



# Raloxifene reduces the risk of local alveolar bone destruction in a mouse model of periodontitis combined with systemic postmenopausal osteoporosis

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## ARTICLE INFO

### Keywords:

Raloxifene  
SERM  
Periodontitis  
Postmenopausal osteoporosis

## ABSTRACT

**OBJECTIVE:** Periodontitis is characterized by local inflammation leading to tooth loss and severe destruction of alveolar bone. Raloxifene is a selective estrogen receptor modulator (SERM) that halts estrogen deficiency-induced systemic bone loss in postmenopausal osteoporosis without the side effects of cancer in breast and uterus. In this study, we examined the effects of raloxifene on alveolar bone mass in a mouse model with estrogen deficiency-induced periodontitis.

**METHODS:** Periodontitis was induced by the injection of lipopolysaccharide (LPS) into the lower gingiva in ovariectomized (OVX) mice, and the alveolar bone and femur bone mineral density (BMD) were analyzed by dual-energy X-ray absorptiometry. To explore the direct osteoclast inhibitory effect of raloxifene, a co-culture system for osteoclast formation and organ culture of alveolar bone was established.

**RESULTS:** When OVX mice were treated with raloxifene, the bone loss in both alveolar bone and femur were abrogated. Interleukin 1 and/or LPS stimulated the osteoclast formation and bone-resorbing activity; however, raloxifene did not show any inhibitory effect on the osteoclast formation or function. *In vivo* local injection of raloxifene also did not prevent bone resorption in a mouse model of periodontitis. However, the systemic treatment of raloxifene using a mini-osmotic pump did prevent the loss of BMD of alveolar bone induced by LPS. **CONCLUSION:** These results suggest that the SERM raloxifene systemically maintain alveolar bone mass in a mouse model of periodontitis with osteoporosis. Increasing the alveolar bone mass by SERMs treatment in patients with postmenopausal osteoporosis may be a useful approach to preventing the destruction of alveolar bone in late-onset periodontitis.

## 1. Introduction

Periodontitis is an inflammatory disease caused by mixed Gram-negative bacterial infection and host immune response that leads to gingival tissue breakdown, alveolar bone destruction and tooth loss (Kinane, Stathopoulou, & Papananou, 2017). The major virulence factor is lipopolysaccharides (LPS), which are outer membrane components of Gram-negative bacteria. In addition, inflammatory molecules, such as

interleukin 1 (IL-1) and tumor necrosis factor- $\alpha$ , stimulate osteoclast differentiation and bone resorption (Komine et al., 2001; Lee, Fujikado, Manaka, Yasuda, & Iwakura, 2010; Tanabe et al., 2005). Recent clinical studies have suggested the association of periodontitis and postmenopausal osteoporosis, suggesting estrogen deficiency as a cause of severe systemic bone loss in women (Guiglia et al., 2013; Juluri et al., 2015; Penoni et al., 2016). As drug treatments of osteoporosis, bisphosphonates increase the bone mineral density (BMD) and reduce the

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risk of bone fractures in patient with post-menopausal osteoporosis. Bisphosphonates directly inhibit the osteoclast activity and induce apoptosis (Hughes et al., 1995). However, it has been reported that these agents induce osteonecrosis in the jaw as a side effect when patients undergo dental surgery. (Ruggiero, Mehrotra, Rosenberg, & Engroff, 2004). Therefore, new approaches are needed to maintain alveolar bone mass in patients with estrogen deficiency, such as those with postmenopausal osteoporosis.

With regard to postmenopausal osteoporosis therapy, estrogen replacement therapy prevents estrogen deficiency-induced bone loss but consequently increases the risk of uterine or breast cancer. Raloxifene is a selective estrogen receptor modulator (SERM) used in the prevention and treatment of osteoporosis of postmenopausal woman without the side effects of estrogen treatment (Brennan, Rizzoli, & Ammann, 2009; Dutertre & Smith, 2000; Gallant et al., 2014). SERMs have been developed as tissue-specific estrogen agonists and are considered beneficial for treating postmenopausal osteoporosis. Raloxifene is a second-generation SERM and is widely used as a drug for the treatment and prevention of bone fracture in postmenopausal osteoporosis (Brennan et al., 2009; Gallant et al., 2014). This agent acts on bone and several other tissues as an estrogen agonist and inhibits bone loss and bone fracture risk; however, it acts on the uterus as an estrogen antagonist and reduces the risk of uterine cancer (Barkhem et al., 1998; Dutertre & Smith, 2000; Lamas et al., 2015). Raloxifene binds to two isoforms of estrogen receptors (ERs): ER $\alpha$  and ER $\beta$ . The tissue selectivity of SERMs derived from ligand-bound structural differences of ERs are considered to determine whether individual ligands act as estrogen agonists or antagonists (Barkhem et al., 1998; Brzozowski et al., 1997; Dutertre & Smith, 2000). In previous studies, estrogen has been reported to promote osteoclast apoptosis through the induction of Fas ligand in osteoblasts and osteoclasts, resulting in the inhibition of osteoclastic bone resorption (Krum et al., 2008; Nakamura et al., 2007). However, the effect of SERMs on the prevention of periodontitis is not known. We previously established a mouse model of periodontitis with osteoporosis and reported that estrogen deficiency enhances the alveolar bone destruction under conditions of inflammatory periodontitis (Kobayashi et al., 2012).

In this study, we examined the effects of the SERM raloxifene on alveolar bone mass decreased by local inflammatory periodontitis in our mouse model of osteoporosis.

## 2. Materials and methods

### 2.1. Animals and reagents

Newborn, 4-week-old and 6-week-old mice of the *ddY* strain were obtained from Japan SLC Inc. (Shizuoka, Japan). All procedures were performed in accordance with the institutional guidelines for animal research. IL-1 was obtained from R&D Systems [Minneapolis, MN, USA]. LPS (E. Coli O55:B5) was purchased from Sigma-Aldrich [St. Louis, MO, USA]. Raloxifene was kindly provided from Eli Lilly [Indianapolis, IN, USA]. Four-week-old female mice were either sham-operated or ovariectomized (OVX). Some of the OVX mice were treated with raloxifene (300  $\mu\text{g}/\text{kg}/\text{day}$ ) by subcutaneous administration using a mini-osmotic pump (Alza Co., Palo Alto, CA, USA) implanted immediately after surgery.

### 2.2. Culture of primary mouse osteoblastic cells

Primary osteoblastic cells (POBs) were isolated from newborn mouse calvariae after 5 routine sequential digestions with 0.1% collagenase (Roche Applied Science, Mannheim, Germany) and 0.2% dispase (Roche Diagnostics GmbH, Mannheim, Germany). Osteoblastic cells collected from fractions 2-4 in 5 routine sequential digestions were combined and cultured in  $\alpha$ -modified MEM ( $\alpha$ MEM), supplemented with 10% fetal bovine serum (FBS) at 37 °C under 5% CO<sub>2</sub> in air.

### 2.3. Co-cultures of mouse bone marrow cells and osteoblasts

Bone marrow cells (BMCs;  $2 \times 10^6$  cells) were isolated from 6-week-old mice and co-cultured with POBs ( $1 \times 10^4$  cells) in  $\alpha$ MEM containing 10% FBS. After being cultured for seven days, the cells adhering to the well surface were stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated cells containing three or more nuclei per cell were counted as osteoclasts.

### 2.4. Organ cultures of mouse calvariae

Mouse calvariae were collected from newborn mice and cultured for 24 h in BGJb medium supplemented with 0.1% bovine serum albumin (BSA) at 37 °C under 5% CO<sub>2</sub> in the air. After 24 h, the bones were treated with IL-1 (2 ng/mL) in the presence or absence of raloxifene (1, 10 and 100 nM) and cultured for 5 days. The bone-resorbing activity was elucidated by measuring the concentration of calcium in the conditioned medium using the o-cresolphthalein complexone (OCPC) method. Conditioned medium was mixed with OCPC in an alkaline solution to form a calcium-OCPC complex exhibiting a violet color, and the absorbance of this mixture was measured at 570 nm.

### 2.5. Organ cultures of mouse alveolar bone

Mandibular alveolar bones were collected from 4-week-old mice under a microscope and cultured for 24 h in BGJb medium supplemented with 0.1% BSA at 37 °C under 5% CO<sub>2</sub> in the air. After 24 h, the bones were treated with LPS (5  $\mu\text{g}/\text{mL}$ ) in the presence or absence of raloxifene (100 nM) and cultured for 5 days. The bone-resorbing activity was determined by measuring the concentration of calcium in the conditioned medium using the OCPC method.

### 2.6. Injection of LPS into the lower gingiva in mice

In the model of experimental periodontitis, LPS (25  $\mu\text{g}/\text{mouse}$ ) and raloxifene (50  $\mu\text{g}/\text{mouse}$ ) were dissolved in 50  $\mu\text{L}$  of the DMSO/PEG300/PBS (9  $\mu\text{L}/36 \mu\text{L}/5 \mu\text{L}$ ) solution and injected into the outside of the lower gingiva of the mice on days 0, 2 and 4. As a vehicle, the DMSO/PEG300/PBS solution was injected into the lower gingiva at the same time points. After seven days of the first injection, the mandibular alveolar bones were collected from mouse molar region, and three molars were removed. The bone mineral density (BMD) of the total area of alveolar bone was measured by dual X-ray absorptiometry (model DCS-600R; Aloka, Tokyo, Japan). The BMD was calculated by dividing the bone mineral content of the measured area by the area.

### 2.7. Statistical analyses

Data were analyzed using one-way ANOVA, followed by Tukey's test for *post hoc* analysis. All data are presented as the means  $\pm$  SEM, and all statistical analyses were performed using IBM SPSS Statistics Ver.23 software.

## 3. Results

### 3.1. Raloxifene restores alveolar bone loss in OVX mice

The structural comparison of raloxifene with estradiol is shown in Fig. 1A. Estrogen and raloxifen differ in the presence of a phenyl 4-piperidinoethoxy side chain (Fig. 1A, dotted square) that changes the positioning of helix12 (H12) in the ligand-ER complex (Brzozowski et al., 1997). We first assessed the effects of raloxifene on estrogen deficiency-induced bone loss in alveoli and femurs using OVX mice. Raloxifene was delivered systemically using a mini-osmotic pump implanted subcutaneously. After four weeks, the body weight, uterine weight and BMD of both the femur and alveolar bone were measured.

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