harvested maxillae tissues with signs of ONJ lesions C) Quantification of number of maxillae with bone exposure D) μCT scan of the maxillae following the experiment showing the bone loss on alveolar ridge. E) Quantification of bone loss on the maxillae measured by distance from CEJ from distal buccal root of first molar. F) Quantification of bone loss on the maxillae measured by distance from CEJ from palatal root of first molar.
5.5. **Figure 5. Removal of ligature decreases ligature- and extraction- induced bone necrosis and BRONJ-like lesions**

A) H&E staining at the extraction site. Empty lacunae are indicated by arrows. B) Quantification of empty lacunae in H&E sections. C) Quantification of necrotic bone area. D) TRAP+ osteoclast quantification in TRAP stained sections
6. References


