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Periodontal disease exacerbates systemic ovariectomy-induced bone loss in mice

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ABSTRACT

Periodontal pathogens and/or inflammatory products from periodontitis participate in the development or progression of systemic diseases. In this context, periodontitis acts as a modifying factor to systemic health, including diabetes and cardiovascular diseases. Osteoporosis is an increasingly prevalent condition in our aging population and considered a risk factor for periodontal disease, but the effect of periodontitis on systemic bone homeostasis is unknown. We thus evaluated the effects of experimental periodontitis (EP) on systemic bone loss and the influence of estrogen deficiency in this context, using a mouse model of combined periodontitis and osteoporosis. Experimental periodontitis (EP) was induced by a ligature insertion around the mandibular first molars and *Porphyromonas gingivalis* infection. Three-dimensional microcomputed tomographic analyses performed 48 days following infection revealed that EP and ovariectomy (OVX) induced a significantly higher femoral and mandibular bone loss compared to EP or OVX alone. EP alone did not induce systemic bone loss. In addition, the EP + OVX and EP groups showed significantly higher levels of tumor necrosis factor (TNF)-α than OVX and control groups at end point. These results suggest that periodontitis could be a risk factor for systemic bone loss, especially in post-menopausal women, and warrant further clinical investigations to confirm this association and propose adapted prophylactic and curative therapies.

1. Introduction

The world population is aging and the proportion of individuals over 65 is increasing [1]. Considering that menopause occurs around the age of 50, millions of women will live many years at risk for osteoporosis and fractures [2].

Osteoporosis is a systemic skeletal disease characterized by low bone density and microarchitectural deterioration of bone tissues, which leads to increased bone fragility and risk of fracture [3]. The influence of osteoporotic fractures in public health includes effects on mortality, functional impairment, quality of life and has a costly financial impact [4].

Periodontitis can be defined as an inflammation of the support tissue around the teeth, leading to local bone tissue loss and destruction of the periodontal ligament, or as an extension of gingivitis to adjacent bone and ligament tissues [5]. From 2009 to 2012, 46% of adults in the US, representing 64.7 million people, had periodontitis, with 8.9% having severe periodontitis [6].

Osteoporosis and periodontitis are inflammatory diseases characterized by bone resorption. Both are silent diseases, being asymptomatic until late stages, and share several risk factors, such as tobacco use and advanced age [7]. Initiation and progression of periodontal disease can be modified by local factors and systemic conditions. Systemic risk factors include gender, smoking and alcohol, as well as medical conditions such as poorly controlled diabetes, obesity, stress and inadequate dietary consumption of calcium and vitamin D [8]. Although osteoporosis has also been considered a risk factor for the progression of periodontal disease [7,9,10], this relationship is controversial [10,11].

Periodontal pathogens and/or inflammatory products from periodontitis participate in the development or progression of systemic diseases [12]. In this context, periodontitis acts as a modifying factor to systemic health, including vascular disorders associated with atherosclerosis, diabetes mellitus, respiratory disorders, as well as several debated associations such as preterm delivery, rheumatoid arthritis, pancreatic cancer and metabolic syndrome [12]. Although a growing body of literature has accumulated to investigate osteoporosis as risk factor for periodontitis [7,9–11], the inverse interaction, i.e., the effect of periodontitis on systemic bone remodeling, is unclear [11,12]. Interestingly, older individuals with both osteoporosis and periodontitis

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are at greater risk for future fractures than those with either osteoporosis or periodontitis alone [13]. This observation led us to hypothesize that periodontitis is a risk factor for systemic bone loss and to evaluate the effects of experimental periodontitis (EP) on ovariectomy-induced systemic bone loss in mice.

There are several well-established models to induce EP in rats and mice, such as the insertion of a ligature around molars [14,15], bacterial infection by oral gavage [15–17], lipopolysaccharide injection [15], or association between ligature and bacterial infection [18–20]. The modalities and the duration of periodontitis induction are very important, since they influence periodontal tissue destruction and serum levels of proinflammatory cytokines as well, and different protocols can lead to diverse results [19]. The ligature model has several disadvantages related to the trauma caused during the ligature insertion and to the decrease in disease severity with time [18]. The oral gavage model on the other hand, first reported by Baker et al. [16], does not induce an acute alveolar bone loss, as opposed to the ligature insertion [15,18]. Ligature associated to *Porphyromonas gingivalis* infection generated more active periodontitis [19], with increased periodontal bone resorption [21], more intense systemic response than ligature or oral *P. gingivalis* infection alone [19] and also led to the maintenance of the disease intensity with time [18]. Therefore, ligature associated to *P. gingivalis* infection was used to experimentally address our hypothesis in mice.

2. Material and methods

Animal care and study protocols were approved by the Institute of Science and Technology of São José dos Campos (UNESP) Ethics Committee (06/2012-PA/CEP). Forty-five 8-week-old specific pathogen free female mice (*Mus musculus*, *BALB/c*), approximately 25 g, were housed in a light- and temperature-controlled environment (12-hlight: 12-h-dark cycle; 22 ± 2 °C). After a 2-week adaptation, they were randomly divided into four groups, as follows: 1) *OVX group*: ovariectomy (OVX) without EP induction (n = 13); 2) *Control group*: OVX-sham surgery without EP induction (n = 10); 3) *OVX + EP group*: EP and ovariectomy (n = 10); 4) *EP group*: induced EP and OVX-sham surgery (n = 12).

Standard pellet diet was offered ad libitum to mice submitted to sham surgery, and ovariectomized animals were pair fed, i.e., restricted to the average amount of food eaten by the sham ones. We measured the daily food intake of sham mice by weighing the amount of food remaining in each cage every 24 h, for two weeks. All animals received water ad libitum. Body weight was measured at the day of OVX/Sham surgery and at euthanasia and these values were converted to percentage of body weight gain.

2.1. Ovariectomy and sham surgery

All animals were anesthetized using injection of ketamine base (100–150 mg/kg)/xylazine hydrochloride (10–15 mg/kg), and submitted to ovariectomy or sham surgery on day 0.

Bilateral ovariectomy was performed following a bilateral longitudinal skin incision in the lateral abdominal region. Sham surgery was performed by exposing the ovaries without excision. Ovariectomy was performed by exposing the ovaries and excision with an electrocautery instrument. The peritoneal muscle was sutured with an absorbable suture thread (catgut 4–0, Ethicon, Johnson & Johnson, São José dos Campos, SP, Brazil) and skin with a silk line (3–0, Ethicon, Johnson & Johnson, São José dos Campos, SP, Brazil). Efficacy of ovariectomy was confirmed by bilateral uterus atrophy at euthanasia.

2.2. Bacterial strains and culture

P. gingivalis (ATCC 33277) was cultured at 37 °C on Fastidious Anaerobe Agar (Acumedia, Lansing, MI, USA) plates with sheep blood, supplemented with 0.1% menadione and hemin (Sigma-Aldrich, Saint Louis, MO, USA) in an oxygen-free atmosphere (80% nitrogen, 10% carbon dioxide and 10% hydrogen), as described previously [22]. After 7 days of growth on blood agar, *P. gingivalis* colonies were selected, and a solution of 10^9 CFU/ml in 100 μl of 2% carboxymethylcellulose [16](Sigma-Aldrich, Saint Louis, MO, USA) was prepared. This inoculum was prepared immediately before use.

2.3. Periodontal disease induction

Periodontal disease was induced by the insertion of a ligature around the lower first molar associated to *P. gingivalis* infection.

All mice were treated with sulfamethoxazole-trimethoprim (Bactrim-Roche Químicos e Farmacêuticos S.A, Rio de Janeiro, RJ, Brazil) (10 ml/l) in water, ad libitum for 10 days (day-11 to day-1; Fig. 1) to reduce native flora [16]. This was followed by a 3-day antibiotic-free period [16](Fig. 1). On day 3, animals from EP + OVX and EP groups were anesthetized again before the insertion of the ligature. A 4-0 silk thread (Ethicon, Johnson & Johnson, São José dos Campos, SP, Brazil) was wrapped around the first inferior molars of the animals, carefully pushing the ligature into the gingival sulcus and knotting mesially. Periodontal disease was not induced in the animals of the OVX and control groups, however, they were anesthetized and manipulated in the same way as the others.

Animals from OVX + EP and EP groups were infected with *P. gingivalis* (10^9 CFU in 100 μl of phosphate-buffered saline with 2% of carboxymethylcellulose) [16] by gavage (50 μl placed into the esophagus and 50 μl into oral cavity) eight times [17] at 2-day intervals (days 3, 6, 9, 12, 15, 18, 21, and 25; Fig. 1). Control and OVX mice received the carboxymethylcellulose gavage without *P. gingivalis*.

On days 18 and 33, the animals were anesthetized, the presence of the ligature was evaluated (groups EP and OVX + EP), and the animals in which it was absent were excluded from the study. At this time, we repositioned the thread in an apical position to maintain the ligature in intimate contact with the marginal tissues.

2.4. Euthanasia

Euthanasia was performed on day 51 (51 days after the day of OVX or sham surgery) [23]. Animals were anesthetized, and blood was collected (BD Vacutainer, BD, São Paulo, São Paulo, Brasil) from the heart ventricle for blood serum analyses. After blood centrifugation (2000 g, 20 min), serum was stored in −20 °C. Urine was also collected and stored in −20 °C for C-terminal telopeptide of type I collagen (CTX) levels evaluation. After decapitation, mandible and femurs were removed and fixed in paraformaldehyde 4% (phosphate buffer pH 7, 4; 0,1 M). A single examiner blind to group allocation of samples and mice performed all following analyses.

2.5. Micro-computed tomography (μCT)

Three-dimensional microcomputed tomography analyses of the distal femoral metaphysis were performed using a Scanco μCT 40 system (Scanco Medical, Bassersdorf, Switzerland). Tomographic images were acquired at 55 kVp and 145 μA with an isotropic voxel size of 12 μm and at an integration time of 300 ms. To segment bone from non-mineralized tissue, a Gaussian noise suppression filter (σ = 0.3, support = 1) was used, and global thresholds were consistent across scans per anatomical site [24].

Five right hemi-mandibles of each group were selected randomly and also scanned in a Scanco μCT40 at 70 kV and 114 μA X-ray source, 500 projections/180° rotation and 300 ms integration, and image were reconstructed with 16 μm isotropic voxels. A three-dimensional (3D) volume of interest (VOI) was standardized and defined by the following points: the apex of the mesial root of the first molar (apical limit); the first molar furcation area (coronary limit); mesial of the mesial root of