Ovariectomy Exacerbates Apical Periodontitis in Rats with an Increase in Expression of Proinflammatory Cytokines and Matrix Metalloproteinases

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Abstract

Introduction: The aim of this study was to evaluate the gene expression of proinflammatory cytokines, matrix metalloproteinases (MMPs), and cathepsin K in apical periodontitis (AP) and the volume of lesions in ovariectomized and sham-operated rats. **Methods:** Twenty 12-week-old female Wistar rats were subjected to ovariectomy (OVX) or sham surgery. After 9 weeks, access cavities were prepared in the maxillary and mandibular first molars, pulp tissue was removed, and canals were exposed to the oral environment during 21 days for the induction of AP. The groups were as follows: sham, OVX, sham+AP, and OVX+AP. Animals were euthanized, and blocks containing the maxillary first molar and the surrounding bone were removed for quantification of proinflammatory cytokines cathepsin K and MMP genes by real-time polymerase chain reaction. The hemimandibles containing the mandibular first molars were used for analysis of the AP lesion volume by micro–computed tomographic imaging. **Results:** AP in OVX rats showed an increased expression of interleukin 1 beta, tumor necrosis factor alpha, interleukin 6, MMP-8, and MMP-13 ($P < .05$). OVX alone, without AP induction, did not affect the expression of the evaluated genes. Additionally, AP induced an increase in cathepsin K expression, without significant differences between AP in the sham and OVX groups ($P > .05$). Micro–computed tomographic imaging showed a significantly greater AP lesion mean volume in OVX compared with sham animals ($P < .05$). **Conclusions:** AP lesions in ovariectomized rats are larger and have an increased expression of proinflammatory cytokines and MMPs, indicating that the infection combined with ovariectomy has an important role in the regulation of these signaling molecules and enzymes during the development of AP. Based on that, it may be assumed that the hypoestrogenic condition aggravates inflammation and degradation of extracellular matrix components in AP, which may provide insight into understanding the development of AP in female postmenopausal patients. *(J Endod 2018;■:1–6)*

Key Words

Apical periodontitis, cytokines, gene expression, matrix metalloproteinases, ovariectomy

Significance

This study is the first to evaluate the gene expression of proinflammatory cytokines cathepsin K and MMPs in AP in ovariectomized rats, beyond the 3-dimensional aspect of AP. We provide insight into understanding the development of AP in female postmenopausal patients.

Apical periodontitis (AP) is a disease characterized by the presence of inflammation and bone resorption (1). This process is mediated by several proinflammatory cytokines secreted by both resident and immunocompetent cells recruited in response to microbial infection from the root canal system. The regulatory mechanisms acting on AP are complex and have not yet been fully elucidated, but tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin-6 (IL -6) contribute to the formation of osteoclasts and periapical bone resorption (1–3). Degradation of the extracellular organic matrix, an important component of the connective tissue (4), is 1 of the first stages of resorption occurring in the periapical region. Matrix metalloproteinases (MMPs) are enzymes that play an essential role in this degradation (5), particularly MMP-8 and MMP-13 collagenases (6).

The pathogenesis of periapical diseases may be influenced by some systemic factors, among them hormones (3,7). Estrogen deficiency, characterized in postmenopausal women, has several effects on oral health (8) and can influence bone remodeling in sites with an inflammatory process, resulting in an exacerbated resorption, as shown in studies in the field of periodontics. The cytokines involved in inflammation-induced remodeling are similar to those suggested to play critical roles in postmenopausal osteoporosis (9). It is known that the inflammation-induced

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0099-2399/$ - see front matter

https://doi.org/10.1016/j.joen.2018.01.010

osteolysis observed in postmenopausal osteoporosis could be a systemic aggravating factor in chronic AP. A significant association between the presence of periapical radiolucencies and low bone mineral density (BMD) was found in a cross-sectional study assessing the relationship between AP and BMD in postmenopausal women (10).

In an experimental rodent model, ovariectomy (OVX) causes estrogen deficiency, which, in turn, is associated with the exacerbation of AP induced experimentally. AP in ovariectomized rats is characterized by greater periapical bone resorption compared with lesions in healthy animals (7, 11). However, there are few studies evaluating AP in this animal model, and none of these previous studies evaluated specifically the AP volume. In addition, little attention has been given to the mediators that regulate this process, and, so far, no study has assessed the role of MMPs.

The aim of this study was to evaluate the gene expression of proinflammatory cytokines, MMPs, and cathepsin K in AP in ovariectomized rats compared with sham animals. Lesion volume was also investigated by micro–computed tomographic (micro-CT) imaging. We hypothesized that estrogen deficiency worsens AP bone loss and inflammation.

Material and Methods

Animals

After approval of the research project by the Animal Care and Use Committee of our institution (protocol #2013.1.1404.58.4), 20 approximately 12-week-old female Wistar rats (*Rattus norvegicus albinus*) were subjected to OVX or sham surgery. The rats were anesthetized by an intramuscular injection of 10% ketamine (55 mg/kg body weight) and 2% xylazine (10 mg/kg). In 10 animals (the OVX groups), the ovaries were excised as a whole bilaterally, and in the other 10 animals (the sham groups) surgery was simulated and the ovaries were manipulated and returned intact to their original position (12). The success of OVX was confirmed by monitoring the animals' body weight and the weight of the uterus on the day of euthanasia, considering that OVX is associated with weight gain and uterine atrophy (13), comparing operated and sham animals. Additionally, the success of OVX was confirmed by BMD analysis. The BMD of the proximal tibias metaphysis was analyzed by a dual-energy x-ray densitometer (Lunar PIXImus Corp, Madison, WI) to record the BMD in g/cm^2.

Induction of AP

The animals were assigned to 4 groups: sham, animals were subjected to sham surgery; OVX, animals were subjected to OVX; and sham+AP and OVX+AP, in which AP was experimentally induced in the animals after surgery. Nine weeks after OVX or sham surgery, the root canals of the maxillary and mandibular first molars were exposed to the oral environment for the induction of AP. The rats were anesthetized and immobilized in a surgical table to retract the mandible and maintain the mouth open for good visualization and access to the teeth. The coronal access cavity was prepared with a round drill (size 1/2) on a low-speed handpiece, and the pulp tissue was removed from the root canal with a size 10 K-file (Dentsply Maillefer, Ballaigues, Switzerland). After 21 days of exposure to oral microbiota for the induction of AP, the animals were euthanized, and the blocks containing the maxillary first molars and the surrounding bone were collected for quantification of proinflammatory cytokines cathepsin K and MMPs genes by real-time polymerase chain reaction (RT-PCR). The samples were maintained in RNAlater solution (Life Technologies Corporation, Carlsbad, CA) and stored frozen at −80° C until the moment of processing. The hemimandibles containing the mandibular first molars were fixed in 10% buffered formalin for 24 hours at room temperature for subsequent analysis by micro-CT imaging.

RNA Extraction and Complementary DNA Amplification by RT-PCR

The tissues were defrosted, macerated, homogenized in tissue homogenizer (BioSpec Products, Inc, Bartlesville, OK), and maintained for 1 minute at room temperature. Total RNA extraction was performed using the Pure Link Mini Kit extraction kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Aliquots of 2 μL were used to evaluate purity and to estimate the RNA concentration (mg/mL) of each sample using a full-spectrum, ultraviolet-visible spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific Inc, Waltham, MA). Complementary DNA was synthesized from 1 μg total RNA using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems).

Quantification of cytokines (IL-1β, IL-6, and TNF-α), MMPs (MMP-8 and MMP-13), and cathepsin K was assessed by gene expression (*Il1b*, *Il6*, *Tnfa*, *Mmp1*, *Mmp8*, *Mmp13*, and *CTSK*). Quantitative analysis of messenger RNA expression was performed with the StepOnePlus Real-Time PCR System (Applied Biosystems) using the TaqMan fluorescence system (Applied Biosystems) for quantification of amplification products. The standard polymerase chain reaction protocol was as follows: 95 °C for 2 minutes followed by 40 cycles of 95 °C for 1 second and 60 °C for 20 seconds. The primers were obtained commercially and are private properties, so the sequences are not available (Applied Biosystems).

For messenger RNA analysis, the relative expression of the target gene was calculated according to the manufacturer's instructions (Applied Biosystems User's Bulletin PN 4303859), using as reference the expression of beta-actin and Gapdh in the same sample using the ΔΔCt method. The mean Ct values obtained from duplicates were used to calculate gene expression, which was compared with the values obtained for the target gene and reference gene of a control sample to determine the relative expression by using the formula $2^{-\Delta\Delta Ct}$.

Micro-CT Imaging

The hemimandibles containing the first mandibular molars were scanned with a high-resolution micro-CT device (SkyScan 1174v2; Bruker-microCT, Kontich, Belgium) operated at 50 kV, 800 mA (0.5-mm Al filter), and an isotropic resolution of 19.7 μm. Scanning was performed by 180° rotation around the vertical axis with a rotation step of 1°. Images of the cross sections were reconstructed with NRecon v.1.6.6.0 software (Bruker-microCT) using a ring artifact reduction of 10. CTAn v.1.12 (Bruker-microCT) software was used for the analyses. Delimitation of the periodontal ligament (1 mm short from the root apex) or the AP lesions was performed in the sagittal plane by a customized delimitation of the region of interest, adjusted manually in 10 by 10 sections, to ensure the inclusion of the entire bone loss volume at the distal root of the rat's first molar. The parameter assessed in this system was the bone volume (mm^3), which represents the 3-dimensional size of the AP lesion.

Statistical Analysis

After normality assumptions (the Shapiro-Wilk test) could be verified, data from body weight, uterine weight, BMD, and volume of AP or the periodontal ligament were subjected to the Student *t* test. The other data were subjected to the Kruskal-Wallis test and the Dunn posttest. All analyses were performed using Graph Pad Prism 4.0 software (Graph Pad Software Inc, San Diego, CA) with a significance level of 5%.

Results

The success of OVX was confirmed by monitoring the animals' body weight and the weight of the uterus at euthanasia. The mean body weight of the sham and OVX animals was 465.1 ± 15.7 g and