Diminished Progression of Periapical Lesions with Zoledronic Acid in Ovariectomized Rats

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Abstract

Introduction: The aim of this study was to investigate the effects of systemically administered zoledronic acid (ZOL) on the progression of periapical lesions in estrogen-deficient rats. **Methods:** Female Wistar rats were divided into the following groups: SHAM-veh, sham surgery treated with vehicle (physiological saline); OVX-veh, ovariectomy treated with vehicle; SHAM-ZOL, sham surgery treated with ZOL; and OVX-ZOL, ovariectomy treated with ZOL. Vehicle or ZOL was administered intravenously once a week for 4 weeks. The pulp of the mandibular first molar of all rats was exposed to the oral environment to induce a periapical lesion, and the lesions were analyzed after 7 and 30 days. The mandibles were examined by micro–computed tomographic imaging and histopathologic, histometric, and immunohistochemical analyses. **Results:** Histopathologically, the OVX-veh group had more severe inflammation and bone loss and a larger number of cells that were positive for tartrate-resistant acid phosphatase compared with the SHAM-veh and OVX-ZOL groups; the SHAM-veh and OVX-ZOL groups were similar to each other. The SHAM-ZOL group had the lowest magnitude of these conditions. Tomographically, the OVX-veh group had greater bone loss than the other groups at both time points. The SHAM-veh, SHAM-ZOL, and OVX-ZOL groups had similar bone loss at both time points. In the sagittal section on day 30, the SHAM-ZOL group had lower bone loss compared with the SHAM-veh and OVX-ZOL groups. **Conclusions:** The hypoestrogenic condition aggravates the progression of periapical lesions. ZOL therapy may help contain bone destruction of periapical lesions. *(J Endod 2015;41:2002–2007)*

Key Words

Bisphosphonate, estrogen deficiency, osteonecrosis

The health problems of the elderly are one of the greatest challenges among a growing elderly population and have encouraged animal model studies (1). Hormones potentially influence the development and integrity of the skeleton and oral cavity (2), and estrogen reduction, found in postmenopausal women, has various effects on oral health (3).

After pulp necrosis, the root infection disseminates in an apical direction, inducing periapical inflammation (4). The periapical tissues suffer bone destruction and are in turn influenced by local (eg, inflammation and necrosis) and systemic factors (eg, estrogen) (5, 6). Estrogen has an effect on the process of bone resorption, and its deficiency could be an aggravating factor in apical periodontitis (7).

In the initial phase of inflammation, the tissue exudate was produced by recruiting chemical mediators, and the activation of the complement system produces chemotactic factors for macrophages, which in turn differentiate into osteoclasts (8). Bisphosphonates (BPs) target osteoclast-mediated bone resorption and ultimately inhibit bone remodeling. The inhibitory effect of BPs on bone remodeling can also potentially interfere with periradicular healing after root canal therapy (9).

Zoledronic acid (ZOL), a potent nitrogen-containing BP, has been used in daily practice for the treatment and prevention of bone diseases and skeletal-related events caused by bone metastasis from diverse tumors (10). These drugs act directly or indirectly on osteoblasts and osteoclasts, resulting in decreased bone turnover, and present inhibitory effects on inflammatory mediators affecting the healing process of bone lesions. Therefore, the healing process of a periapical lesion can be potentially affected by BPs (11).

Little information is available concerning the effect of BP on periapical lesion progression in estrogen-deficient organisms. The aim of this study was to evaluate periapical lesions in ovariectomized rats treated with ZOL.

Materials and Methods

Animals

Forty female Wistar rats (6 months of age) from Japan SLC (Hamamatsu, Japan) were used. The Animal Research Committee of the University of Fukui approved the experiments (No. 26125). The groups were divided according to the pulp exposure time point, ovariectomy (OVX), and treatment as follows: sham surgery and vehicle (physiological saline) treatment at 7 days (SHAM-veh 7 d, $n = 5$) or 30 days (SHAM-veh 30 d, $n = 5$) after pulp exposure, sham surgery and ZOL treatment at 7 days (SHAM-ZOL 7 d, $n = 5$) or 30 days (SHAM-ZOL 30 d, $n = 5$) after pulp exposure, OVX and vehicle

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treatment at 7 days (OVX-veh 7 d, $n = 5$) or 30 days (OVX-veh 30 d, $n = 5$) after pulp exposure, and OVX and ZOL treatment at 7 days (OVX-ZOL 7 d, $n = 5$) or 30 days (OVX-ZOL 30 d, $n = 5$) after pulp exposure.

Estrous Cycle

Vaginal swabs were collected every morning and examined using an optical microscope for 12 days (12). Rats with regular estrous cycles were selected and randomly divided into the experimental groups. Rats with irregular estrous cycles were excluded.

Ovariectomy

After selecting the rats with regular estrous cycles, ovariectomies or sham surgeries (the ovaries were exposed but not removed) were performed under general anesthesia with isoflurane. After surgery, all animals received an intraperitoneal injection of ampicillin (50 mg/body) for infection prevention.

ZOL and Vehicle Treatment

The treatment started 10 days after the ovariectomy or sham surgery. The rats in the control and intravenously treated groups received 0.3 mL physiological saline or 0.04 mg/body ZOL (ZOMETA; Novartis Pharma, Tokyo, Japan), respectively, according to the previous protocol (13). The injection was performed via the jugular vein once a week for 4 weeks.

Induction of the Periapical Lesion

Under general anesthesia with intraperitoneal pentobarbital sodium (30 mg/kg), the crowns of the mandibular first molars were drilled using a steel round bur (#1/4) and remained open. These procedures were performed 7 or 30 days before sacrifice. The pulp exposures were standardized to a 0.1-mm diameter.

Sample Collection

Thirty days after the initial treatment, the animals were euthanized by an anesthetic overdose of intraperitoneal pentobarbital sodium. The mandibles were examined using micro-computed tomographic (micro-CT) scanning, hematoxylin-eosin staining, and immunohistochemical techniques. The uteri were also removed and weighed.

TABLE 1. Uterus Weight, Periapical Lesion Areas, and Tartrate-resistant Acid Phosphatase–positive Cells Stratified by Group

Experimental group	Uterus weight (g)	Periapical lesion (mm^2)	TRAP ($cells/mm^2$)
SHAM-veh 7 d	0.65 ± 0.17	0.32 ± 0.04	3.42 ± 1.43
OVX-veh 7 d	0.18 ± 0.02	0.47 ± 0.06	6.61 ± 1.32
SHAM-ZOL 7 d	0.80 ± 0.09	0.11 ± 0.05	1.12 ± 1.53
OVX-ZOL 7 d	0.19 ± 0.08	0.30 ± 0.07	2.38 ± 1.38
SHAM-veh 30 d	0.61 ± 0.02	1.28 ± 0.24	4.59 ± 1.47
OVX-veh 30 d	0.30 ± 0.09	2.63 ± 0.26	8.67 ± 1.69
SHAM-ZOL 30 d	0.66 ± 0.04	0.58 ± 0.22	2.61 ± 1.29
OVX-ZOL 30 d	0.24 ± 0.09	1.09 ± 0.21	3.41 ± 1.56

OVX-veh, ovariectomy treated with vehicle; OVX-ZOL, ovariectomy treated with zoledronic acid; SHAM-veh, sham surgery treated with vehicle (physiological saline); SHAM-ZOL, sham surgery treated with zoledronic acid.

Micro-CT Scanning

The mandibles were fixed in 4% neutral-buffered formalin for 24 hours and were imaged using 3-dimensional micro–x-ray CT imaging (R_mCT; Rigaku, Tokyo, Japan) at 400-μm slices, with the calibration at 90 kV and 100 μA. The sagittal, coronal, and axial sections through the mandibular molars were generated to measure the amount of periapical bone loss. The areas of the periapical lesions of the molars were quantified in pixels. A straight line distance between the root apex to the periapical alveolar bone in the sagittal and coronal slices and between the lingual to the vestibular surface of the periapical alveolar bone in the axial slice were delimited. The data were converted to square millimeters by using 1 mm^2 = 256 pixels, as determined by a 3-dimensional micro–x-ray CT program.

Histopathologic, Histometric, and Immunohistochemical Analyses

The mandibles were separated, decalcified in 10% EDTA, routinely embedded in paraffin, and sliced into 5-μm sections. The sections were stained with hematoxylin-eosin or immunohistochemically prepared using an indirect immunoperoxidase technique (ImmPRESS; Vector Laboratories, Burlingame, CA) for the detection of tartrate-resistant acid phosphatase (TRAP) (goat anti-TRAP antibody; SC30832, Santa Cruz Biotechnology, CA) following the previously described protocol

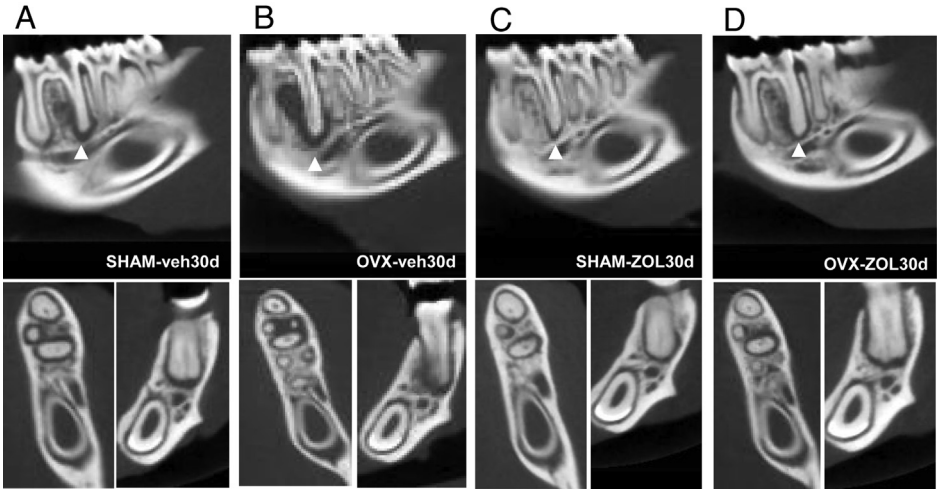

Figure 1. Tomographic and histologic aspects of periapical lesions at 7 and 30 days after pulp exposure to the oral environment. (*A–D*) Sagittal, coronal, and axial sections through the mandibular first molars in groups (*A*) SHAM-veh 30 d, (*B*) OVX-veh 30 d, (*C*) SHAM-ZOL 30 d, and (*D*) OVX-ZOL 30 d. *Arrowheads* indicate periapical lesions. The OVX-veh group had greater bone loss compared with the other groups (*continued*).

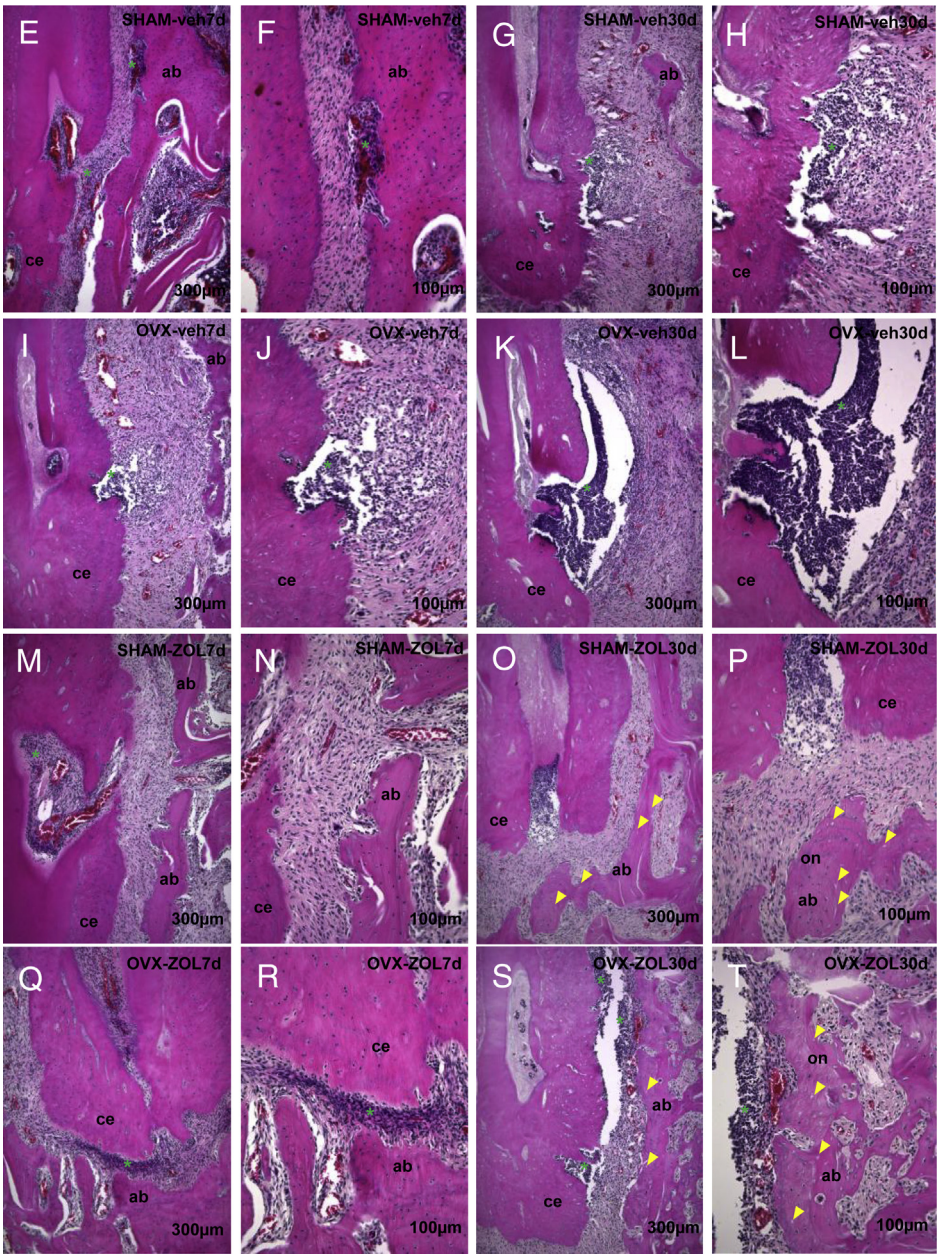

Figure 1. *Continued.* (*E–T*) Photomicrographs showing the histologic appearance and magnitude of inflammation in periapical lesions at 7 and 30 days in the (*E* and *F*) SHAM-veh 7 d, (*G* and *H*) SHAM-veh 30 d, (*I* and *J*) OVX-veh 7 d, (*K* and *L*) OVX-veh 30 d, (*M* and *N*) SHAM-ZOL 7 d, (*O* and *P*) SHAM-ZOL 30 d, (*Q* and *R*) OVX-ZOL 7 d, and (*S* and *T*) OVX-ZOL 30 d groups. The severity of inflammation was greater in the OVX-veh group compared with other groups, whereas the SHAM-ZOL group had a lower degree of inflammation. The magnitude of the inflammatory reaction was similar between the SHAM-veh and OVX-ZOL groups. ab, alveolar bone; ce, cementum; on, osteonecrosis. *Arrowheads* indicate empty lacunae of osteocytes. *Asterisk* (*) indicates inflammatory infiltrate. Hematoxylin counterstaining. *(E, G, I, K, M, O, Q,* and *S)* Scale bars = 300 μm, original magnification ×100. (*F, H, J, L, N, P, R,* and *T*) Scale bars = 100 μm, original magnification × 250.

(14). Positive and negative controls were used for the immunohistochemical analyses.

The histologic parameters were the extent of inflammation and necrosis, the state of the vasculature, and the cellularity pattern of the dental and periodontal tissues. The total numbers of empty lacunae and osteocytes were quantified.

Histometric analysis was conducted using Leica Microsystems software (Leica, Wetzlar, Germany). All samples were analyzed by 2 evaluators who were previously calibrated. The distal root of the mandibular first molar was examined. The limits of the periapical lesions were the external cementum surface, the periodontal ligament, and the external alveolar bone surface. In 5 equidistant sections, the widest area was selected to calculate the lesion area (mm^2).

The number of only the mature osteoclasts as TRAP-positive multinucleated cells was quantified in the perimeter of the lesion. The results were expressed as the number of multinucleated TRAP-positive cells per mm^2.

Statistical Analysis

The data were analyzed using Sigmaplot software (San Jose, CA). The Kruskal-Wallis test followed by the Dunn test was performed for

TABLE 2. Bone Loss around the Distal Root of the Mandibular First Molar in the Axial, Coronal, and Sagittal Sections (mm) by Tomographic Analysis Stratified by Group

Experimental group	Axial section (mm)	Coronal section (mm)	Sagittal section (mm)
SHAM-veh 7 d	0.98 ± 0.18	0.90 ± 0.22	0.06 ± 0.01
OVX-veh 7 d	1.15 ± 0.05	1.13 ± 0.10	0.13 ± 0.02
SHAM-ZOL 7 d	0.82 ± 0.03	0.86 ± 0.05	0.05 ± 0.01
OVX-ZOL 7 d	0.97 ± 0.10	0.94 ± 0.07	0.05 ± 0.02
SHAM-veh 30 d	1.07 ± 0.04	1.04 ± 0.05	0.18 ± 0.07
OVX-veh 30 d	1.34 ± 0.05	1.24 ± 0.08	0.37 ± 0.06
SHAM-ZOL 30 d	0.87 ± 0.04	0.85 ± 0.08	0.12 ± 0.02
OVX-ZOL 30 d	0.98 ± 0.04	0.96 ± 0.05	0.17 ± 0.07

OVX-veh, ovariectomy treated with vehicle; OVX-ZOL, ovariectomy treated with zoledronic acid; SHAM-veh, sham surgery treated with vehicle (physiological saline); SHAM-ZOL, sham surgery treated with zoledronic acid.

nonparametric data. Analysis of variance followed by the Tukey test was performed for parametric data. The level of significance was 5%.

Results

Uterus Weight

The OVX-veh and OVX-ZOL groups had smaller uteri weights in comparison with the SHAM-veh and SHAM-ZOL groups at both time points ($P < .0001$). The uterus weight was not affected by the ZOL treatment in the OVX-ZOL group ($P > .05$) (Table 1).

Micro-CT Analysis

Micro-CT sections through the apical area of the mandibular molars were created (Fig. 1*A–D*, Table 2). At both time points, bone loss in all sections was significantly higher in the OVX-veh group than in the other groups ($P < .0001$) and in the coronal and axial sections, bone loss in the SHAM-veh, SHAM-ZOL, and OVX-ZOL groups was similar ($P > .05$). However, in the sagittal section on day 30, the SHAM-ZOL group had less bone loss than the other groups ($P < .0001$). Bone loss increased in all groups when comparing day 7 to day 30 after pulp exposure to the oral environment.

Histopathologic Analysis

Figure 1*E–T* shows the histologic aspects of the periapical lesions in the different experimental groups.

On day 7, the root canal had a large amount of inflammatory cells, and the pulp was still vascularized; however, in the OVX-veh group, the vascularization was diminished. In the periapical region, the predominantly polynuclear inflammatory cells were sparsely distributed, reaching the alveolar bone, where osteoclast-promoting resorption was noted.

On day 30, most of the root canals showed pulp necrosis. Chronic inflammatory cells were sparsely distributed in greater quantities around the periapical region. Larger extensions of the periapical lesions and an increased amount of osteoclasts resorbing the alveolar bone were observed. The ZOL treatment resulted in empty osteocytic lacunae, featuring osteonecrosis. In evaluating these areas, osteonecrosis was present only in the drilled site of BP-treated animals on day 30. The empty lacunae/total osteocytes percentages were 12.2% and 14.3% for the SHAM-ZOL 30 d and OVX-ZOL 30 d groups, respectively, whereas empty lacunae were absent in the SHAM-veh 7 and 30 d, OVX-veh 7 and 30 d, SHAM-ZOL 7 d, and OVX-ZOL 7 d groups.

At both time points, the OVX-veh group had greater inflammation and bone loss than the SHAM-veh and OVX-ZOL groups, which were similar to each other; the SHAM-ZOL group presented with the lowest magnitude of inflammatory reaction and bone loss.

The most severe root resorption was noted in the vehicle-treated groups, whereas in the ZOL-treated groups, root resorption was observed to a lesser extent. When comparing the SHAM with the OVX rats, there were higher resorption lacunae in the OVX group in both the vehicle and ZOL groups. The resorptive process was aggravated when comparing day 7 with day 30.

Histometric Analysis

At both time points, the OVX-veh group had greater periapical lesion sizes compared with the other groups ($P < .0001$), the periapical lesion areas of the SHAM-veh and OVX-ZOL groups were similar ($P < .05$), and the SHAM-ZOL group had the smallest periapical lesion size ($P < .0001$). The periapical lesion size increased in all experimental groups when comparing day 7 with day 30 (Fig. 2*A*, Table 1).

TRAP Immunohistochemistry

The immunohistochemical technique for TRAP was highly specific to osteoclasts (Fig. 2*B–J* and Table 1). Labeling was predominantly confined to the cytoplasm of multinucleated cells. The negative control showed no immunolabeling.

The OVX-veh group had a higher number of TRAP-positive multinucleated cells per mm^2 in the periapical lesion than the other groups ($P < .05$). The SHAM-ZOL group had the fewest TRAP-positive cells ($P < .05$). The number of osteoclasts on day 30 after pulp exposure significantly increased in all groups ($P < .05$).

Discussion

Menopause is associated with significant adverse changes in both the systemic (uteri atrophy) and orofacial complexes (15, 16). In the present study, the uteri of ovariectomized rats weighed less than those of the sham surgery rats, confirming the efficacy of the ovariectomy. BP treatment did not cause alterations in the uteri weight.

The micro-CT analysis showed that the OVX-veh group had greater bone loss, and this loss was diminished with ZOL treatment at both time points according to previous studies (11). In the sagittal section, the SHAM-ZOL 30 d group had less bone loss in comparison with all of the other groups, most likely as a result of the beneficial effect of estrogen and ZOL in decreasing bone resorption (17).

Periapical bone destruction in the experimental groups was indicated by the presence of TRAP-positive cells displaying osteoclast activities. The OVX-veh group had greater bone loss and root resorption with a large number of TRAP-positive cells in comparison with the other groups, which is consistent with other studies (18, 19). After an ovariectomy, there was an imbalance in the bone remodeling with an increased bone turnover that is highlighted by the presence of a large number of active osteoclasts and resorption lacunae and the larger lesion size in comparison with that of the sham rats. However, the group that had the least bone loss among all of the groups was the SHAM-ZOL group, suggesting that the estrogen acted together with ZOL in preventing bone loss. The minimum occurrence of root resorption in the ZOL group can be explained by its incorporation into the mineralized tissue and remaining available for a long time, acting specifically on osteoclasts and inhibiting the root resorption process in patients using BPs in the same way as observed in the previous study (20–22).

The OVX-ZOL group had a smaller number of osteoclasts and consequently a smaller lesion area and less root resorption, preventing the deleterious effects that result from an ovariectomy similar to the SHAM-veh group in a previous study (23). This suggests that ZOL

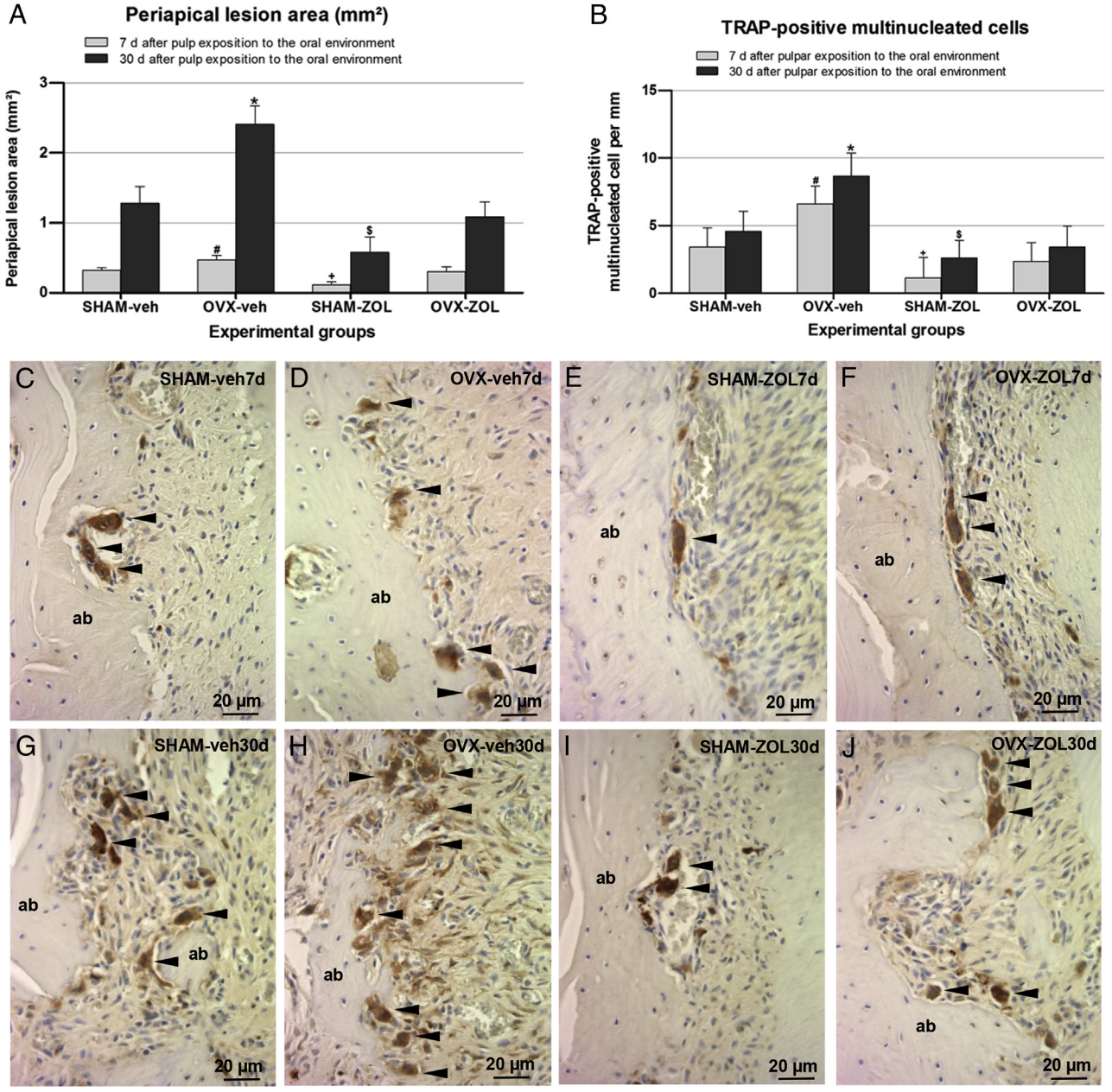

Figure 2. (*A*) The periapical lesion area (mm^2) in the distal root of the mandibular first molar in the experimental groups. (*B*) The number of TRAP-positive multinucleated cells per mm^2 in the perimeter of a periapical lesion in the distal root of the mandibular first molar in the experimental groups. (*C–J*) Photomicrographs showing the histologic appearance of immunolabeling for TRAP-positive multinucleated cells (*arrowheads*) at 7 and 30 days after pulp exposure in the (*C*) SHAM-veh 7 d, (*D*) OVX-veh 7 d, (*E*) SHAM-ZOL 7 d, (*F*) OVX-ZOL 7 d, (*G*) SHAM-veh 30 d, (*H*) OVX-veh 30 d, (*I*) SHAM-ZOL 30 d, and (*J*) OVX-ZOL 30 d groups. Note the greater immunolabeling in the OVX-veh group at both time points after pulp exposure to the oral environment. ab, alveolar bone. Hematoxylin counterstaining. (*C–J*) Scale bars = 20 μm, original magnification ×1000. #Statistically significant difference between the indicated group and the SHAM-veh 7 d, SHAM-ZOL 7 d, and OVX-ZOL 7 d groups ($P < .05$). *Statistically significant difference between the indicated group and the SHAM-veh 30 d, SHAM-ZOL 30 d, and OVX-ZOL 30 d groups ($P < .05$). +Statistically significant difference between the indicated group and the SHAM-veh 7 d, OVX-veh 7 d, and OVX-ZOL 7 d groups ($P < .05$). $Statistically significant difference between the indicated group and the SHAM-veh 30 d, OVX-veh 30 d, and OVX-ZOL 30 d groups ($P < .05$).

mimicked the beneficial effects of estrogen, inducing osteoclast apoptosis, inhibiting their differentiation and resorption activity, and consequently reducing the number of TRAP-positive cells (24). These conditions are favorable for the occurrence of osteonecrosis once the antiresorptive property of ZOL impairs the bone resorption that occurs in the first days after periapical lesion formation; the coordinated osteoclast and osteoblast activity is central to the healing process of the involved osseous structures (25).

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) has emerged as a significant complication in a subset of patients receiving BP and has been most frequently observed in patients with an oral infection, such as periodontitis (26, 27). Dental evaluations and procedures preceding BP therapy are recommended to prevent BRONJ (28, 29).

In the present study, an osteonecrosis area was noted in the SHAM-ZOL and OVX-ZOL groups on day 30, which is in agreement with previous studies (30, 31). This finding supports the theory that the

suppression of bone remodeling by ZOL is a critical step in osteonecrosis formation. Many hypotheses have been proposed to explain the occurrence of osteonecrosis of the jaw, including BP toxicity to the oral epithelium, bone remodeling imbalance, oral infection and inflammation, and the suppression of angiogenesis and bone turnover (32, 33). The hypoestrogenic condition aggravates the bone resorption associated with periapical lesion. ZOL therapy may help contain bone destruction of periapical lesions.

The importance of dental disease to the pathogenesis of osteonecrosis must be emphasized, and this rat osteonecrosis model without tooth extraction can be used in further studies for exploring the relevant pathophysiological mechanisms.

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The authors deny any conflicts of interest related to this study.

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