## Title

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# Pre-existing Periapical Inflammatory Condition Exacerbates Tooth Extraction–induced BRONJ Lesions in Mice

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## Abstract

**Introduction—**Surgical interventions such as tooth extraction increase a chance of developing osteonecrosis of the jaw (ONJ) in patients receiving bisphosphonates (BPs) for treatment of bone-related diseases. Tooth extraction is often performed to eliminate pre-existing pathological inflammatory conditions that make the tooth unsalvageable; however, the role of such conditions on bisphosphonate-related ONJ (BRONJ) development following tooth extraction is not clearly defined. Here, we examined the effects of periapical periodontitis on tooth extraction-induced BRONJ development in mice.

**Methods—**Periapical periodontitis was induced by exposing the pulp of the maxillary first molar for 3 weeks in C57/BL6 mice that were intravenously administered with BP. The same tooth was extracted, and after 3 additional weeks, the mice were harvested for histological, histomorphometric, and histochemical staining analyses.

**Results—**Pulp exposure induced periapical radiolucency as demonstrated by increased inflammatory cells, TRAP+ osteoclasts, and bone resorption. When BP was administered, pulp exposure did not induce apical bone resorption despite the presence of inflammatory cells and TRAP+ osteoclasts. While tooth extraction alone induced BRONJ lesions, pulp exposure further increased tooth extraction-induced BRONJ development as demonstrated by the presence of more bone necrosis.

**Conclusion—**Our study demonstrates that pre-existing pathological inflammatory condition such as periapical periodontitis is a predisposing factor that may exacerbate BRONJ development following tooth extraction. Our study further provides a clinical implication whereby periapical

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periodontitis should be controlled before performing tooth extraction in BP-users in order to reduce the risk of developing BRONJ.

**Keywords**

Bisphosphonate; BRONJ; periapical periodontitis; inflammation; tooth extraction

## INTRODUCTION

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a devastating side effect that predominantly occurs in patients who is undergoing or has undergone therapy with bisphosphonates (BPs) (1). Clinical presentation includes exposed or “probable” bone that persists more than 8 weeks without a history of radiation exposure (2). Increasing lines of evidence support a notion that such clinical presentations also occur in users of other drugs such as anti-resorptive or anti-angiogenic medications, and hence re-defined as medication-related osteonecrosis of the jaw (MRONJ) (3). Because there are currently no reproducible and predictable therapeutic modalities to cure BRONJ, it imposes significant problems in both medical and dental communities.

Dentoalveolar trauma such as tooth extraction is one of the major known risk factors for BRONJ development. Studies showed that more than 50% of patients having ONJ experienced a history of having tooth extraction (4, 5). Also, tooth extraction was reported to increase the risk of developing ONJ by 33-folds (3, 5). Interestingly, but intuitively, the vast majority of tooth extraction procedures is performed to resolve prior existing pathological lesions such as periodontal or periapical diseases that make the tooth unsalvageable. Such observation implies that these pre-existing pathological problems may already have predisposed the affected areas to BRONJ development following tooth extraction.

Bacterial infection and inflammation are almost always found universally in ONJ lesion (Reid & Cornish, 2011) and periodontal and periapical diseases are associated with bacterial infection and host’s inflammation (6, 7). Indeed, these pathological inflammatory conditions have been known to be the precipitating factors for ONJ (3, 8, 9). Periapical diseases are typically initiated by bacterial penetration through the infected pulp followed by local inflammation and bone destruction at the root apex. Nonetheless, the extent to which periapical diseases affect ONJ development following dentoalveolar trauma remains to be elucidated.

Previously, we have established the mouse model for BRONJ and showed that tooth extraction alone induces ONJ (10). Here, we hypothesize that pre-existing inflammatory conditions exacerbate BRONJ development following tooth extraction. To do so, we established a mouse model for periapical periodontitis by performing pulp exposure and examined the effects of prolonged periapical endodontic lesion in ONJ development following tooth extraction.

## MATERIALS AND METHODS

### Animal

C57BL/6 mice (six-week-old female) were purchased from the Jackson Laboratory (Bar Harbor, ME) and kept in a pathogen-free vivarium in the 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).

### The mouse model of periapical lesion

Mice were anesthetized with ketamine/xylazine (100 and 5 mg/kg body weight, respectively) by intraperitoneal injection. Pulp exposure was made on the left maxillary first molar using a high-speed ¼ round bur on a portable dental unit (Aseptico Inc., Woodinville, WA) under ×10 magnification of an endodontic microscope (BM-LED stereo microscope, MEIJI Techno, Japan). Exposed teeth were left open to the oral environment without any coverage. The right maxillary first molar was used as a control without pulp exposure. Mice were sacrificed and maxillae were harvested at 3, 7, 21, and 42 days after pulp exposure (n = 3).

### The BRONJ mouse model with pulp exposure and tooth extraction

The BRONJ mouse model with pulp exposure and tooth extraction was performed as described previously with modifications (10). Briefly, a total of 20 mice were divided into 2 groups (n = 10); one group intravenously administered with vehicle (Veh) solution (0.9% NaCl saline) and the other group with 125 mg/kg Zometa (ZOL; Novartis Oncology, East Hanover, NJ). Under general anesthesia, periapical periodontitis was induced by exposing the pulp of the maxillary first molar for 3 weeks, and the same tooth was extracted. The mice were allowed to heal for additional 3 weeks, during which maxilla and femur were harvested from each mouse for further analysis.

### Tissue preparation

The harvested maxilla were fixed with 4% paraformaldehyde in PBS, pH 7.4, at 4° overnight and stored in 70% ethanol solution. Fixed tissues were subjected to μCT scanning, after which maxillae were decalcified with 5% EDTA and 4% sucrose in PBS, pH 7.4 for 2 weeks at 4 degree. The decalcification solution was changed daily for 3 weeks, and decalcified tissues were sent to the UCLA Translational Procurement Core Laboratory (TPCL) for paraffin embedding.

### uCT Scan and Three-Dimensional Volumetric Analysis

μCT scanning (Scanco μCT 40; Scanco Medical, Brüttisellen, Switzerland) of maxilla and femur was performed using a voxel size of 20 $\mu m^3$ 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). Resolution was set to medium (1024 × 1024 × 148 pixels). Two-dimensional images were reconstructed using μCT v6.1 software (Scanco Medical, Brüttisellen,

Switzerland) and three-dimensional images obtained by the CTvol software (Bruker microCT, Kontich, Belgium).

Morphological parameters of trabecular bone microarchitecture in femur were assessed using the CTAn software (Bruker microCT, Kontich, Belgium) in accordance with the recommended guidelines (11). The size of a region of interest (ROI) was determined by calculating three-tenths of total femur length and the starting point of ROI was defined as epiphyseal growth plate. From each femur, bone volume fraction (BV/TV; %) was measured.

### Tartrate-resistant acid phosphatase (TRAP) histochemical staining

TRAP staining and quantification were performed as described previously (10). The sectioned slides (n = 3, every 5 cuts) were incubated with TRAP solution (Sigma-Aldrich), and Osteoclasts were identified the presence of multiple nuclei (n>5). Osteoclasts number quantification was measured using ImageJ software version 1.48 (NIH) on digital pictures taken through Olympus microscope (model DP72; Olympus) at 100× magnification.

### Histomorphometric Analysis

Empty lacunae counts and necrotic bone areas measurement were performed as described previously (10). Four slides per sample were stained with H&E, and the total bone surface area was measured using ImageJ software version 1.48 (NIH, Bethesda, MD). Empty lacunae were counted per total bone area (#/$mm^2$) in the ROI. Necrotic bone was defined as an area containing five or more empty lacunae per 1 $mm^2$ and the percentage of necrotic bone was defined as necrotic bone areas divided by total bone areas.

### Statistical Analysis

μCT data were analyzed using t-test for comparison of Veh and ZOL on trabecular bone measurements (BV/TV). 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, Somers, NY) with a significance level of 0.05.

## RESULTS

### Pulp exposure induces periapical radiolucency (PARL) with increased inflammatory cells and activated osteoclasts in mice

To experimentally examine whether pre-existing inflammatory lesion exacerbate development of BRONJ after tooth extraction, we first developed a periapical disease model in mice by creating a pulp exposure. As expected, periapical radiolucency (PARL) was developed at the apex of the pulp-exposed tooth in a time-dependent manner (Fig. 1*A*, white dotted lines). Histological examination revealed increased infiltration of inflammatory cells and marked bone resorption around the apex of the tooth (Fig. 1*B*). Increased expression of TNF-α was also observed (Suppl. Fig. 1). Because bone resorption is primarily mediated by osteoclasts, we used TRAP staining to identify the presence of multi-nucleated mature osteoclasts. Indeed, the TRAP staining showed a drastic increase in the numbers of

osteoclasts as early as Day 3 (Fig.1*C* and *1D*, $p < 0.05$). These data suggests that pulp exposure activated osteoclast differentiation, induced bone resorption, and caused PARL lesion. Because infiltration of inflammatory cells and PARL lesion was clearly evident at Day 21, we used this time point to examine the effect of periapical pre-existing inflammatory condition in ONJ development.

### Bisphosphonate prevents bone resorption at the apex of the pulp-exposed tooth despite the presence of inflammatory cells and osteoclasts

Because BPs inhibit bone resorption by targeting osteoclasts, we next examined whether BPs can prevent pulp exposure-induced PARL in mice. Indeed, the presence of BPs significantly prevented development of PARL that is comparable to the contralateral control group (Fig. 2*A*). Interestingly, histological examination revealed that there is intense infiltration of inflammatory cells at the apex in both Veh- and ZOL-treated mice (Fig. 2*B*). Consistent with this finding, TRAP+ mature osteoclasts were activated in both Veh- and ZOL-treated mice, albeit statistically more so in ZOL-treated mice (Fig.2*C* and 2*D*, $p < 0.05$). These findings imply that bone resorption in the apex of the pulp-exposed tooth did not occur even in the presence of inflammation and osteoclasts.

### Periapical periodontitis exacerbate tooth extraction-induced BRONJ development in mice

To evaluate the direct role of pre-existing periapical periodontitis in exacerbating BRONJ development following tooth extraction, we induced the periapical lesion for 3 weeks and then extracted the same tooth. After additional 3 weeks during which mice are allowed to heal, we harvested the maxilla (Fig. 3*A*). μCT analysis femur confirmed that BP administration significantly enhanced bone volume as demonstrated by BV/TV ratio (Fig. 3*B*, $p < 0.05$). Consistent with our previous study (10), μCT images of the tooth-extracted sites in ZOL-treated mice showed unfilled sockets, which was more prominent in pulp-exposed sites in both groups (Fig 3*C*). Histochemical staining for TRAP showed that ZOL-treated mice exhibited significantly more TRAP+ osteoclasts regardless of pulp exposure (Fig. 3*D* and *E*, $p < 0.05$). Further analysis showed that extraction alone induced osteonecrosis in ZOL-treated group as demonstrated by the presence of necrotic bone and empty lacunae (Fig. 4*A, B* and *C*). Evidently, we found a marked increase in necrotic bone and empty lacunae when the pulp-exposed tooth was extracted (Fig. 4*A, B* and *C*, $p < 0.05$), indicating that pre-existing pathological inflammatory lesion exacerbated bone necrosis following tooth extraction in the presence of BP.

## DISCUSSION

Pre-existing pathological inflammatory conditions such as periapical diseases has been speculated to be an additional risk factor that increases BRONJ development. In this study, we performed pulp exposure before tooth extraction in mice receiving BPs and provide experimental evidence that pre-existing periapical periodontitis exacerbates BRONJ development following tooth extraction. To the best of our knowledge, this study is the first report to demonstrate the combined effects of periapical periodontitis and tooth extraction on BRONJ development.

Currently, our understanding is limited as to why pre-existing periapical periodontitis exacerbates tooth extraction-induced BRONJ development. Periapical lesion develops as a result of overwhelming inflammatory responses in the infected dental pulp (7). Bacterial infection, physical or iatrogenic trauma can cause the pulpal inflammation and pulpal necrosis, which is followed by inflammatory infiltrates and increased osteoclasts and bone resorption around the periapical tissues (12). These pathological inflammation typically causes destructive environments as activated inflammatory cells release multiple secretor proteins including cytokines (e.g, IL-6 or TNF-α) and degrading proteins (e.g., collagenase or proteinases) that interfere with wound healing (13, 14). Indeed, high amounts of cytokines, collagenases and MMPs are readily found in periapical lesion (15, 16), and our study demonstrated an increased expression level of TNF-α following pulp exposure (Supplement Fig. 1). As BPs are known to activate inflammatory signals (17, 18), it is possible that BPs may further elevate inflammation activated by local pathological condition and prevent proper wound healing process following tooth extraction.

Our TRAP staining showed that the numbers of osteoclasts is consistently high in ZOL-treated mice regardless of pulp exposure (Fig. 2*C*, 2*D*, 3*D*, and 3*E*). A closer examination also revealed that osteoclasts in the ZOL-treated group seemed to be larger in size and detached from bone surfaces, which is in line with other studies (19). This notion is also consistent with the previous report that abnormal osteoclasts were increased in the bones from the patients taking BP (12). Therefore, the increased numbers of osteoclasts in response to BPs may be, in part, due to proinflammatory effects of BPs (18, 20) in addition to pulp exposure, leading to recruitment of inflammatory cells including macrophages, the pre-osteoclastic cells.

Interestingly, we also found an increased amount of alkaline phosphatase (ALP) at the local level (Supplement Fig. 2). While it seems to be in sharp contrast to our previous finding in that the serum level of ALP was significantly suppressed in ZOL-treated mice, bone formation is known to be induced at the local level in the presence of ZOL despite the systemic suppression of bone remodeling (21, 22). Further studies warrant closer examination.

Clinically, periapical lesion is exhibited as periapical radiolucency (PARL), a radiographic presentation that reflects bone destruction at the apex of the affected tooth. The presence of PARL is an indicative of a doomed tooth and the “gold standard” in assessing the tooth for root canal therapy (RCT). However, our study showed that PARL may not always be a good indicator for RCT in BP-users; in the presence of BPs, no PARL was detected despite prominent pulp exposure and the histological presence of inflammatory cells and osteoclasts (Fig.2*A*). Indeed, such reports were previously documented in other clinical and preclinical observations (23, 24). These findings imply that pulpal infection may be gone unnoticed during routine dental examination due to no radiographic lesions and that such conditions may be falsely classified as “spontaneous ONJ” or the “staging 0 ONJ.”

Tooth extraction is a traumatic event that creates large structural defects. Tooth removal results in discontinuity in soft tissues and exposure of hard tissues, requiring orchestrated tissue regeneration for complete wound closure and proper healing. Previously, we

demonstrated that woven bone formation after tooth extraction may play a pivotal role in osteomucosal healing – simultaneous healing of the soft and hard tissues in the oral cavity – as woven bone is a specialized intermediate entity that mediates between soft and hard tissues by establishing highly collagen-enriched structures onto which mineralization occurs to form bone (10). As such, it is tempting to speculate that, in the presence of BPs, certain pre-existing pathologic inflammatory signals interfere with osteomucosal healing by preventing proper woven bone formation following creation of large structural defects such as tooth extraction.

In conclusion, we demonstrated here that pre-existing periapical periodontitis exacerbates tooth extraction-induced BRONJ development. Tooth extraction is often indicated as a last resort to immediately resolve pre-existing pathologic inflammatory conditions on the unsalvageable tooth. Generally, tooth-extracted sites are predictably healed with better prognosis. However, our study provides experimental evidence that, in BP-users, a tooth with periapical lesion that is otherwise doomed to be removed due to its existing pathological condition should be controlled before extraction to prevent increased risk of developing BRONJ lesion. Careful evaluation should be implemented when managing periapical inflammatory conditions on patients with history of the BP uses.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Highlights

- Pulp exposure induced periapical periodontitis and PARL in mice.
- In the presence of bisphosphonate, pulp exposure induced periapical periodontitis without causing PARL.
- Periapical periodontitis exacerbated tooth extraction-induced BRONJ development in mice.

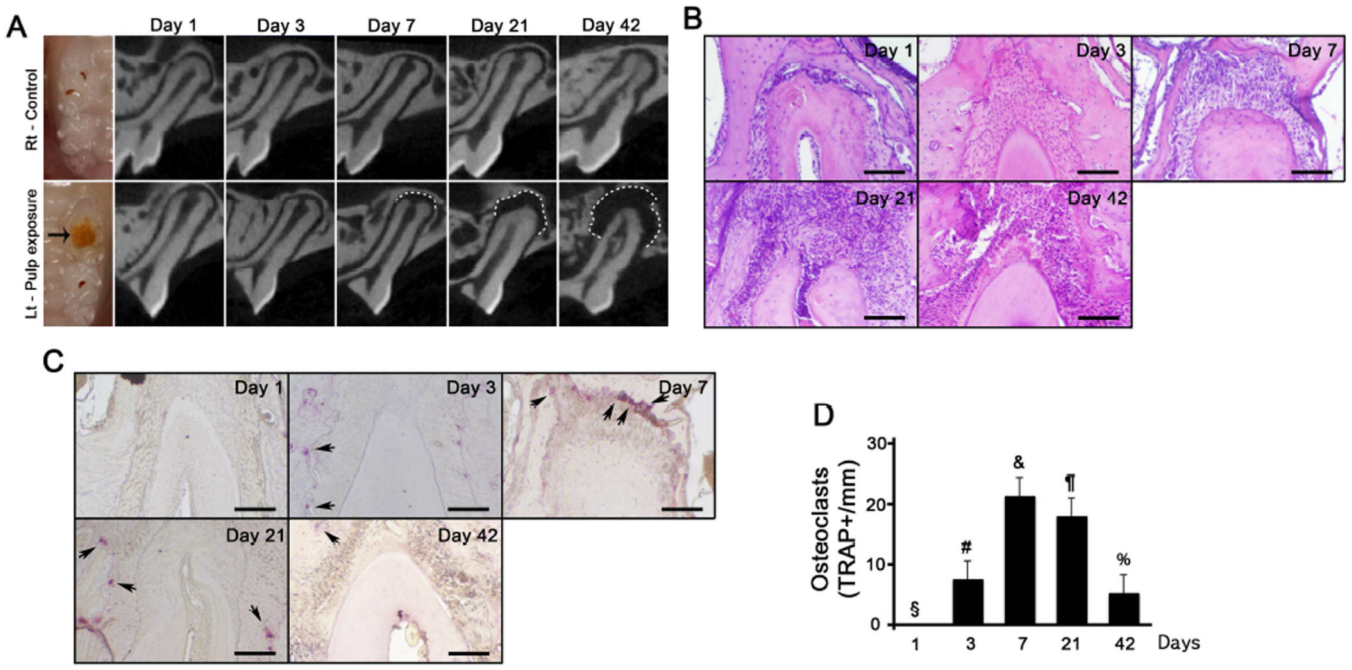


**Figure 1. Pulp exposure induces periapical radiolucency (PARL) with increased inflammatory cells and activated osteoclasts in mice**

Pulp was exposed using a round bur on the first molar in mice (left panels). The exposed pulp was left as is for 1, 3, 7, 21, and 42 days. **(A)** The μCT scans at the distopalatal root. **(B)** H&E staining at the apex. **(C)** TRAP staining at the apex. Arrows indicate TRAP+ osteoclasts. **(D)** Quantification of TRAP+ osteoclasts around the apex of the root ($p<0.05$). Different symbols are significantly different by ANOVA and Tukey's post hoc test ($P<0.05$). Bar = 100 μm.

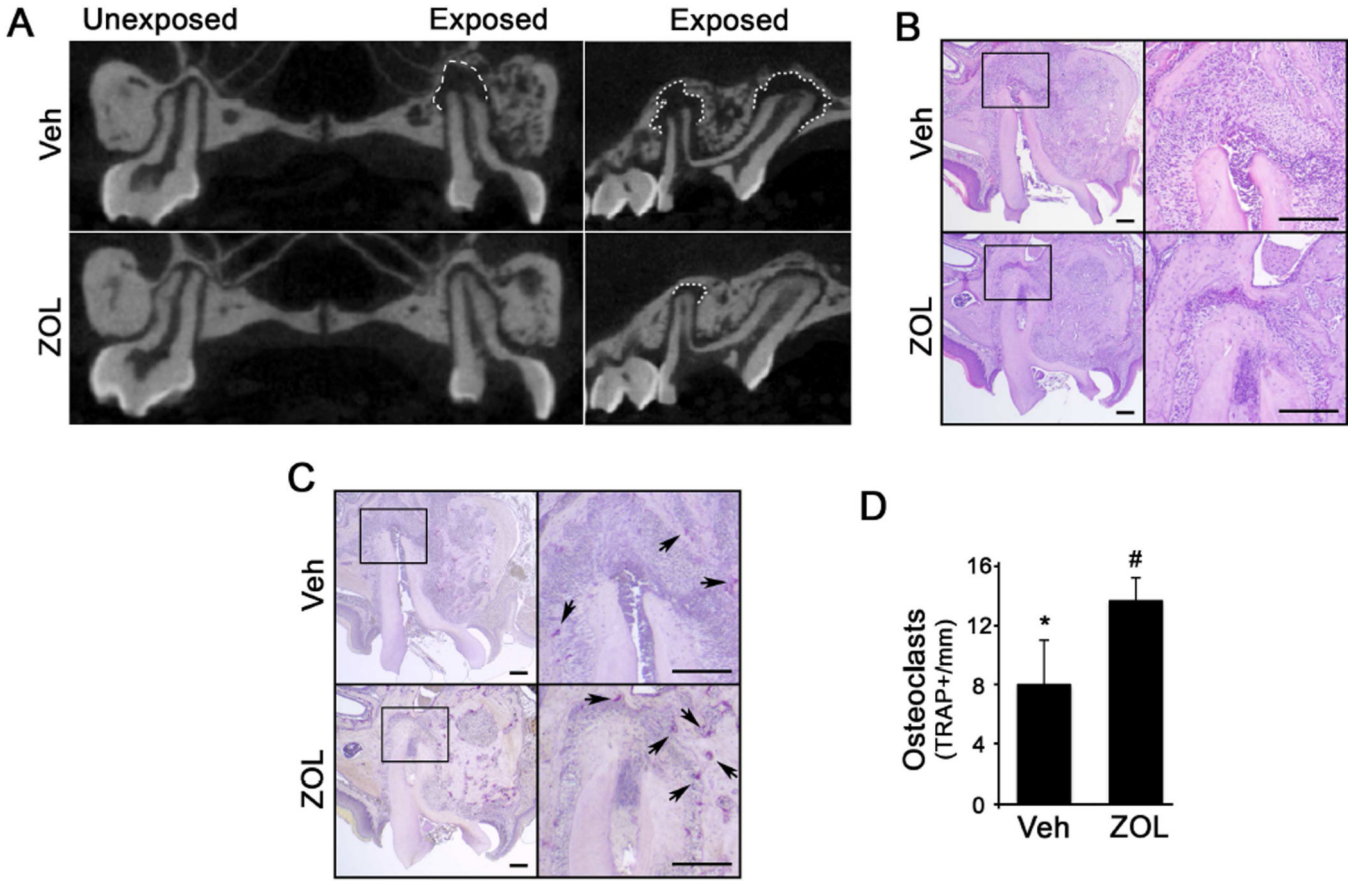


**Figure 2. Bisphosphonate prevents bone resorption at the apex of the pulp-exposed tooth despite the presence of inflammatory cells and osteoclasts**

Mice were intravenously administered with BP twice a week throughout the study. One week after initial administration, pulp was exposed and left open for 3 weeks, and maxillae were harvested. **(A)** The μCT scans of pulp-exposed (right) and -unexposed (left) tooth. **(B)** H&E staining and **(C)** TRAP staining at the apex of the pulp-exposed and –unexposed tooth. **(D)** Quantification of TRAP+ osteoclasts. Different symbols are significantly different by t-test ($P<0.05$). Bar = 100 μm.

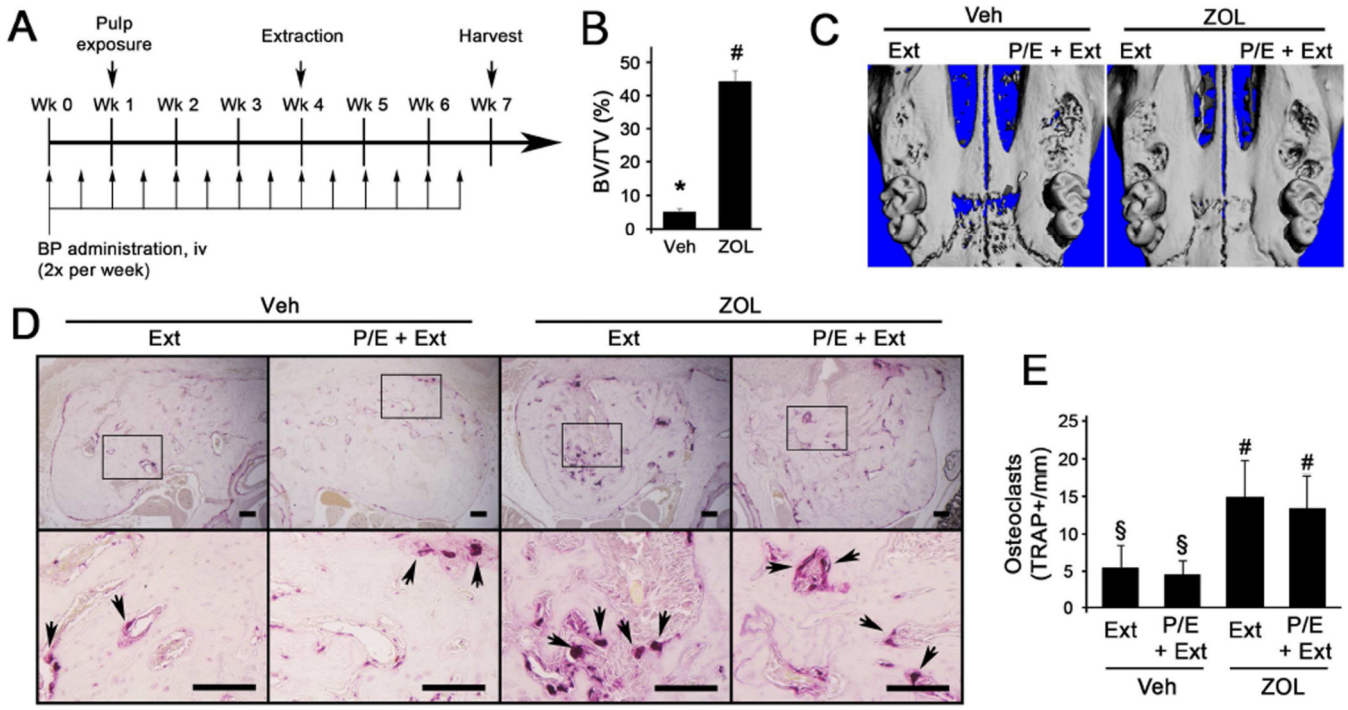


**Figure 3. Establishment of pulp exposure and tooth extraction BRONJ model in mice**

**(A)** Schematic diagram of the experiment. **(B)** Quantification of bone volume ($p<0.05$). Different symbols are significantly different by t-test. **(C)** μCT scan of the maxillae at the end of experiment. **(D)** TRAP staining at the tooth extracted site. Bar = 100 μm. **(E)** Quantification of TRAP+ osteoclasts. Different symbols are significantly different by ANOVA and Tukey's post hoc test ($P<0.05$).

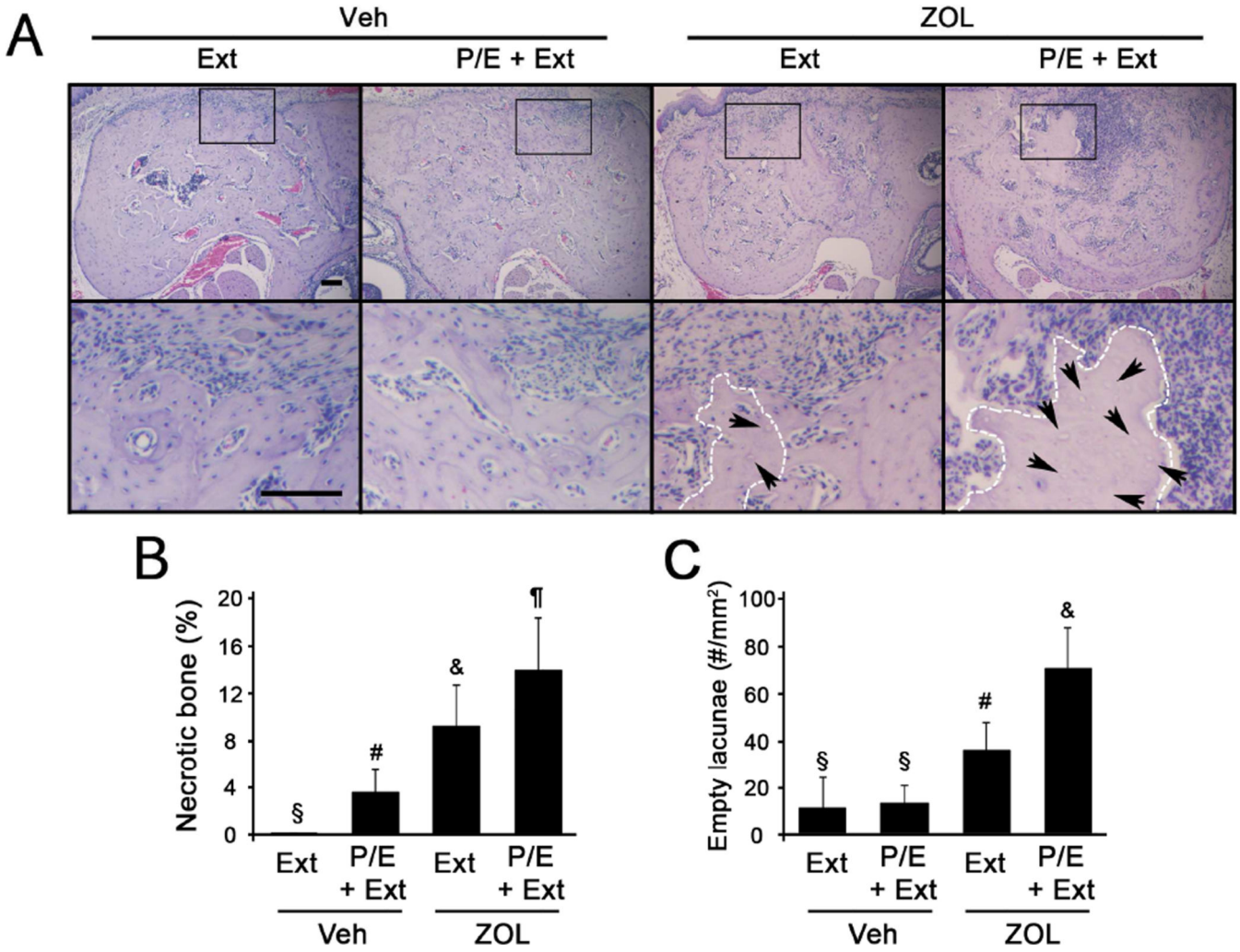


**Figure 4. Pulp exposure exacerbates tooth extraction-induced BRONJ development in mice**

**(A)** H&E staining at the tooth extraction site Bar = 100 μm. **(B)** Quantification of necrotic bone. Dotted lines indicate necrotic bone areas. **(C)** Quantification of empty lacunae. Arrows indicate empty lacunae. Different symbols are significantly different by ANOVA and Tukey's post hoc test ($P<0.05$).