Initial changes in alveolar bone volume for sham-operated and ovariectomized rats in ligature-induced experimental periodontitis

Jing Dai[1] · Yihui Ma[1] · Miusi Shi[1] · Zhengguo Cao[1] · Yufeng Zhang[1,2] ·
Richard J. Miron[3]

Abstract

Objectives Osteoporosis is a disease characterized by a reduction in bone mass, poor bone strength, and microarchitectural deterioration primarily in postmenopausal women. With respect to periodontal disease, osteoporosis is thought to contribute to pre-existing alveolar degeneration although the association between both diseases is not fully characterized. The aim of the present study was to observe the initial changes in mandibular alveolar bone for sham-operated and ovariectomized (OVX) rats in ligature-induced experimental periodontitis.

Materials and methods A total of 64 Wistar rats (7 weeks of age, 180–200 g) were used in this study (32 control sham-operated animals+ligature placement, 32 OVX animals+ligature placement). Following an 8-week period to induce an OVX model, micro-CT analysis was performed to calculate vertical and furcation bone loss of mandibular first molars at time points 0, 3, 7, and 11 days following ligature placement (six animals per group per time point). Furthermore, histological analysis was performed to calculate the loss of alveolar bone crest height from the cemento-enamel junction, and tartrate-resistant acid phosphatase (TRAP) staining was utilized to calculate the number of osteoclasts.

Results The results from the present study demonstrate that OVX animals showed significant vertical bone loss at all time points when compared to control sham-operated animals. In the furcation area, no significant difference in bone loss was observed between sham-operated and OVX animals at 0, 3, and 7 days; however by 11 days, a significant decrease in bone volume/total volume and trabecular thickness was observed in the OVX group. The histological analysis also revealed that alveolar bone crest height was significantly reduced in OVX animals, and TRAP staining further revealed the greater number of multinucleated osteoclasts peaking at 3 days postligature placement.

Conclusion The results from the present study demonstrate a direct correlation between the osteoporotic phenotype and the progression of periodontal breakdown in a diseased-induced animal model.

Clinical relevance It may be suggested that an osteoporotic phenotype has the potential to speed periodontal breakdown and thus contributes to the overall degeneration of the periodontium in patients suffering from postmenopausal bone loss. Future research from human clinical studies are necessary to further understand the relationship between periodontal disease and osteoporosis.

Keywords Osteoporosis · Periodontal disease · Periodontitis · Ovariectomy

✉ Yufeng Zhang
zyf@whu.edu.cn

[1] The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) and Key Laboratory of Oral Biomedicine Ministry of Education, School and Hospital of Stomatology, Wuhan University, 237 Luoyu Road, Wuhan 430079, People's Republic of China

[2] Department of Oral Implantology, School of Stomatology, Wuhan University, Wuhan 430079, China

[3] Department of Periodontology, University of Bern, Bern, Switzerland

Introduction

Osteoporosis is a worldwide health care issue characterized by reduction in bone mass, poor bone strength, and microarchitectural deterioration in trabecular and cortical

bone. The disease is known to progress with increasing age, and the primary cause is thought to be derived by an imbalance between bone-forming osteoblasts and bone-resorbing osteoclasts due primarily from postmenopausal estrogen deficiencies [1–5]. Over 200 million people are now estimated to be suffering from the disease worldwide with the great majority being white or Asian women over the age of 65 [6]. Commonly utilized pharmacological agents for the treatment of osteoporosis include anabolic agents capable of increasing bone formation such as parathyroid hormone and the use of anti-resorbing agents such as bisphosphonates, calcitonin, RANKL inhibitors, raloxifene, and estrogen replacement therapy [7]. Despite these many options, there exist no single option capable of fully restoring lost bone, and the use of bisphosphonate therapy (the leading treatment options for prevention of bone mass in osteoporotic patients) is associated with a number of side effects including halting the bone remodeling cycle.

Interestingly, growing clinical evidence has suggested that a relationship between osteoporosis and periodontal disease exist [8–14]. In one of the first well-documented studies investigating this relationship, it was found that osteoporosis had no significant effect with respect to plaque and gingival bleeding but was associated with a significantly greater loss of attachment in osteoporotic women [10]. Passos et al. compared different diagnostic criteria for periodontal disease that were used to study the association between periodontal disease and osteoporosis [8]. The results from their work emphasize that the frequency of periodontal disease may be influenced by different outcome measurements associated between osteoporosis and periodontal disease. More recently, Takahasi et al. studied the association between periodontitis and systemic bone mineral density in Japanese community-dwelling postmenopausal women [13]. They evaluated the association between loss of attachment and bone mineral density (BMD) of the lumbar vertebrae and femur in 374 postmenopausal women aged 55–74 [13]. The results from this study demonstrated that a significant negative correlation was observed between periodontal disease and truncal bone BMD further confirming the relationship between osteoporosis and periodontal disease [13]. Further research groups studying populations from Jordan [12], India [9], and USA [11] demonstrate various relationships between periodontal disease and osteoporosis. Noteworthy, while a positive correlation is observed in some studies, other studies have failed to demonstrate either a relationship exists or have concluded that osteoporosis is not a causal factor in the development of moderate to severe chronic periodontitis [15, 16].

It may thus be concluded that the relationship between periodontal disease and osteoporosis is one area of research needing a more developed understanding. It is believed that osteoporosis is able to contribute to periodontal breakdown given that it may increase bone resorption and prevent proper healing ultimately increasing the severity of the pre-existing periodontal disease; however, very little evidence is available demonstrating such a relationship. Therefore, the aim of the present study was to investigate with an animal model the relationship between periodontal disease progression and osteoporosis. An ovariectomized rat was chosen as an osteoporosis model, and periodontal progression was induced by ligature placement of the rat mandibular first molars. Micro-CT and histological assessment were performed at 0, 3, 7, and 11 days following ligature placement in sham-operated and ovariectomized (OVX) animals to assess the rate of bone loss.

Materials and methods

Animal experiments

Sixty-four female Wistar rats (7 weeks of age, 180–200 g) were used in this study. At the start of the experiment, no clinical signs of gingival inflammation were observed among the animals. The animals were housed in individual wire cages in a temperature- and humidity-controlled room (23 ± 1 °C and 60 ± 5 % relative humidity) with a 12-h light/dark cycle as previously described [17]. All animals were allowed to acclimate to the laboratory environment for 1 week before surgical procedures were carried out. For 32 of the 64 animals, ovariectomy (OVX) surgery was performed at 8 weeks of age as previously described [18, 19]. Briefly, general anesthesia was achieved by intraperitoneal injection of chloral hydrate (10 %, 4 ml/kg body weight); the rats were operated with a 10-mm linear bilateral lumbar lateral skin incisions. Then, the enterocoelia was exposed by blunt dissection of muscle and peritoneum. The bilateral ovaries were removed gently following ligation of the ovarian artery and vein. Then, the small incisions were sutured stratified. Postoperatively, penicillin (40,000 IU/ml, 1 ml/kg) was injected for 3 days, and there was no sign of inflammation or other notable anomaly. Thirty-two control animals were subject to sham operations as previously described [20].

At 8 weeks following the surgery, cotton thread ligatures (no. 3-0) were tied around the cervixes of the bilateral first mandibular molar (M1) under anesthesia with chloralic hydras for all 64 rats. At time points 0, 3, 7, and 11 after ligature placement, 12 animals were euthanized (six control sham-operated animals with ligatures and six OVX animals with ligatures). The mandible was harvested and fixed in 10 % formalin solution for 48 h. After fixation, alveolar bone was examined using a micro-CT scanner and embedded in paraffin for histological analysis.

μCT scanning and analysis

A μCT imaging system (μCT50, Scanco Medical, Basersdorf, Switzerland) was used to scan the mandibular molars and alveolar bone of all animals. Scanning was performed at 70 kV and 114 μA with a thickness of 15 μm per slice in medium-resolution mode and 300-ms integration time. A Gaussian filter (sigma=0.8 and support=1) was used to remove noise. Vertical bone loss was evaluated by measuring the distance between the cemento-enamel junction (CEJ) and the alveolar bone crest (ABC) at six points (lingual-mesial, lingual-middle, lingual-distal, buccal-mesial, buccal-middle, buccal-distal) of the first mandibular molar. The architectures of interradicular bone of the first molar were evaluated by means of 3D reconstruction. An area with a height of 2.4 μm was defined as the 3D regions of interest (ROIs), starting coronally by a 2D slice 15 μm below the furcation of the first molar. Bone volume fraction was calculated from the bone volume (BV) and total volume (TV) as BV/TV. Trabecular thickness (Tb.Th) was calculated by applying the distance transformation by filling maximal spheres in the bone structures as determined by the micro-CT supplier protocol.

Histological analysis and tartrate resistant acid phosphatase stain

After fixation, the mandibles were decalcified in 10 % EDTA, dehydrated, embedded in paraffin, sectioned to 4-μm thickness, and stained with hematoxylin and eosin (H&E) as previously described [21]. Vertical alveolar bone loss of the first mandibular molar were assessed for inflammation and alveolar bone destruction as assessed by measuring the length (mm) of the CEJ to ABC between the first and second mandibular molars.

Then, tartrate-resistant acid phosphatase (TRAP) (Sigma #387A; Sigma-Aldrich, St. Louis, USA) was performed to determine osteoclast activity as previously described [21]. Analysis of the furcation area was assessed for the presence of osteoclasts as defined by cells with greater than three nuclei and counted at the various time points as previously described [22]. Samples were assessed for their staining intensity with respect to alveolar bone loss adjacent to the periodontal ligament in the furcation area along the PDL-bone perimeter.

Statistical analysis

All data analysis was performed using SPSS software and statistically significant values were adopted as $p < 0.05$. All experiments were displayed as mean and standard deviation (SD) and were calculated by one-way ANOVA and Newman Keuls t test.

Results

Micro-CT analysis

Micro-CT was performed to analyze the rate of alveolar bone loss in both sham-operated and OVX animals (Fig. 1, 64 total animals, six per group per time point). At day 0, the roots embedded within alveolar bone demonstrate no differences in the vertical bone loss as assessed by micro-CT (Fig. 2). Furthermore, cross-sectional analysis from the micro-CT images reveal large areas of bone in both sham-operated (Fig. 1e) and OVX groups (Fig. 1m) with no differences in furcation bone loss between sham-operated and OVX groups (Fig. 3). At 3 days following placement of the ligature on the first mandibular molar, small noticeable changes were observed specifically on the distal root where the micro-CT images begin to demonstrate slight bone loss (Fig. 1b, i). Significantly greater vertical bone loss was observed between OVX and sham-operated animals, and this observation was maintained throughout the remaining study time points ($p < 0.01$, Fig. 2). At both 3 and 7 days, no significant differences in furcation bone loss were observed (Fig. 3). By 11 days, micro-CT images depict significantly greater bone loss in both vertical height and within the furcation in the OVX group ($p < 0.01$, Figs. 1l, 2, and 3) when compared to sham-operated animals (Fig. 1d). Cross-sectional analysis from micro-CT further reveals the loss of bone volume in the furcation area (Fig. 1p), and computation analysis from the micro-CT demonstrated that BV/TV as well as trabecular thickness is now significantly reduced in the furcation area in OVX animals when compared to control sham-operated animals ($p < 0.01$, Fig. 3).

Histological analysis

Histological analysis was performed in the furcation areas of first mandibular molars to further confirm the rate of bone loss between OVX and sham-operated animals as well as to assess osteoclast activity via TRAP staining. The histological analysis demonstrates that both sham-operated and OVX animals show healthy bone in furcation areas with little to no changes between groups (Fig. 4a, e). At 3 days postligature placement, noticeable changes in the morphology of trabecular bone became apparent with loss of bone beginning in the furcation area in both sham-operated and OVX animals demonstrating the effectiveness of the ligature-induced periodontitis model (Fig. 4b, f). At 7 days, this trend was also consistent with more bone loss taking place in the OVX group (Fig. 4c, g). By 11 days, a significantly greater amount of bone was lost in the furcation areas between OVX and sham-operated groups further confirming the micro-CT results (Fig. 3).

Then, histological quantification was used to measure the rate of vertical bone loss by calculating the distance between the CEJ and the ABC between the first and second mandibular
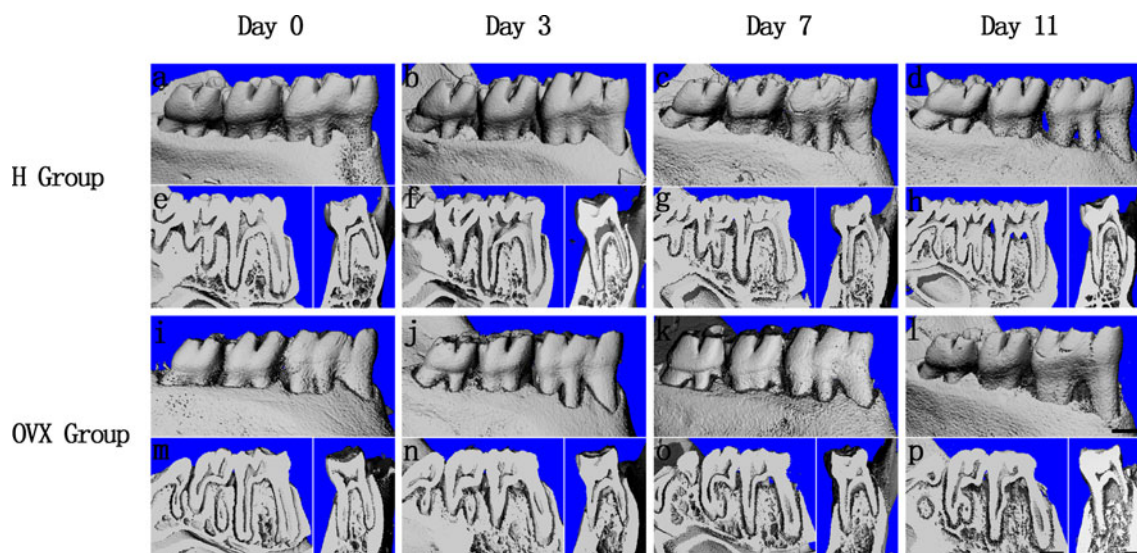
Fig. 1 μCT images of alveolar bone loss in both sham-operated and OVX animals at 0, 3, 7, and 11 days following ligature-induced periodontitis (*bar*= 1 mm). Lingual view (*a, b, c, d, i, j, k,* and *l*) and cross sections (*e, f, g, h, m, n, o,* and *p*) of mandibles were observed for both groups

molars (Fig. 5). The histological measures further confirm micro-CT results by demonstrating a significantly greater loss of vertical bone at 3, 7, and 11 days postligature placement ($p<0.05$, Fig. 5). TRAP staining was then utilized to assess the involvement of osteoclast activity (Fig. 6). It was observed that at 3 days following ligature placement, osteoclast activity peaked with a significantly higher number of multinucleated cells and staining intensity observed in the OVX group when compared to control sham-operated samples at all time points ($p<0.01$, Fig. 6c).

Discussion

The aim of the present study was to investigate the relationship between osteoporosis and periodontal disease. There

Fig. 2 Representative bar graph demonstrating vertical bone loss of the first mandibular molars as assessed by μCT images. Significantly greater vertical bone loss is observed in OVX animals at 3, 7, and 11 days postligature-induced periodontitis. Data are expressed as mean ± standard deviation (mm) (**p<0.05*)

exists accumulating evidence that a relationship may exist [8–14]; however to date, no animal or histological study has fully confirmed this possible relationship. Therefore, we sought to utilize an animal model to perform histological assessment and to characterize alveolar bone loss in an osteoporotic model by creating OVX animals and comparing them to control sham-operated animals for periodontal changes from 0 to 11 days following a ligature-induced periodontitis animal model.

The animal model chosen was selected as it has a long history of being utilized to examine periodontal change in periodontitis [23–25]. It is noteworthy to mention that in the present study, periodontal breakdown was calculated by evaluating alveolar bone loss as the key changes associated between mucositis and periodontitis are the loss of alveoloar bone and the direct clinical attachment loss was not calculated. Instead, the distance between the CEJ and the alveolar bone crest were measured to represent this parameter. The model was confirmed as the rate of periodontal change even in sham-operated animals was significantly altered following the placement of the ligature on the first mandibular molar thus demonstrating its effectiveness as a study model. It was observed that within only 3 days, bone loss had occurred in both control sham-operated and OVX animals confirming the fast rate of bone loss in the present animal model (Fig. 2). The group containing OVX animals without a ligature-induced model was not considered as the rate of periodontal breakdown is thought to be insignificant. As the OVX animals are left for 8 weeks following removal of their ovaries with no bone loss present, including an additional group allowing an additional 10 days of induced OVX rats was not considered. At day 0, no difference in alveolar bone loss or tissue inflammation was apparent in OVX animals when compared to
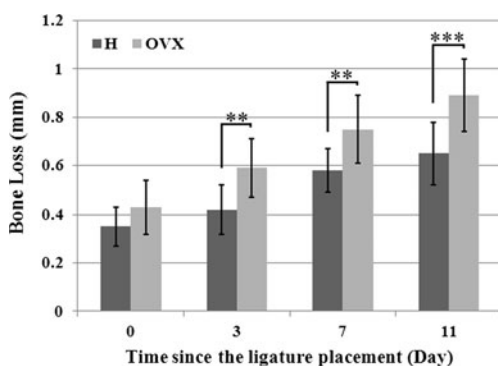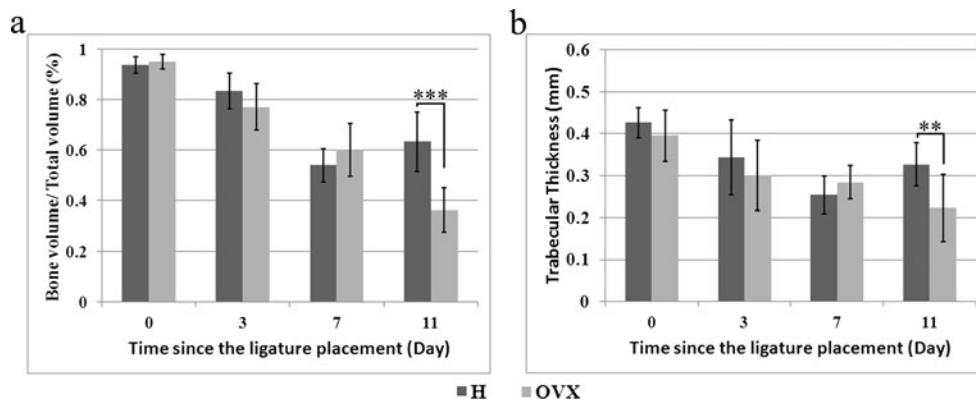
Fig. 3 **a** Bone volume/total volume and **b** trabecular thickness of alveolar bone located within the furcation area of the first mandibular molar. While no significant difference was observed between 0 to 7 days however at 11 days postligature-induced periodontitis, both BV/

TV and trabecular thickness were significantly reduced in OVX animals when compared to control. Data are expressed as mean ± SD (mm) (*$p < 0.05$)

control sham-operated animals. Including an additional 32 animals to the experimental design was thus not warranted as the rate of periodontal breakdown and alveolar bone loss is considered to be negligible. The changes occurring in the present model are apparent once the ligature-induced state was placed with more alveolar bone loss taking place in OVX animals.

The results from the present study also demonstrate the severe complication associated with the progression of both diseases. Although the present results are drawn from an animal model with a much shorter life expectancy than humans, the investigation demonstrates how quickly the degeneration of periodontal tissues may arise with a simultaneous combination of both periodontal disease and osteoporosis. It is known that periodontal disease is thought to contribute to osteoclast activation

[26–28] and the combination with a disease such as osteoporosis known to cause a hyper-activity of osteoclasts is subject to pose many future problems. Furthermore, it is known that both osteoporosis and periodontal disease are age-related diseases and the increasing life expectancy in humans is thought to contribute to both diseases. As patients become older, bone loss tends to increase with little therapy available to re-establish proper bone volume. Similarly, as patient age increases, their dexterity decreases making it increasingly difficult to brush their teeth efficiently and maintain proper oral hygiene. Thus, the combination of these two age-related disorders is one that necessitates full clinical attention and involvement as patients reach the later years of their lives.

We also studied the time necessary to reach the tooth furcation, an area specifically located between

Fig. 4 Representative sections of H&E staining demonstrate periodontal breakdown of sham-operated (sham, *a–e*) and ovariectomized (OVX, *f–j*) rats at 0, 3, 7, and 11 days postligature-induced periodontitis. More alveolar bone loss is observed in the OVX group at 11 days as depicted by the loss of bone in the furcation area of first mandibular molars (*j*). *Black scale bar*: 200 μm
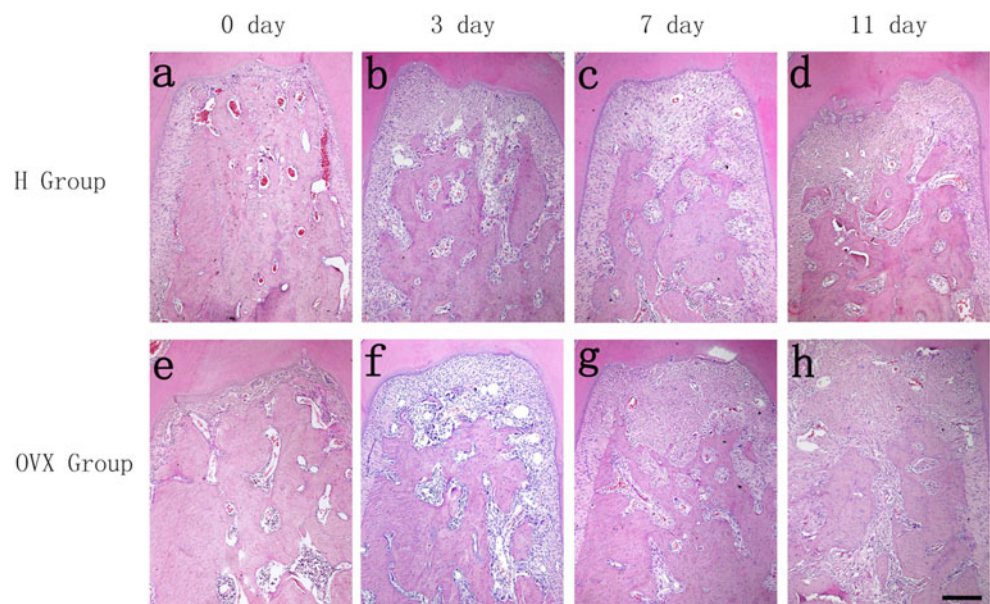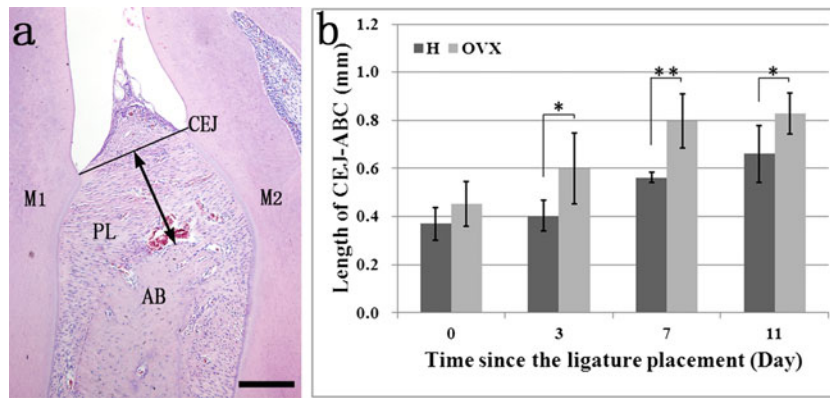
Fig. 5 The mandibular vertical alveolar bone loss in the interproximal region assessed histologically. **a** Histological appearance of the region between the first molar (M1) and second molar (M2) roots (*bar*= 200 μm). The *double-headed arrow* shows the vertical distance from the tooth roots (Fig. 5). Once bone loss exceeds the alveolar bone crest to the amelocemental junction. *CEJ* cemento-enamel junction, *AB* alveolar bone, *PL* periodontal ligament. **b** Quantitative analysis of interproximal bone loss assessed in histological sections. Data are expressed as mean ± SD (mm) (*$p<0.05$, **$p<0.01$)

the tooth roots (Fig. 5). Once bone loss exceeds the furcation area, it is known that the regenerative potential of these teeth are much more limited with no guarantee that even with the use of the best available periodontal growth factors, these teeth maintain many uncertainties involving their ability to be restored [29–31]. Thus, the results from the present study demonstrate that there is a small window from which patients can change/modify their habits which may facilitate their necessary future periodontal regeneration further illustrating the need for medical practitioners to be aware of the patient's periodontal status and act upon the diseased state in a timely manner before furcation involvement is reached.

Fig. 6 Alveolar bone resorption through TRAP(+) osteoclast identification. Multinucleated osteoclast-like cells (*black arrows*) were observed at the PDL-bone interface for SHAM (**a**) and OVX (**b**) groups at 3 days following ligature placement (*bar*=200 μm). *AB* alveolar bone, *D* dentin, *C* cementum, *PL* periodontal ligament. **c** The number of multinucleated osteoclasts within root furcation of the first molar (M1) was manually counted along the PDL-bone perimeter, and ratios of osteoclast count to perimeter were calculated (*$p<0.05$, **$p<0.01$, ***$p<0.001$)
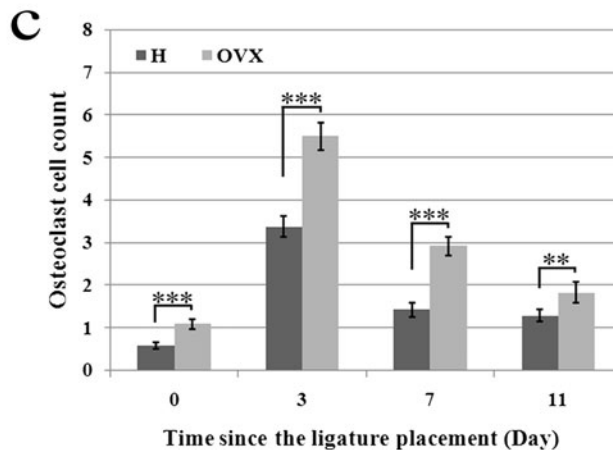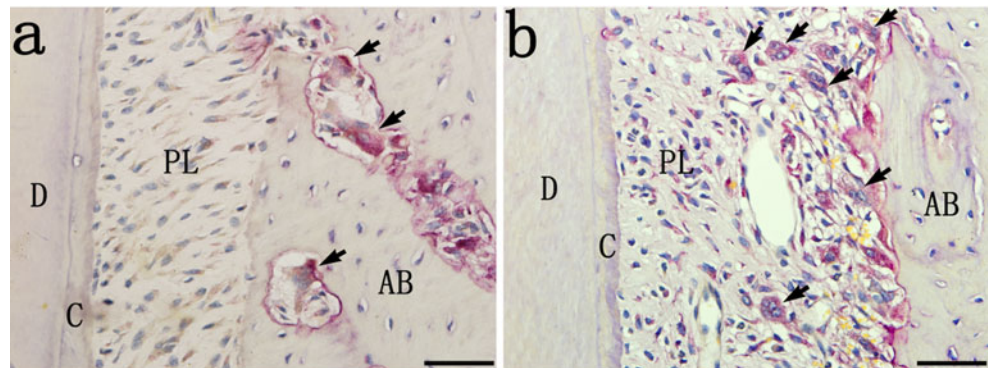
Conclusion

The results from the present investigation demonstrate a correlation between osteoporosis and bone loss following ligature placement. Although osteoporosis is not thought to induce periodontal disease, we show in the present study that the disease progression was apparent in OVX animals by demonstrating significantly greater vertical bone loss, furcation bone loss, and loss of alveolar bone crest height when compared to sham-operated animals. The histological analysis reveal that OVX animals were associated with higher TRAP staining indicative that osteoclast activity was highly active in periodontal tissues when compared to their respective time point controls.

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