

# Bis-enoxacin blocks alveolar bone resorption in rats with ovariectomy-induced osteoporosis

XI ZHANG<sup>1\*</sup>, XINCHEN XU<sup>1,2\*</sup>, XUQIANG LIU<sup>3\*</sup>, CHUANYUAN MAO<sup>1</sup>, AN QIN<sup>4</sup> and ERYI LU<sup>1</sup>

<sup>1</sup>Department of Stomatology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127; <sup>2</sup>Department of Stomatology, Xuzhou Central Hospital, Xuzhou, Jiangsu 221009; <sup>3</sup>Department of Orthopedics, The First Affiliated Hospital of Nanchang University, The Artificial Joint Engineering and Technology Research Center of Jiangxi Province, Nanchang, Jiangxi 330200; <sup>4</sup>Department of Orthopedics, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Orthopedic Implants, Shanghai 200011, P.R. China

Received May 16, 2016; Accepted April 7, 2017

DOI: 10.3892/mmr.2017.8223

**Abstract.** Postmenopausal osteoporosis is a common systemic skeletal disease that is associated with estrogen-deficiency. Bone loss associated with bisphosphonates therapy can increase the risk of developing oral osteonecrosis. Recent studies have indicated that enoxacin may inhibit osteoclast formation and bone resorption via a different mechanism from that of bisphosphonates. Therefore, the authors hypothesized that the use of an enoxacin such as bis-enoxacin (BE) in association with bisphosphonates may be effective in the treatment of postmenopausal osteoporosis-associated alveolar bone resorption and reduce the risk of oral osteonecrosis by allowing the dose of bisphosphonates to be reduced. A total of 30 6-month-old female Sprague-Dawley rats were randomly assigned to five groups: The Sham, Vehicle, zoledronic acid (ZOL), low concentrations of BE (BE-L) and high concentrations of BE (BE-H) groups. The results demonstrated that the ZOL, BE-L and BE-H groups had an increased bone volume/tissue volume, trabecular thickness, mineral apposition rate, mineralizing surface/bone surface and a decreased trabecular separation when compared with the Vehicle group. The microscopic evaluation of histological sections clearly supported the results of the

micro-computed tomography. The number of tartrate-resistant acid phosphatase-positive osteoclasts was markedly decreased in the ZOL, BE-L and BE-H groups, indicating that BE may inhibit osteoclast formation. The anti-resorptive effect in the BE-H group was close to or better than that exhibited by the ZOL group; however, this effect was poorer in the BE-L group. In conclusion, BE has the potential to block alveolar bone resorption resulting from ovariectomy-induced osteoporosis in rats in a dose-dependent manner.

## Introduction

Oral health status has become one of the most important determinants of quality of life in elderly people for a variety of physical, social and psychological reasons (1). Elderly individuals are now increasingly more likely to seek dental treatment, including implant restoration, periodontal and orthodontic treatment (2). All of these treatments involve the alveolar bone, which is the major tissue supporting the teeth. Osteoporosis is a common systemic skeletal disease associated with aging (3). The effect of osteoporosis on the alveolar bone and on oral health in general has been the focus of clinical dentistry and of basic research for a number of years. A number of studies have provided evidence that osteoporosis can affect human alveolar bones (4-6). For example, osteoporotic women had less mandibular bone mass and density and a thinner cortex at the gonion compared with non-osteoporotic women (5).

The most destructive effects of alveolar bone resorption result from postmenopausal osteoporosis induced by excessive osteoclast activation (7). Therefore, it is plausible that anti-resorptive agents could be employed in patients with postmenopausal osteoporosis to prevent alveolar bone resorption (8). Pharmacological agents for the treatment of osteoporosis may be classified as either antiresorptive or anabolic, and mainly include estrogen, selective estrogen-receptor modulators (SERMs), bisphosphonates, denosumab and teriparatide (9). Concerns regarding the nonskeletal risks associated with estrogen use, including breast cancer and coronary, cerebrovascular and thrombotic events, have led to recommendations against using estrogen as a first-line

---

*Correspondence to:* Professor Eryi Lu, Department of Stomatology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, 1630 Dongfang Road, Shanghai 200127, P.R. China  
E-mail: lueryi222@126.com

Dr An Qin, Department of Orthopedics, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai Key Laboratory of Orthopedic Implants, 639 Zhizaojiu Road, Shanghai 200011, P.R. China  
E-mail: dr\_qinan@163.com

\*Contributed equally

**Key words:** bis-enoxacin, ovariectomy, osteoporosis, alveolar bone resorption, osteoclast

therapy for osteoporosis (10). Raloxifene, a SERM that has been approved by the Food and Drug Administration (FDA) to treat osteoporosis, increases the risk of thromboembolic events (11,12). The long-term use of bisphosphonates presents a risk for the development of oral osteonecrosis and atypical femoral fracture (13,14). Denosumab inhibits bone resorption by binding to the receptor activator of nuclear factor- $\kappa$ B ligand, thereby decreasing the differentiation of osteoclasts. The most common side effects of denosumab include urinary and respiratory tract infections, cataracts, constipation, rashes and joint pain (15). Teriparatide is an anabolic agent that functions primarily by increasing bone formation rather than by decreasing resorption (16). Ongoing toxicology studies in rats have demonstrated that a longer duration and larger dose of therapy is a risk factor for osteosarcoma (9,16). For this reason, the FDA has limited the use of teriparatide to 2 years and use is not permitted in patients with a history of Paget's disease or any type of cancer. Therefore, the authors hypothesized that a novel class of anti-resorptive agents that inhibit osteoclast activation via a mechanism different from that of the currently used bisphosphonates may be beneficial in reducing the occurrence of oral osteonecrosis.

Enoxacin, a fluoroquinolone antibiotic, is widely used to treat patients with bacterial infections. A recent study has indicated that enoxacin can inhibit osteoclast formation and bone resorption by blocking the interactions between the V-ATPase B2-subunit or V-ATPase  $\alpha$ 3-subunit and microfilaments in osteoclasts (17). Enoxacin has a stronger affinity for bone than bisphosphonates, and inhibits bone resorption via a mechanism different from that of bisphosphonates. Therefore, it is possible that a combination of bisphosphonates and enoxacin in the form of bis-enoxacin (BE) could be used to treat alveolar bone resorption in postmenopausal osteoporosis. The use of BE is advantageous as it reduces the risk of oral osteonecrosis with bisphosphonates and dysbacteriosis with enoxacin.

BE is a bisphosphonate derivative of enoxacin. Enoxacin is widely used to treat patients with chronic infections associated with osteomyelitis. However, enoxacin has a weaker ability to bind to, concentrate in, and/or be retained by infected bone, a site often difficult to treat clinically. Therefore, in 2002, Herczegh *et al.* (18) synthesized a novel antibacterial drug, BE, by conjugating bone-binding bisphosphonate groups to enoxacin. However, whether BE is able to prevent osteoporosis *in vivo* has yet to be elucidated. Based on this research, our group proposed the hypothesis that BE is able to prevent osteoporosis *in vivo* via the anti-bone resorptive property of bisphosphonates and enoxacin. Hence, the aim of the present study was to investigate the effect of BE on the maxillary alveolar bone in a rat model of osteoporosis.

## Materials and methods

**Animals and study design.** All animal care and experimental procedures were carried out in strict accordance with the recommendations provided in the Guide for Ethical Conduct in the Care and Use of Nonhuman Animals in Research produced by the American Psychological Association (<http://www.apa.org/science/leadership/care/guidelines.aspx>) and were approved by the Animal Care Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of

Medicine [Shanghai, China; HKDL (2014)125]. A total of 30 6-month-old female Sprague–Dawley rats (approximately 300–330 g) were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All rats were housed in filter-top cages in a temperature and humidity controlled room ( $23\pm 1^\circ\text{C}$  and  $60\pm 5\%$ , respectively) with a 12-h light/dark cycle and free access to food and water.

The rats were randomly divided into 5 groups of 6 rats each: Group 1, Sham saline control (Sham); group 2, ovariectomized (OVX) group (Vehicle); group 3, OVX rats treated with  $50\ \mu\text{g}/\text{kg}/\text{day}$  zoledronic acid (ZOL); group 4, OVX rats treated with  $50\ \mu\text{g}/\text{kg}/\text{day}$  BE (BE-L; low concentration group); group 5, OVX rats treated with  $100\ \mu\text{g}/\text{kg}/\text{day}$  BE (BE-H; high concentration group).

The rats were either sham operated or bilaterally OVX through a vertical dorsal incision. In the OVX groups (groups 2–5), the bilateral ovaries were ligated and ablated under aseptic conditions. The remaining rats in the Sham group were subjected to sham operations in which the ovaries were not removed. At 8 weeks following the ovariectomy or Sham surgery, rats in the Sham and Vehicle groups were injected intraperitoneally with vehicle (0.9% saline) and used as controls. Rats in the ZOL group were treated with  $50\ \mu\text{g}/\text{kg}/\text{day}$  ZOL (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and those in BE-L and BE-H groups received 50 and  $100\ \mu\text{g}/\text{kg}/\text{day}$  BE (SynQuest Laboratories, Inc., Alachua, FL, USA), respectively. All treatments were performed on alternate days over a period of 8 weeks (Table I). At 16 weeks post-surgery, all rats were sacrificed by exsanguination under 10% chloral hydrate anesthesia and both sides of the maxillae were removed from each rat. The surgical procedures were performed according to FDA guidelines (19). All rats were housed under the conditions stated above for 16 weeks and body weight was measured once a month during the course of the study.

A polychrome sequential fluorescent bone labeling method was performed to label the mineralized tissue and to assess the time course of new bone mineral apposition rate (MAR) and mineralizing surface/bone surface (MS/BS) of the maxilla alveolar bone. Following the initiation of BE, ZOL or vehicle treatment, rats were injected intraperitoneally with  $10\ \mu\text{g}/\text{kg}$  calcein (Sigma-Aldrich; Merck KGaA) on day 20 and  $20\ \mu\text{g}/\text{kg}$  alizarin red (Sigma-Aldrich; Merck KGaA) on day 40.

**Micro-computed tomography (CT) scanning and assessment of the alveolar bone.** The two sides of the maxillae were scanned using a high-resolution micro-CT scanner ( $\mu\text{CT}80$ ; Scanco Medical, Brüttisellen, Switzerland). The scanning protocol was set using an isometric resolution of  $20\ \mu\text{m}$ , and X-ray energy settings of 70 kV and 1,170 mA. Scans were reconstructed to generate three-dimensional digitized models, and microstructure parameters of bone volume/tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp) were calculated in a three-dimensional region of interest (ROI) using a calculated Hounsfield Unit grayscale threshold value. The ROI was a cuboidal bone body that encompassed the roots of M1 and M2. The length of the ROI extended from the most mesial aspect of the M1 root to the most distal aspect of the M2 root. The width of the ROI extended from the most buccal aspect of either root of the two molars to the most palatal aspect of either root.

Table I. Experimental design and the associated treatments in each group of rats.

Group no.	Surgery type	Treatment type	Dose applied	Duration	Group abbreviation
1	Sham	Vehicle	Saline	8 weeks	Sham
2	OVX	Vehicle	Saline		Vehicle
3	OVX	Zoledronic acid	50 $\mu\text{g}/\text{kg}/\text{day}$ ZOL		ZOL
4	OVX	Low concentration of BE	50 $\mu\text{g}/\text{kg}/\text{day}$ BE		BE-L
5	OVX	High concentration of BE	100 $\mu\text{g}/\text{kg}/\text{day}$ BE		BE-H

OVX, ovariectomy; BE, bis-enoxacin; ZOL, zoledronic acid; BE-L, low concentration of bis-enoxacin (50  $\mu\text{g}/\text{kg}/\text{day}$ ); BE-H, high concentration of bis-enoxacin (100  $\mu\text{g}/\text{kg}/\text{day}$ ).

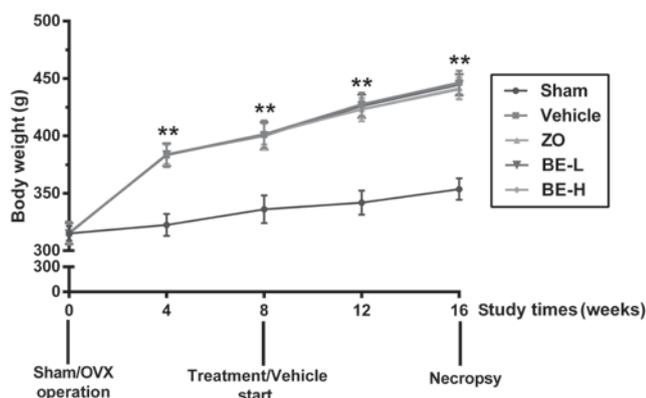


Figure 1. Monthly body weight results for rats receiving OVX or Sham surgery. OVX, ovariectomy; ZOL, zoledronic acid; BE-L, low concentration of bis-enoxacin (50  $\mu\text{g}/\text{kg}/\text{day}$ ); BE-H, high concentration of bis-enoxacin (100  $\mu\text{g}/\text{kg}/\text{day}$ ).

The height of the ROI extended from the most apical aspect of either root to the most coronal part of the alveolar bone crest. The ROI was generated as an indicator of the border of the volumetric analysis. The 3D reconstructed images were processed to 2D parasagittal images, with a single investigator drawing the outline of the desired alveolar bone region in order to maximize the quantification of bone and minimize the inclusion of roots.

**Bone histology.** Following the completion of micro-CT scanning, decalcified sections were prepared from the left maxilla specimens and undecalcified sections were prepared from the right maxilla specimens.

The left maxillae specimens were decalcified with 10% ethylenediaminetetraacetic acid disodium salt for ~2 months and then embedded in paraffin. Sections were prepared from the occlusal surface of the tooth crown to the alveolar bone mesiodistally along a plane parallel to the long axis of the tooth, and then cut into 5- $\mu\text{m}$  thick serial sagittal sections. Half of these sections were stained with hematoxylin (200  $\mu\text{g}/\text{l}$ ) and eosin (500  $\mu\text{g}/\text{l}$ ; H&E; Sigma-Aldrich; Merck KGaA) at 37°C for 2 h for descriptive analysis. The remaining sections were stained with tartrate-resistant acid phosphatase (7%; TRAP; Sigma-Aldrich; Merck KGaA) at 37°C for 2 h to identify osteoclasts on the bone surface. Trabecular bone volume fractions (expressed as the percentage of BV/TV)

in H&E-stained sections were analyzed by Image-Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) using an IX71 inverted microscope (Olympus Corporation, Tokyo, Japan). The average number of TRAP-positive multinucleated osteoclasts per  $\text{mm}^2$  was counted by randomly selecting 5 regions of each section.

The right maxilla specimens were processed and embedded without decalcification in methyl methacrylate (35g/l; MMA; M55909; Sigma-Aldrich; Merck KGaA) at 37°C for 1 month. A confocal laser-scanning microscope (Leica TCS SP5; Leica Microsystems GmbH, Wetzlar, Germany) was used to examine the fluorescence labeling of the undecalcified sections (~20  $\mu\text{m}$ ). The excitation/emission wavelengths used for fluorescence were 488/517 nm (calcein) and 543/617 nm (alizarin red). Three section slides from each specimen were selected for histomorphometric analysis. Trabecular bone dynamic parameters were measured in a 1- $\text{mm}^2$  square area positioned at the inter-radicular region of M1 at magnification, x200, using calcein and alizarin red as labels. The dynamic parameters included single-label perimeter (sL.Pm), double-label perimeter (dL.Pm), trabecular bone perimeter (Tb.Pm) and interlabel width (Ir.L.Wi). The mean Ir.L.Wi, was obtained dynamically by averaging the distances between points randomly selected from 5 intervals. MAR (The mean Ir.L.Wi/interval period,  $\mu\text{m}/\text{day}$ ) was measured using BioQuant OSTEO II software (version 8.00.20; BioQuant Image Analysis Corporation, Nashville, TN, USA). The mineralizing surface [MS/BS=(dL.Pm + sL.Pm/2)/Tb.Pm x 100%] was analyzed using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.). The sections were then stained with van Gieson's picro fuchsin (Sigma-Aldrich; Merck KGaA) at 37°C for 4 h for histological observation.

**Statistical analysis.** Results are expressed as the mean  $\pm$  standard deviation. One-way analysis of variance followed by the Bonferroni post hoc test was used to measure statistically significant differences among groups. SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all analyses.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Body weight.** Monthly records of body weight are shown in Fig. 1. The rats in each group had a similar initial mean body

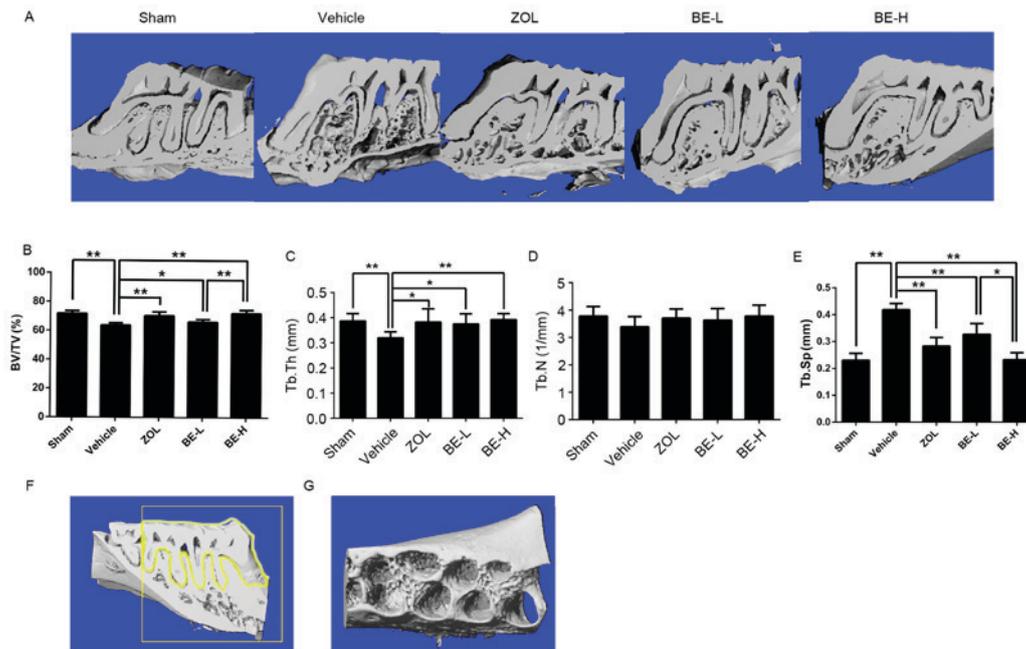


Figure 2. The effect of BE on the maxilla assessed by micro-CT analysis of bone volumetric parameters. (A) Longitudinal-sectional micro-CT images of the first molar (M1) and the second molar (M2) in the maxilla. Analysis of micro-CT volumetric parameters: (B) BV/TV, (C) Tb.Th, (D) Tb.N and (E) Tb.Sp. (F) The contour of the desired alveolar bone region was transposed on to two-dimensional parasagittal images. (G) The region of interest was a cuboidal bone body that encompassed the roots of the M1 and M2 molars. Values are expressed as the mean  $\pm$  standard deviation (n=6). \*P<0.05 and \*\*P<0.01 cf. Vehicle. BE, bis-enoxacin; CT, computed tomography; BV/TV, bone volume/tissue volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; ZOL, zoledronic acid; BE-L, low concentration of bis-enoxacin (50  $\mu$ g/kg/day); BE-H, high concentration of bis-enoxacin (100  $\mu$ g/kg/day).

weight (315 $\pm$ 10 g) and the difference between the groups was insignificant. Ovariectomy generally resulted in weight gain. At 4, 8, 12 and 16 weeks post-ovariectomy, the body weights of the Vehicle group were 383.25 $\pm$ 10.3, 401.42 $\pm$ 11.53, 427.58 $\pm$ 10.1 and 446.5 $\pm$ 10.16 g, respectively. When compared with the Sham group at the same time points, these differences were all significant (all P<0.01). The trend in body weight among the ZOL or BE administration groups (groups 3-5) and the Vehicle group was similar throughout the course of the experiment and the differences between them were not statistically significant.

#### Micro-CT analysis of alveolar bone microarchitecture.

The three-dimensional images provided a clear view of the microarchitecture of the inter-radicular alveolar bone in rats. Statistical analysis of the microarchitectural parameters demonstrated that the BV/TV and Tb.Th were significantly reduced, whereas Tb.Sp was significantly increased, in the Vehicle group when compared with the Sham group (P<0.01; Fig. 2B, C and E). Notably, no significant difference in Tb.N was identified between the groups (P>0.05; Fig. 2D). The trabecular and cortical thicknesses were greater in the Sham group than in the Vehicle group. Taken together, these results indicate that there was a loss of bone and deterioration of the trabeculae of the alveolar bone in the OVX rats. The ZOL, BE-L and BE-H groups had an increased BV/TV and Tb.Th, and a decreased Tb.Sp, when compared with the Vehicle group (P<0.05; Fig. 2B, C and E). There was a statistically significant difference between the BE-L and BE-H groups in BV/TV (P<0.01; Fig. 2B) and Tb.Sp (P<0.05; Fig. 2E). The BE-H group had a significantly lower Tb.Sp value compared with the ZOL group (P<0.05; Fig. 2E).

*Histomorphometric and histological analysis of the alveolar bone.* Analysis of the van Gieson-stained sections and H&E-stained sections from the maxilla revealed that the trabecular bone was affected by the ovariectomy operation, with the Sham group having an increased bone marrow volume when compared with the Vehicle group (Fig. 3A). The alveolar bone from the Sham group had a thicker interconnected trabecula and in the Vehicle group the alveolar bone exhibited lower connectivity and a thinner trabecular; however, it did exhibit an abundance of bone marrow (Fig. 3A). The amount of bone tissue was higher in all of the ZOL and BE administration groups when compared with the Vehicle group, indicating a potential protective effect of BE administration in the preservation of alveolar bone (Fig. 3A). In the H&E-stained sections, analysis of the trabecular bone fraction (percentage of BV/TV) revealed a higher value for the Sham group when compared with the Vehicle group (~15%; Fig. 3B). The trabecular bone area increased in the ZOL, BE-L and BE-H groups when compared with the Vehicle group (~12, 8 and 14%, respectively; Fig. 3B). The microscopic evaluation of the histological sections clearly supported the results of the micro-CT.

Osteoclasts were defined as multinuclear cells that were stained wine-red by TRAP staining (Fig. 3A). In alveolar bone, osteoclasts were observed lining the bone surface of the marrow space or in the periodontal ligament. While the osteoclasts were densely clustered in the Vehicle group, a significant decrease was observed in the ZOL and BE administration groups when compared with the Sham group, where only sporadic osteoclasts were visible (Fig. 3A). The number of TRAP-positive osteoclasts were significantly decreased in the ZOL, BE-L and BE-H groups when compared with the Vehicle

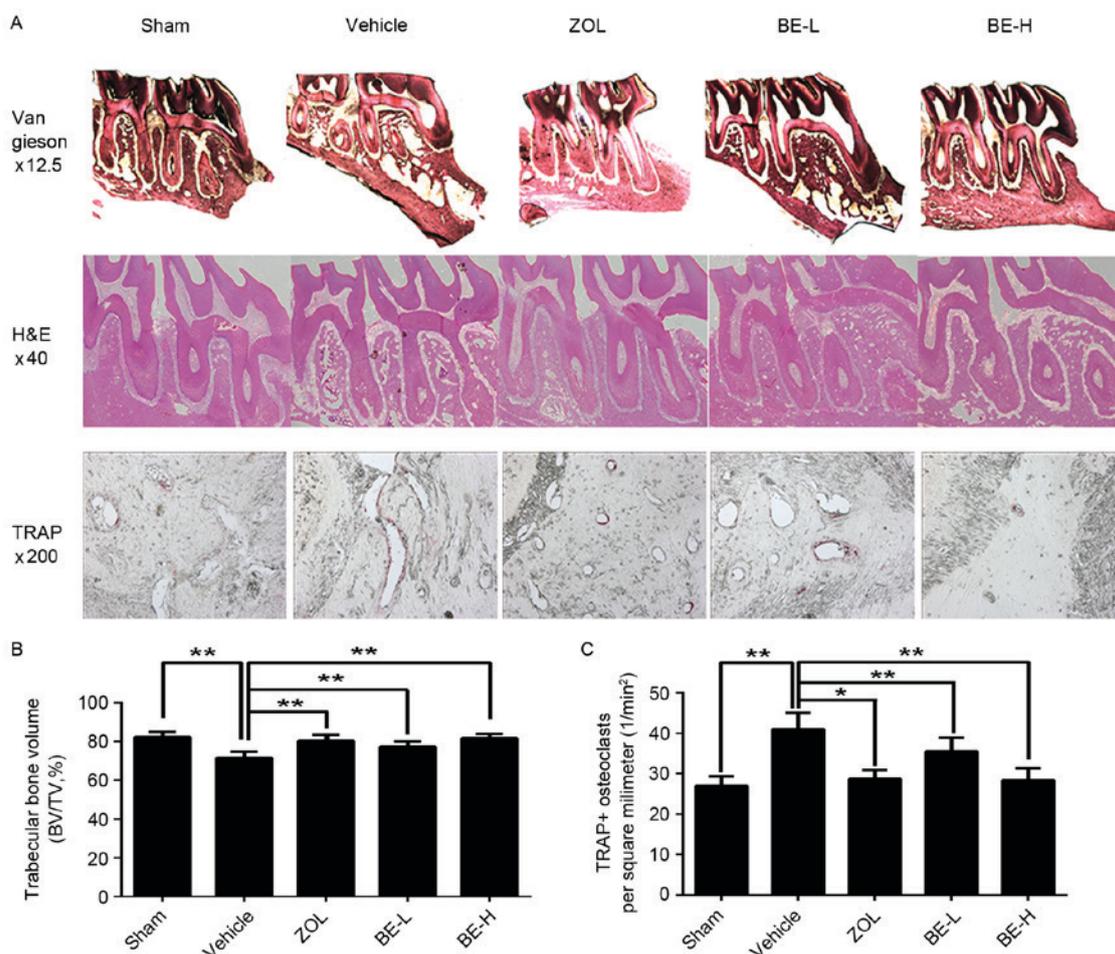


Figure 3. Histomorphometric and histological analysis of the alveolar bone. (A) The images show sagittal sections of each group using van Gieson staining (magnification,  $\times 12.5$ ), H&E staining (magnification,  $\times 100$ ) and TRAP staining (magnification,  $\times 200$ ). (B) Analysis of the trabecular bone fraction in the H&E-stained sections. (C) Analysis of the number of osteoclasts in a  $1 \text{ mm}^2$  area. Values are expressed as the mean  $\pm$  the standard deviation ( $n=6$ ).  $^*P<0.05$  and  $^{**}P<0.01$  cf. Vehicle. H&E, hematoxylin and eosin; TRAP, tartrate-resistant acid phosphatase; ZOL, zoledronic acid; BE-L, low concentration of bis-enoxacin ( $50 \mu\text{g}/\text{kg}/\text{day}$ ); BE-H, high concentration of bis-enoxacin ( $100 \mu\text{g}/\text{kg}/\text{day}$ ).

group ( $\sim 30$ ,  $13$  and  $31\%$ , respectively), suggesting that BE may inhibit osteoclast formation ( $P<0.05$  and  $P<0.01$ ; Fig. 3C).

**Fluorochrome microscopy.** The deposition of mineralized bone matrix was observed, which was demonstrated by calcein (green) and alizarin red S (red) staining. The distance between the labels decreased notably in the Vehicle group compared with the other four groups (Fig. 4A). The Vehicle group exhibited a significantly lower MAR and MS/BS when compared with the Sham group ( $\sim 38$  and  $55\%$ , respectively; Fig. 4B and C). A significantly increased MAR and MS/BS were observed in the ZOL, BE-L and BE-H groups when compared with the Vehicle group ( $\sim 101$ ,  $52$  and  $131\%$ , and  $355$ ,  $124$  and  $382\%$ , increases, respectively; Fig. 4B and C).

## Discussion

Ovarian hormone deficiency leads to postmenopausal osteoporosis as a result of increased bone turnover and a rapid loss of cancellous bone (20,21). However, the rate and magnitude of such changes vary markedly between different skeletal sites (22,23). Alveolar bone is an irregular protuberance of the jawbone that encompasses the roots of the teeth. Although

alveolar bone forms a relatively small part of the bone system, it serves the most important role in supporting the teeth, and its anatomic study is therefore important for therapies including implant restoration, periodontal, endodontic, orthodontic and maxillofacial treatments (24). As a consequence, research into the connection between postmenopausal osteoporosis and alveolar bone is receiving an increasing amount of attention. A previous study examined the effects of osteoporosis on periodontal disease, subsequent tooth loss and on the capacity of the maxilla and mandible to integrate endosseous dental implants (25). In orthodontics, consolidation therapy in estrogen-deficient patients usually takes longer, and relapse and therapeutic failures are more common (26). Osteoporosis in the jaw may present a risk for accentuation of alveolar bone loss in patients with full dentures (27). Taken together, these findings suggest that postmenopausal osteoporosis may have a substantial effect on alveolar bone. Thus, identifying therapeutic agents to alleviate osteoporosis in alveolar bone would have significant clinical value. To investigate this, the present study established an OVX rat model; such models have been widely used to study postmenopausal osteoporosis in humans and have helped to reveal the underlying mechanisms.

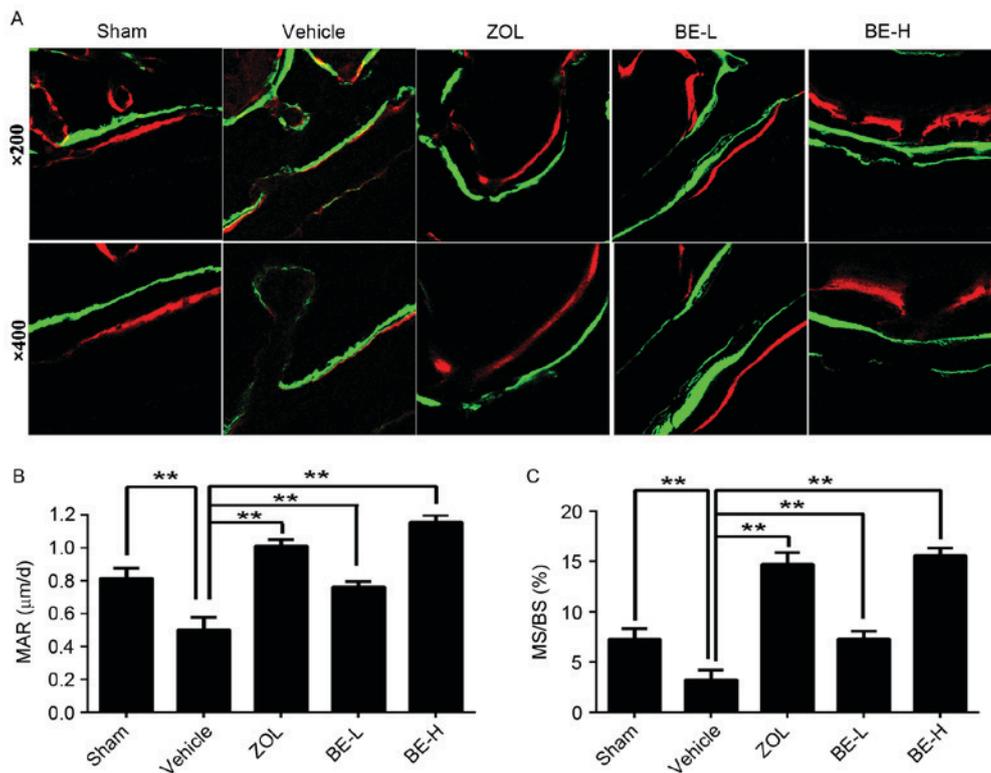


Figure 4. New alveolar bone formation and mineralization were determined by fluorescence analysis. (A) Analysis of the alveolar bone. Images of sequential fluorescently labeled maxilla bone, showing Alizarin red (red) and calcein (green) staining. (B) Analysis of (MAR) and (C) MS/BS. Values are expressed as the mean  $\pm$  standard deviation (n=6). \*\*P<0.01. MAR, bone mineral apposition rate; MS/BS, mineralizing surface/bone surface; ZOL, zoledronic acid; BE-L, low concentration of bis-enoxacin (50  $\mu$ g/kg/day); BE-H, high concentration of bis-enoxacin (100  $\mu$ g/kg/day).

The primary purpose of the present study was to characterize the alveolar bone changes in the maxilla of OVX rats by simultaneously assessing micro-CT and histomorphometry. The micro-CT results revealed a significant decrease in the BV/TV, and a significant increase in the Tb.Th and Tb.Sp, parameters in the Vehicle group when compared with the Sham group. No significant differences between the groups were observed in the microarchitecture parameter of Tb.N (P>0.05). This suggests that ovariectomy may not affect the density of the trabecular bone. The microscopic evaluation of histological sections using H&E and van Gieson's staining clearly supported the findings obtained by micro-CT. In the present study, animal body weights in OVX rats (including the Vehicle, ZOL, BE-L and BE-H groups) were notably different at the first observation time point of 4 weeks. A rapid increase in body weight was observed in OVX rats when compared with those in the Sham group at every point following surgery (\*\*P<0.01). Ovariectomy is associated with a disorder in fat metabolism that is induced by estrogen deficiency, resulting in an increase in body fat rather than lean body mass, which is in line with previous reports (19,28).

In 2002, Herczegh *et al* (18) synthesized a novel antibacterial drug, BE, by conjugating bone-binding bisphosphonate to enoxacin. BE has been demonstrated to have the ability to bind to bone and to inhibit the growth of bacteria, and may offer additional treatment options for highly concentrated targeting of an antibacterial to the site of a pathogen (18). A recent study (29) has identified other unexpected properties of enoxacin. Enoxacin has been shown to inhibit osteoclast formation and function by interfering with the interactions

between the V-ATPase B2 subunit or V-ATPase a3 subunit and microfilaments. Based on previous studies, it was hypothesized that BE may prevent osteoporosis disease *in vivo* via the combined anti-bone resorptive properties of bisphosphonates and enoxacin (18,29). As the long-term use of bisphosphonates may present a certain degree of risk for the development of oral osteonecrosis, the authors of the present study speculated that BE may be beneficial in reducing the occurrence of oral osteonecrosis through enoxacin replacement therapy by allowing for a reduction in the dose of bisphosphonates.

To the best of our knowledge, the present study has confirmed for the first time that BE has the potential to restore maxilla alveolar bone mass induced by OVX, and has further demonstrated its role in inhibiting osteoclasts *in vivo*, suggesting that its use may have potential therapeutic benefits for the treatment of patients with alveolar bone osteoporosis. This conclusion is in agreement with the results of two previous studies, which demonstrated that BE administration had a protective effect on alveolar bone for orthodontics and the treatment of periodontitis (30,31). In the present study, micro-CT measurement demonstrated that the ZOL and BE administration groups exhibited an increased BV/TV, an increased Tb.Th and a decreased Tb.Sp when compared with the Vehicle group. Consistent with these results, histological observation of the region between M1 and M2 in the maxilla alveolar bone demonstrated that ovariectomy induced trabecular bone destruction; however, ZOL and BE administration alleviated this process. In addition, analysis of the TRAP-stained paraffin sections and the line distance

data from fluorochrome labeling demonstrated that ZOL and BE administration was protective against the deterioration of trabecular bone induced by excessive osteoclast production, and also accelerated the rate of alveolar bone formation. BE exhibited dose-dependent bone-resorption inhibiting effects. Specifically, the anti-resorptive effect in the BE-H group was close to, or better than, that of the ZOL group, while the effect was poorer in the BE-L group. It is possible that the unique anti-resorptive mechanism of BE made it particularly useful in blocking the alveolar bone resorption triggered by osteoporosis in the present study (30).

In conclusion, BE, a bisphosphonate derivative of enoxacin, inhibits osteoclast formation and bone resorption in a manner that resembles the novel mechanism by which enoxacin functions (30). Notably, the present study demonstrated that BE inhibits maxilla osteoporosis, a process that is dependent on osteoclast activity *in vivo*. However, the conclusions of the present study on BE are confined to oral medicine, and so whether it will improve local or systemic bone osteoporotic changes has yet to be elucidated. The present study proposes that BE has the ability to reduce alveolar bone resorption following periodontal infection and osteoporosis induced by ovariectomy, as well as reducing orthodontic tooth movement, which makes it a promising candidate for clinical use (30,31).

### Acknowledgements

The present was supported by grants from the Science and Technology Commission of Shanghai Municipality (grant no. 17140903400), the Shanghai Municipal Commission of Health and Family Planning (grant no. M20170365), the Opening Project of Shanghai Key Laboratory of Orthopaedic Implant (grant no. KFKT2015002) and the National Natural Science Foundation of China (grant no. 81570948).

### References

- McGrath C and Bedi R: The importance of oral health to older people's quality of life. *Gerodontology* 16: 59-63, 1999.
- Howson CP: Perspectives and needs for health in the 21st century: 20th-century paradigms in 21st-century science. *J Hum Virol* 3: 94-103, 2000.
- Dempster DW, Birchman R, Xu R, Lindsay R and Shen V: Temporal changes in cancellous bone structure of rats immediately after ovariectomy. *Bone* 16: 157-161, 1995.
- Kribbs PJ, Chesnut CH III, Ott SM and Kilcoyne RF: Relationships between mandibular and skeletal bone in an osteoporotic population. *J Prosthet Dent* 62: 703-707, 1989.
- Kribbs PJ: Comparison of mandibular bone in normal and osteoporotic women. *J Prosthet Dent* 63: 218-222, 1990.
- Jeffcoat MK: Osteoporosis: A possible modifying factor in oral bone loss. *Ann Periodontol* 3: 312-321, 1998.
- Purdue PE, Koulouvaris P, Potter HG, Nestor BJ and Sculco TP: The cellular and molecular biology of periprosthetic osteolysis. *Clin Orthop Relat Res* 454: 251-261, 2007.
- Sun W, Wang YQ, Yan Q, Lu R and Shi B: Effects of Er-Zhi-Wan on microarchitecture and regulation of Wnt/ $\beta$ -catenin signaling pathway in alveolar bone of ovariectomized rats. *J Huazhong Univ Sci Technol Med Sci* 34: 114-119, 2014.
- Black DM and Rosen CJ: Postmenopausal osteoporosis. *N Engl J Med* 374: 254-262, 2016.
- Cauley JA, Robbins J, Chen Z, Cummings SR, Jackson RD, LaCroix AZ, LeBoff M, Lewis CE, McGowan J, Neuner J, *et al*: Effects of estrogen plus progestin on risk of fracture and bone mineral density: The Women's Health Initiative randomized trial. *JAMA* 290: 1729-1738, 2003.

- Cummings SR, Tice JA, Bauer S, Browner WS, Cuzick J, Ziv E, Vogel V, Shepherd J, Vachon C, Smith-Bindman R and Kerlikowske K: Prevention of breast cancer in postmenopausal women: Approaches to estimating and reducing risk. *J Natl Cancer Inst* 101: 384-398, 2009.
- Crandall CJ, Newberry SJ, Diamant A, Lim YW, Gellad WF, Booth MJ, Motala A and Shekelle PG: Comparative effectiveness of pharmacologic treatments to prevent fractures: An updated systematic review. *Ann Intern Med* 161: 711-723, 2014.
- Khosla S, Bilezikian JP, Dempster DW, Lewiecki EM, Miller PD, Neer RM, Recker RR, Shane E, Shoback D and Potts JT: Benefits and risks of bisphosphonate therapy for osteoporosis. *J Clin Endocrinol Metab* 97: 2272-2282, 2012.
- Shane E, Burr D, Abrahamsen B, Adler RA, Brown TD, Cheung AM, Cosman F, Curtis JR, Dell R, Dempster DW, *et al*: Atypical subtrochanteric and diaphyseal femoral fractures: Second report of a task force of the American Society for Bone and Mineral Research. *J Bone Miner Res* 29: 1-23, 2014.
- Tella SH and Gallagher JC: Prevention and treatment of postmenopausal osteoporosis. *J Steroid Biochem Mol Biol* 142: 155-170, 2014.
- Black DM, Bilezikian JP, Ensrud KE, Greenspan SL, Palermo L, Hue T, Lang TF, McGowan JA and Rosen CJ: PaTH Study Investigators: One year of alendronate after one year of parathyroid hormone (1-84) for osteoporosis. *N Engl J Med* 353: 555-565, 2005.
- Ostrov DA, Magis AT, Wronski TJ, Chan EK, Toro EJ, Donatelli RE, Sajek K, Haroun IN, Nagib MI, Piedrahita A, *et al*: Identification of enoxacin as an inhibitor of osteoclast formation and bone resorption by structure-based virtual screening. *J Med Chem* 52: 5144-5151, 2009.
- Herczegh P, Buxton TB, McPherson JC III, Kovács-Kulyassa A, Brewer PD, Sztaricskai F, Stroebel GG, Plowman KM, Farcasiu D and Hartmann JF: Osteoadsorptive bisphosphonate derivatives of fluoroquinolone antibacterials. *J Med Chem* 45: 2338-2341, 2002.
- Thompson DD, Simmons HA, Pirie CM and Ke HZ: FDA Guidelines and animal models for osteoporosis. *Bone* 17 (4 Suppl): 125S-133S, 1995.
- Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. Report of a WHO Study Group. *World Health Organ Tech Rep Ser* 843: 1-129, 1994.
- Tuck SP and Francis RM: Osteoporosis. *Postgrad Med J* 78: 526-532, 2002.
- Yang J, Pham SM and Crabbe DL: Effects of oestrogen deficiency on rat mandibular and tibial microarchitecture. *Dentomaxillofac Radiol* 32: 247-251, 2003.
- Mavropoulos A, Rizzoli R and Ammann P: Different responsiveness of alveolar and tibial bone to bone loss stimuli. *J Bone Miner Res* 22: 403-410, 2007.
- Dai QG, Zhang P, Wu YQ, Ma XH, Pang J, Jiang LY and Fang B: Ovariectomy induces osteoporosis in the maxillary alveolar bone: An *in vivo* micro-CT and histomorphometric analysis in rats. *Oral Dis* 20: 514-520, 2014.
- Al Habashneh R, Azar W, Shaweesh A and Khader Y: The relationship between body mass index and periodontitis among postmenopausal women. *Obes Res Clin Pract* 10: 15-23, 2016.
- Salazar M, Hernandez L, Ramos AL, Salazar Bde O, Micheletti KR, Paranhos LR, de Mendonça MR and Cuoghi OA: Effect of alendronate sodium on tooth movement in ovariectomized rats. *Arch Oral Biol* 60: 776-781, 2015.
- von Wowern N: General and oral aspects of osteoporosis: A review. *Clin Oral Investig* 5: 71-82, 2001.
- Chen H, Xu X, Liu M, Zhang W, Ke HZ, Qin A, Tang T and Lu E: Sclerostin antibody treatment causes greater alveolar crest height and bone mass in an ovariectomized rat model of localized periodontitis. *Bone* 76: 141-148, 2015.
- Liu X, Qu X, Wu C, Zhai Z, Tian B, Li H, Ouyang Z, Xu X, Wang W, Fan Q, *et al*: The effect of enoxacin on osteoclastogenesis and reduction of titanium particle-induced osteolysis via suppression of JNK signaling pathway. *Biomaterials* 35: 5721-5730, 2014.
- Toro EJ, Zuo J, Gutierrez A, La Rosa RL, Gawron AJ, Bradaschia-Correa V, Arana-Chavez V, Dolce C, Rivera MF, Kesavalu L, *et al*: Bis-enoxacin inhibits bone resorption and orthodontic tooth movement. *J Dent Res* 92: 925-931, 2013.
- Rivera MF, Chukkappalli SS, Velsko IM, Lee JY, Bhattacharyya I, Dolce C, Toro EJ, Holliday LS and Kesavalu L: Bis-enoxacin blocks rat alveolar bone resorption from experimental periodontitis. *PLoS One* 9: e92119, 2014.