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Original Article

Estrogen deficiency aggravates apical periodontitis by regulating NLRP3/caspase-1/IL-1β axis

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Abstract: Estrogen plays critical roles in apical periodontitis and subsequent bone loss, however the mechanism is not clear yet. In this study, we aimed to study the underlying mechanism of estrogen in apical periodontitis using both clinic samples and animal model. Clinically, as estrogen physiologically declines in elder female patients (premenopausal verses postmenopausal patients), we found that the expression level of NLRP3/Caspase-1/IL-1β signaling pathway was elevated in the infected apical tissues of postmenopausal patients as compared to the premenopausal patients, suggesting that this pathway is involved in the estrogen-mediated apical periodontitis. Furthermore, by analyzing the well-established OVX (estrogen deficiency model) animal model, we confirmed that the expression level of NLRP3/Caspase-1/IL-1β signaling pathway was also elevated in the infection areas of apical periodontitis in OVX animals. Importantly, as the periodontitis progressed, the subsequent bone loss was aggravated significantly. Thus, taken all these data together, our results demonstrated that the NLRP3/Caspase-1/IL-1β signaling pathway is involved in the estrogen-mediated apical periodontitis and the consequent bone loss in both human being and animal model. This study may provide a potential target for female apical periodontitis therapy.

Keywords: Estrogen, apical periodontitis, bone loss, inflammation, NLRP3/Caspase-1/IL-1β signaling pathway

Introduction

Apical periodontitis (AP) is a common infectious disease worldwide, and already impacted people's normal life. It occurs in the periradicular tissues, and primarily caused by bacterial infection of the dental pulp, therefore results in alveolar bone resorption in periradicular tissues [1, 2]. The pathogenesis of AP is mainly related to host's inflammatory and immunological responses [3]. Like other inflammatory reactions, upon the constant stimuli of bacteria, the immune cells in apical lesions will recruit the inflammatory cells and then generate the cytokines in order to defense the attack of inflammatory cells subsequently [3-5]. Moreover, systemic factors, such as hormones, also play a key role in the pathogenesis of AP [6]. Using ovariectomized rat model, previous studies have demonstrated that the shortage of estrogen can aggravate apical periodontitis, for instance leading to larger periapical lesions. However, the underlying mechanism of how estrogen is regulating the local immune response against the inflammation (in this case, the apical periodontitis) is not completely understood yet.

Nod-like receptors (NLRs), as the cytosolic pattern recognition receptors (PRRs), play pivotal roles in autoimmune diseases and inflammatory diseases [7, 8]. Generally, NLRs family is comprised of 22 members, including 14 NLRP (nucleotide-binding domain and leucine-rich rep protein) members, five NLRC subfamily members, NAIP, NLRX, and CIITA [9, 10]. Among them, NLRP1, NLRP3, NLRP6, NLRP7, and NLRC4 are capable of forming inflammasomes [11, 12]. Inflammasome is a platform that links the sensing of pathogen and danger signals to pro-IL-1β processing [13]. Compared with other inflammasomes, NLRP3 inflammasomes have attracted the most attention. It can be activated by a diverse array of stimuli such as bacterial

peptidoglycan, lipopolysaccharides, adenosine triphosphate and endogenous proteins released from damaged cells [14]. Once being activated, NLRP3 inflammasomes (also known as Nalp3, cryopyrin) start to oligomerize and form a multiprotein complex which is associated with apoptosis-associated speck-like protein (ASC) and procaspase-1, then activate the caspase (Casp)-1 cascade, which in turn produces the active pro-inflammatory cytokines, interleukin (IL)-18 and IL-1β [15]. Previous studies have shown that the activity of NLRP3 inflammasome is abnormal in rheumatoid arthritis, atherosclerosis, chronic obstructive pulmonary disease, Parkinson's disease and cancer [16-20], indicating that NLRP3 inflammasomes are involved in the pathogenesis of human diseases.

Reports have illustrated the presence of NLRP3/Caspase-1/IL-1β axis in AP and demonstrated that this axis is contributing to the aggravation of AP [21, 22]. Interestingly, recent studies have reported the relationship between estrogen deficiency and NLRP3/Caspase-1/IL-1β axis in mouse brain. In OVX, the deficiency of estrogen activates the NLRP3/Caspase-1/IL-1β axis in the hippocampus of brain [23, 24]. In addition, in the induced osteoblasts derived from OVX-BMSCs, the protein levels of NLRP3 showed a similar decreased tendency as ASC and Caspase-1 in comparison with the Sham-BMSCs [25]. Furthermore, estrogen deficiency aggravate airway inflammation via up-regulating the expression of the NLRP3, ASC and cleaved caspase-1/pro-caspase-1 [26]. Given that the NLRP3/Caspase-1/IL-1β axis is either involved in the pathogenesis of apical periodontitis or modulated by estrogen deficiency, it would be great interesting to investigate if estrogen deficiency aggravates apical periodontitis lesions via regulating the expression/activity of the NLRP3/Caspase-1/IL-1β axis.

In this study, we evaluated the potential roles of estrogen-regulated NLRP3/Caspase-1/IL-1β axis in apical periodontitis lesions using two models: patients' samples and OVX animal model. First, with patients' samples, by comparing the expression levels of NLRP3, Caspase-1 and IL-1β in apical lesions of postmenopausal female patients with that of premenopausal female patients, we found that postmenopausal female patients have higher expression levels of NLRP3, Caspase-1 and IL-1β. Secondly, in OVX/apical periodontitis animal model, we found that the deficiency of estrogen aggravates apical periodontitis by up-regulating the expression levels of NLRP3, Caspase-1, and IL-1β, suggesting a potential role of NLRP3/Caspase-1/IL-1β axis in estrogen-regulated apical periodontitis.

Materials and methods

Patients

Tissue samples: Periapical lesion tissues were obtained during tooth extraction from 30 female patients with apical periodontitis. Among them, 15 female participants were 28-35 years of age (premenopausal female patients, designed as control group, pre), and the other participants were postmenopausal women aged from 58-65 (postmenopausal group, post). Women with any additional diseases, such as diabetes, immune disorders, malignant diseases, endocrine disorders and apical periodontitis were excluded from our experiments. None of the women has ever experienced hormone replacement therapy, smoking, or addiction to alcohol.

Of note, before the samples were collected, we explained to all participants in great details about the purpose of this study, and the patients' consents were signed up as well. The study was approved by the Ethical Committee of Stomatology School, Xi'an Jiao Tong University (2019-1018) and was conducted in accordance with all requirements of the Helsinki Declaration. Periapical tissues were either fixed in 4% paraformaldehyde or promptly frozen in liquid nitrogen and stored at -80°C until later use.

Western blot: Tissues from periapical lesions stored at -80°C were solubilized on ice. Briefly, the tissue was blotted, weighed on a microbalance, cut into small pieces (1-2 mm^3). Then the tissues were homogenized in RIPA buffer including protease inhibitor cocktail (Roche) and phosphatase protease inhibitor, PhosSTOP. The homogenate was centrifuged at 13.000 rpm for 10 min at 4°C, and the supernatant was transferred into fresh tubes. Protein concentration was determined using a Bradford Assay Reagent kit (Bio-Rad, PA, USA). Thirty-five micrograms of total protein from each samples were subjected to run in a 12% SDS-PAGE.

After the proteins were transferred to polyvinylidene fluoride membranes (0.45 μm), the membranes were firstly blocked in 5% nonfat milk at room temperature (RT) for 1 hr, and then incubated with different primary antibodies: anti-NLRP3 (1:150 dilution, Bioss, China), anti-Caspase-1 (1:150 dilution, Bioss, China), anti-IL-1β (1:100 dilution, Bioss, China), anti-GAPDH (1:1000 dilution, Bioss, China) overnight at 4°C, rotated. GAPDH blotting was performed as loading control. The membranes were washed with 1× TBST for 3×10 min at RT, rotated; then incubated with HRP-conjugated secondary antibody at RT for 1 hr. Following 3×10 min washes in 1× TBST, protein bands were visualized using enhanced chemiluminescent reagents. The original western blot membranes corresponding with the representative images are present in Figure S2.

Immunohistochemistry: For immunohistochemical analysis, the embedded specimens were cut at 4 μm thickness. First, the sections were deparaffinized and rehydrated, then followed by 1, incubating with 3% hydrogen peroxide in order to eliminate the endogenous peroxidase activity; 2, washing with 1× PBS for 3×10 min; 3, incubating with different primary antibodies: anti-NLRP3 (1:150 dilution, Bioss, China), anti-Caspase-1 (1:150 dilution, Bioss, China) and anti-IL-1β (1:100 dilution, Bioss, China) antibodies at 4°C for overnight, respectively; 4, washing with 1× PBS for 3×10 min; 5, developing the immunohistochemical staining using the anti-rabbit SP kit (Maixin, Fuzhou, China) according to the manufacturer's introduction; 6, counterstaining with hematoxylin for light microscopy. Negative controls were set by omitting the primary antibodies in step 3.

Animals

Establishment of experimental apical periodontitis model: Animal care and experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee, Xi'an Jiao Tong University.

A total of 50 female Sprague-Dawley rats (12-16 week old, and 200-220 g body weight) were purchased from the laboratory animal center, Xi'an Jiao Tong University. The rats were randomly assigned into ovariectomized (OVX) and Sham groups, 25 rats per group. After anesthesia with an intraperitoneal injection of 3 mg/ml pentobarbital sodium (10 ml/kg; Veterinary Institute of Military Supplies University, Changchun, China), the bilateral ovariectomies were performed in the OVX group; for the sham group, the surgeries were performed by removing the same-size of fatty tissues near the ovaries. After the surgery, all animals were let to recover for 7 days. For establishing the apical periodontitis model, rats from both groups were first anesthetized as previously described, then the pulps of the mandibular first molars were exposed with a #1/4 round bur. To avoid the furcal perforation, the depth of the exposion should be equal to the diameter of the bur. The pulps of the teeth were exposed to the oral environment without any restoration during the entire experimental period.

Measurement of serum E2: On week 0, 1, 2, 3, 4 and 5 after ovariectomize surgery, the venous blood of rats were collected and promptly centrifuged at 10.000 rmp for 10 min under 4°C. The serum level of E2 was measured within 48 hours by the immunochemiluminescent assay with UniCel D ×1800 Immunoassay System (Beckman Coulter Inc.) according to the manufacturer's instructions.

Tissue preparation: 7, 14, 21 and 28 days after the lesion induction, 5 rats from each groups were anesthetized as previously described and sacrificed by cervical dislocation. Bilateral mandibles were removed and fixed with 4% paraformaldehyde at 4°C for 2 days. Then the left mandibles were subjected to radiological analysis. Meanwhile, the right mandibles were decalcified in 10% EDTA for 4 weeks at 4°C for sectioning. These decalcified specimens were cut at 4 μm thickness in order to obtain the mesial-distal serial sections, which were then subjected to histologic, enzyme histochemical, and immunohistochemical analysis.

Radiological examination: A dental x-ray unit (CCX digital; Trophy Radiologic UK Ltd, London, UK) and 31×41 mm dental radiographic film (Insight Film; Eastman Kodak, Rochester, NY) were used to take the radiographs. Uniform positioning of the mandibles was achieved by using a mold made of dental impression materials. To analyze the bone loss induced by apical periodontitis, we quantify the bone loss areas from the radiographs using SPOT RT software v3.5 (Spot Diagnostic Instruments, Ster-

ling Heights, MI), and then compared the results between the apical periodontitis group and the control group. This quantification analysis was conducted in a blind manner.

Histologic analysis: Serial paraffin slices with the complete root canal, including the apical foramen, were selected for hematoxylin/eosin staining. After hematoxylin/eosin staining, sections were photographed using a light microscope (Olympus, Tokyo, Japan). The bone loss areas of periapical lesions were measured by SPOT RT software, version 3.5 (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). For comparison, position matched images from the apical periodontitis group and the control group were quantified in a blind manner.

Enzyme histochemistry examination: Tartrate-resistant acid phosphatase (TRAP) is a biochemical marker specific for osteoclasts [27]. To detect TRAP activity and identify osteoclast, a TRAP kit (Sigma, St. Louis, MO) was used. The sections with periapical region were selected and subjected to TRAP activity examination. Briefly, the sections were rehydrated first and then incubated with the TRAP staining solution for 1 hour at 37°C, according to the manufacturer's protocol (Acid Phosphatase Kit; Sigma-Aldrich, Inc., St. Louis, MO). After incubation, sections were stained with hematoxylin. Certain sections were incubated in a substrate free medium, which were used as control for TRAP activity.

Immunohistochemistry: For immunohistochemistry, the sections were first deparaffinized and rehydrated, and then subjected to the following procedure: 1, incubating with 3% H_2O_2 to eliminate the activity of endogenous peroxidase; 2, performing the antigen retrieval by probing the sections with hyaluronidase (Sigma-Aldrich, St Louis, MO, USA) for 20 min at 37°C; 3, blocking the sections with 5% BSA; 4, incubating with the primary antibodies (anti-NLRP3, anti-Caspase-1, and anti-IL-1β (all from Santa Cruz Biotechnology, Santa Cruz, CA) at dilution of 1:100, respectively) for 20 hr at 4°C; 5, after 3×10 min washing, the sections were incubated with anti-rabbit straptavidin peroxidase (SP) (Maixin, Fuzhou, China), according to the manufacturers' instructions; 6, developing with the fresh 3,3'-diaminobenzidine (DAB) (Maixin, Fuzhou, China); 7, counter-staining with hematoxylin for taking images with light microscopy.

For negative controls, the sections went through the entire procedure described above excepting no primary antibodies in step 4.

Statistical analysis

All of the experiment was performed in triplicate. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Data were presented as ±SEM. Data from different groups were compared using the one-way analysis of variance (ANOVA) test. $P<0.05$ was considered as statistically significant. The graphic software used was GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

NLRP3, ASC, Caspase-1 and IL-1β is up-regulated in apical tissues of periapical periodontitis in postmenopausal patients

To study the effects of estrogen on the expression of NLRP3/Caspase-1/IL-1β axis during apical periodontitis, we performed IHC and western blot experiments using apical tissues of periapical periodontitis from two groups of patients: the premenopausal and postmenopausal patients with apical periodontitis. We found that in the postmenopausal apical tissues of periapical periodontitis, due to the decreased levels of estrogen in postmenopausal female patients as compared to that in premenopausal female patients, the expression levels of NLRP3, Caspase-1, IL-1β were up-regulated (**Figure 1A**), respectively. The statistical analysis showed that the differences were significant (P<0.05) (**Figure 1B**). Interestingly, not only the expression level of cleaved Caspase-1 was increased in postmenopausal female patients, but also the level of the precursor of cleaved Caspase-1, proCaspase-1, was increased as well (**Figure 1A**), indicating that probably estrogen regulates the translation of Caspase-1. Next, we performed IHC staining using the sections from the apical tissues of periapical periodontitis to detect the cellular expression levels of NLRP3, Caspase-1 and IL-1β. We observed that there were more immunostaining positive cells in the apical lesion areas in the postmenopausal patients as compared to the premenopausal patients, as showed by quantification analysis (P<0.05) (**Figure 2**). Thus, clinically, this data demonstrated that estrogen may be regulating the expression/activity of NLRP3/Caspase-1/IL-1β

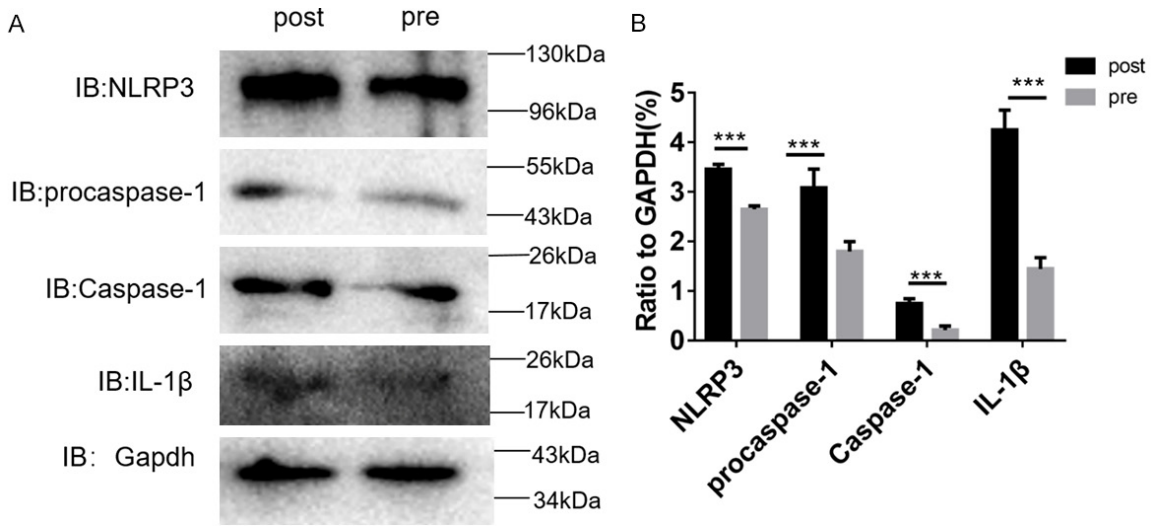

Figure 1. The expression levels of NLRP3, proCaspase-1, active Caspase-1 and IL-1β are increased in periapical tissues of periapical periodontitis in postmenopausal patients. The tissue lysates from the patients were separated on SDS-PAGE, and then immunoblotted with different specific antibodies. A. Representative immunoblotting images of different antibodies as indicated. B. Quantification analysis of the expression levels of the indicated proteins based on the immunoblotting results. The expression levels of NLRP3, Caspase-1, IL-1β were markedly up-regulated in periapical tissues of periapical periodontitis in postmenopausal patients. Data are presented as mean ± SEM (n=15 per group). ***P<0.001, *P<0.05. pre, premenopausal patients; post, postmenopausal patients.

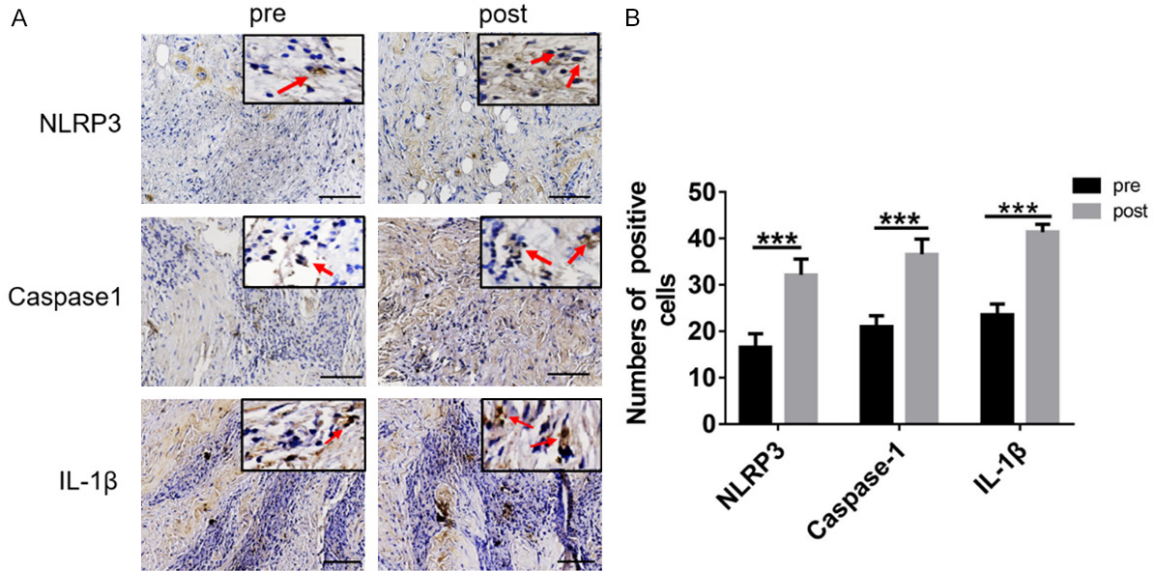

Figure 2. Immunohistochemic analysis of the cellular expression of NLRP3, Caspase-1 and IL-1β in periapical tissues of periapical periodontitis in premenopausal and postmenopausal patients. A. Representative images of immunohistochemical staining using anti-NLRP3, Caspase-1 and IL-1β antibodies, respectively. The red arrows in the insert point to the immunoreactive positive cells. Scale bar, 100 μm. B. Quantification of the NLRP3, Caspase-1 and IL-1β positive cells in the periapical lesions of periapical periodontitis in postmenopausal patients vs premenopausal patients. The number of NLRP3, ASC, Caspase-1 and IL-1β immunostaining positive cells were significantly increased in the postmenopausal patients (grey bars) as compared to the premenopausal patients (black bars). Data are presented as mean ± SEM (n=15 per group). ***P<0.001, *P<0.05. Original magnification, 200×; Scale bar, 100 μm.

axis during apical periodontitis development. In addition, we further found that the expression level of apoptosis-associate speck-like protein (ASC), an upstream interacting molecule of

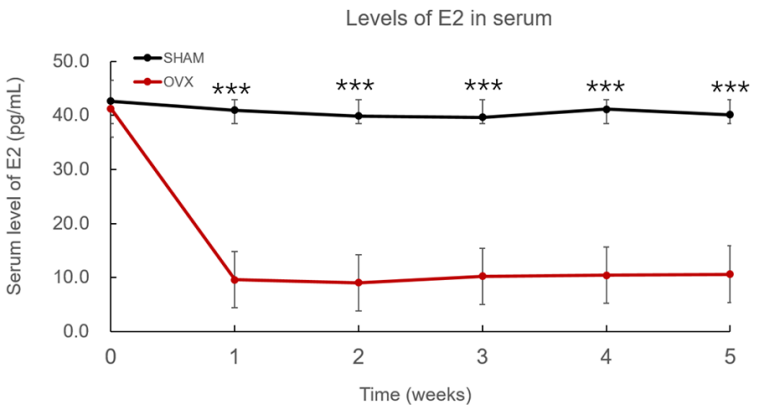

Figure 3. Measurement of serum levels of estrogen (E2) in Sham and OVX rats. Serum samples were prepared at different time points as indicated from both Sham and OVX groups, respectively. The serum level of estrogen was measured using immunochemiluminescent assay. At the start point right after ovariectomy (week 0), the serum levels of E2 in Sham and OVX rats are alike (P>0.05). However, during the first week after ovariectomy, the serum level of E2 was gradually reduced in the OVX group as compared to that in Sham group. After 1 week up to 5 weeks of ovariectomy, the serum levels of E2 were significantly reduced in the OVX groups as compared to that in Sham group, but no significant changes found within each group itself. Data are presented as mean ± SEM (n=5), ***P<0.001.

NLRP3/Caspase-1/IL-1 signaling pathway, in the infected apical tissues of the postmenopausal patients was also increased as compared to that of the premenopausal patients (Figure S1), providing another evident that NLRP3/Caspase-1/IL-1 axis is involved in the apical periodontitis mediated by estrogen deficiency.

Estrogen deficiency regulates the expression of NLRP3/Caspase-1/IL-1β axis in OVX animal model

Establishing OVX model: The ovariectomy rat model is known to result in characteristics of bone loss similar to those found in post-menopausal women [28]. In order to study the function of estrogen during the development of apical periodontitis, we also employed this model in our study.

To verify the efficacy of the ovariectomy procedure in OVX model, we measured the serum levels of E2 at different time points: week 0, 1, 2, 3, 4, 5 after ovariectomy. At week 0, there was no significant difference on the serum levels of E2 between OVX and Sham groups (P>0.05) (**Figure 3**). 1 week after ovariectomy, the serum levels of E2 in OVX group decreased to~26% of the sham group, whereas no significant changes on the serum levels of E2 in the sham group from week 0 up to week 1 (**Figure 3**). Afterwards, from 1 week to 5 weeks, the serum levels of E2 in both groups remained stable, respectively. However, starting from 1 week, the mean serum level of E2 in OVX group was approximately 4-times less than that in the Sham group (P<0.01) (**Figure 3**).

Estrogen mediates the proliferation of osteoclasts: Osteoclasts play very important roles during the alveolar bone loss resulted from periapical periodontitis [29]. Thus, we also investigated if estrogen deficiency impacts the development of osteoclasts using TRAP staining. In general, we found that, on day 0 after surgery, only a very few osteoclasts were observed in both groups. However, on day 7, the number of osteoclasts increased as compared to that on day 0, and kept increasing until day 14, where it reached the peak (**Figure 4A**). On day 21, the number of osteoclasts decreased, and persisted to day 28, the last time point we examined in both groups. Specifically, on day 7, 14, and 21, we observed that there are significant more osteoclasts in the OVX group as compared to the Sham group in the apical periodontitis lesion areas, but not on day 0 and 28 (**Figure 4B**). Our data suggest that estrogen may aggravate the alveolar bone loss via regulating the proliferation of osteoclasts during periapical periodontitis.

Estrogen deficiency up-regulates the expression levels of NLRP3/Caspase-1/IL-1β axis in experimental apical periodontitis: The NLRP3/Caspase-1/IL-1β axis has been demonstrated to be involved in the development of inflammatory periapical lesions [21, 22]. To address the effects of estrogen on the expression levels of NLRP3/Caspase-1/IL-1β axis in apical periodontitis, we performed immunohistochemic experiments on sections from day 7, day 14, day 21, and day 28. Our results showed that the numbers of immunoactive cells for the three components of NLRP3/Caspase-1/IL-1β

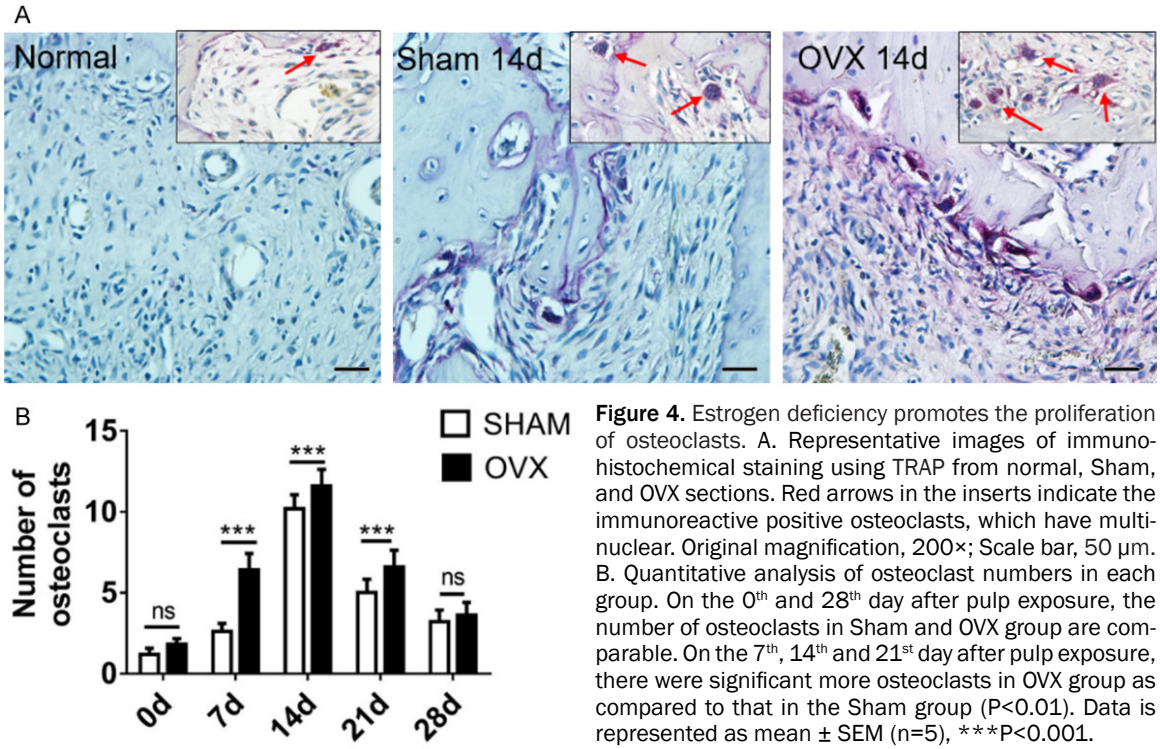

Figure 4. Estrogen deficiency promotes the proliferation of osteoclasts. A. Representative images of immunohistochemical staining using TRAP from normal, Sham, and OVX sections. Red arrows in the inserts indicate the immunoreactive positive osteoclasts, which have multinuclear. Original magnification, 200×; Scale bar, 50 μm. B. Quantitative analysis of osteoclast numbers in each group. On the 0th and 28th day after pulp exposure, the number of osteoclasts in Sham and OVX group are comparable. On the 7th, 14th and 21st day after pulp exposure, there were significant more osteoclasts in OVX group as compared to that in the Sham group (P<0.01). Data is represented as mean ± SEM (n=5), ***P<0.001.

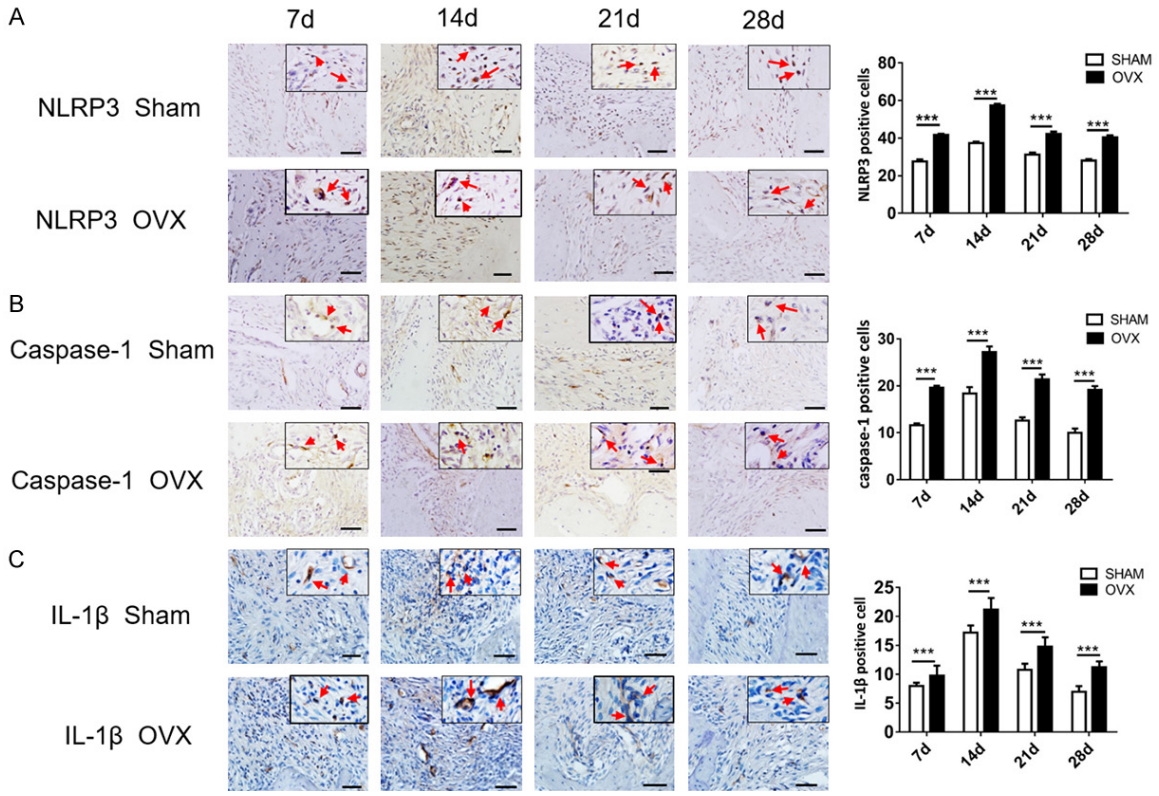

Figure 5. Estrogen deficiency upregulates the expression levels of NLRP3, capase-1 and IL-1β in periapical tissues of periapical periodontitis in OVX rats. A. Representative images of Immunohistochemical staining using anti-NLRP3 antibody at different time points as indicated (right panel), and the quantification analysis of the NLRP3 immunoactive positive cells in periapical tissues of periapical periodontitis of sham and OVX groups (left panel). B. Represen-

tative images of Immunohistochemical staining using anti-caspase-1 antibody at different time points as indicated (right panel), and the quantification analysis of the caspase-1 immunoactive positive cells in periapical tissues of periapical periodontitis of sham and OVX groups (left panel). C. Representative images of Immunohistochemical staining using anti-IL-1β antibody at different time points as indicated (right panel), and the quantification analysis of the IL-1β immunoactive positive cells in periapical tissues of periapical periodontitis of sham and OVX groups (left panel). The red arrows in the inserts point to the immunoreactive positive cells. Original magnification, 200×; Scale bar, 100 μm. Data are presented as mean ± SEM. ***P<0.001.

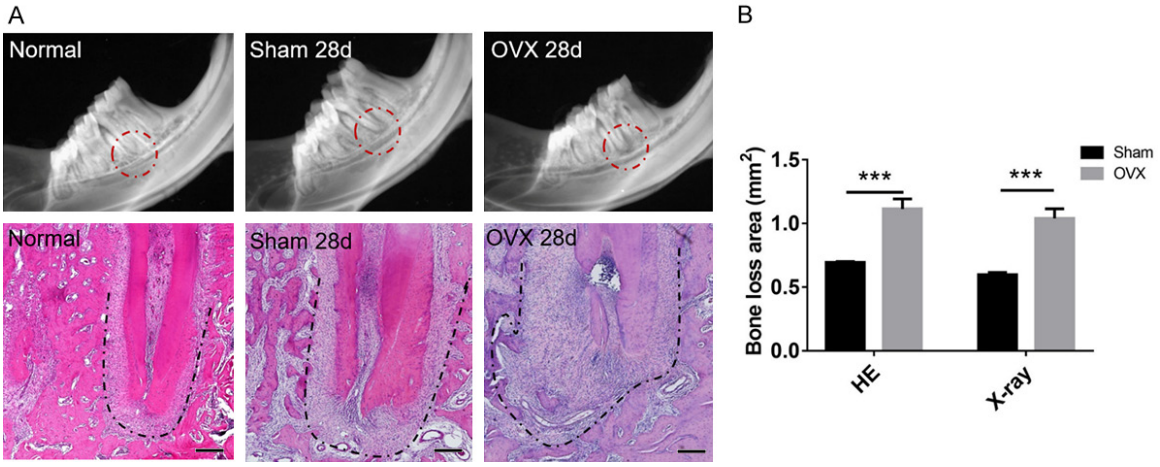

Figure 6. Estrogen deficiency aggravates the alveolar bone loss induced by apical periodontitis. A. Representative images of the radiologic photographs (upper panel) and the histological staining (lower panel) after 28 days of OVX. Original magnification, 400×; Scale bar, 50 μm. The periapical lesion areas were marked with the dot lines. B. Quantitative analysis of the bone loss area in periapical lesions of each group. Compared to the sham group, based on both radiologic and histological data, the bone loss area is significantly greater in the OVX group. Data is represented as mean ± SEM. ***P<0. 001.

axis in the apical periodontitis lesion areas of OVX group are greater than the numbers of Sham group from day 7 to day 28 (**Figure 5A-C**). This alteration is dynamic: from day 7 to day 14, the increase of the immunopositive cells reached the peak on day 14, and then started to decrease (**Figure 5A-C**), which is in line with the dynamic changes of osteoclasts (**Figure 4**). Taken all these data together, we found that estrogen deficiency probably aggravates the development of apical periodontitis lesion via up-regulating the expression/activity of the NLRP3/Caspase-1/IL-1β axis.

Estrogen deficiency aggravates the alveolar bone loss induced by apical periodontitis

The consequent results of apical periodontitis are the alveolar bone loss, so next we examined whether in the OVX model, estrogen deficiency results in the bone loss using radiographic image and HE staining. On day 0 after pulp exposure, no alveolar bone loss was observed in both OVX and Sham groups. However, on day 28 after pulp exposure, as the apical periodontitis is getting worse and worse, we found that there was significant alveolar bone loss in OVX group as compared to the Sham group (**Figure 6A**). The data from the two different measurements are comparable (**Figure 6B**). Thus, these findings indicate that estrogen may play important roles in the alveolar bone loss induced by apical periodontitis.

Discussion

It has been demonstrated that estrogen deficiency is strongly associated with bone loss and osteoporosis [30, 31]. Apical periodontitis, which is the major causative factor of bone loss in the periodontitis lesion, is a worldwide common oral disease. Previous studies have established a close relationship between estrogen deficiency and the bone resorption during the development of apical periodontitis [32, 33]. However, to date, the specific underlying mechanisms of how estrogen deficiency regulates the bone loss induced by apical periodontitis are still unclear. In the present study, using the clinic samples of postmenopausal woman pa-

tients and OVX rat model, we found that estrogen deficiency accelerated the apical periodontitis-induced bone loss via up-regulating the expression of NLRP3/Caspase-1/IL-1β axis.

In human females, the ovarian functions become dysfunction or failure gradually when they are entering the perimenopause [34], and after menopause, the estrogen level is suddenly dropped [35, 36]. This estrogen decrease due to ovarian failure is known to significantly affect bone homoeostasis and increases bone loss, and this antiresorptive action of estrogen is thought to be related to its ability to regulate modulating factors of osteoclastgenesis, but not due to its direct action on osteoclasts, for instance, the regulation of cytokine release in hypoestrogenism accelerates bone resorption [37, 38]. Cytokines, such as IL-1β, are found to be increased in ovariectomized rats and postmenopausal women [39, 40]. In consistent with these previous findings, we also demonstrated that, in postmenopausal women, the decreased estrogen level accelerated the bone resorption via up-regulating the expression of NLRP3/Caspase-1/IL-1β axis in an inflammation condition, providing evidences that the NLRP3/Caspase-1/IL-1β axis is involved in estrogen-regulated bone resorption.

It is well known that the ovariectomy model used in this study can result in characteristics of bone loss very similar to those found in postmenopausal women, and providing an experimental model to study the effects of estrogen on bone loss or resorption [27]. Before the surgical procedure, the estrous cycle was evaluated to select animals with normal cycle, and the efficacy of the ovariectomy procedure was confirmed by measuring the reduced serum levels of estrogen in the OVX groups (**Figure 3**). Several studies have used the OVX animal model to address the effects of estrogen deficiency and related molecules on bone loss issues [28, 29]. NLRP3, Caspase-1, and IL-1β are components of NLR signaling pathway, and Caspase-1 and IL-1β are downstream molecules of NLRP3. Mature IL-1β is synthesized as pro-IL-1β first, and then cleaved into mature forms by activated Caspase-1 [10]. Studies have shown that IL-1β is highly expressed in apical granulomas and that the overwhelming release of IL-1β leads to the destruction of local tissues by stimulating bone resorption [41, 42]. NLRP3 inflammasomes have been demonstrated to play an important role in inflammation. Tran et al. and Besnard et al. showed that the increased expression levels of NLRP3 and IL-1β in the allergic airway inflammation of OVX model, indicating that NLRP3 activation was required in allergic airway inflammation, which was further confirmed by the observation of that NLRP3-specific inhibitor could reverse neutrophilic inflammation in allergic airway disease [43-45]. Very recently, the results of Cheng et al. demonstrated that after OVX challenge, the expression levels of NLRP3 and Caspase-1 were increased in allergic airway inflammation, further confirmed the association of allergic airway inflammation with activation of NLR signaling [26]. Besides the roles of NRLP3 in the allergic airway inflammation mentioned above, Ran et al. and Liu et al. also demonstrated that in apical periodontitis patients and rats, the expression levels of NRLP3, Caspase-1, and IL-1β were up-regulated, and may be involved in the process of inflammation and associated with the degree of inflammation in apical periodontal lesions [21, 22]. In the present study, using apical periodontitis model with or without bilateral ovariectomies, we documented that NLRP3 expression level was increased accompanied by increased levels of active Caspase-1 and IL-1β, suggesting that estrogen deficiency would lead to activation of NLR signaling, therefor result in the bone resorption during the development of apical periodontal lesions.

To further confirm the effects of estrogen on the expression/activity of NLRP3 and its downstream molecules, including Caspase-1 and IL-1β, investigators performed the rescue experiments using E2. In mouse brain, Xu et al. indicated that administration of E2 or its receptor (ERβ) agonists can reverse the activity of NLRP3 caused by estrogen deficiency [24]. Thakkar et al. (2016) showed that E2 can noticeably inhibit NLRP3 activation and pro-inflammatory cytokine production in the brain after global cerebral ischemia. In the mouse model of amyotrophic lateral sclerosis (ALS), Heitzer et al. showed that E2 reduced the expression levels of NLRP3 protein, activate Caspase-1 and mature IL-1β [46]. Cheng et al. also showed that E2 markedly inhibited the mRNA and protein expression of NLRP3, as well cleaved Caspase-1 in inflammatory lung tissue [26]. Taken together, these studies further demonstrated that NLR signaling pathway plays an important role during the development of

estrogen-mediated inflammation. However, the interaction between estrogen and NLRP3/Caspase-1/IL-1β axis in estrogen-mediated development of apical periodontitis is still unclear. Our current study provided evidences showing that the NLRP3/Caspase-1/IL-1β axis is playing a critical role during the development of estrogen-mediated apical periodontitis.

In conclusion, our current study revealed a potential mechanism underlying the development of apical periodontitis lesion mediated by estrogen deficiency, and indicated that pharmacological inhibition of NLR signaling might be beneficial for the treatment of inflammatory diseases in postmenopausal woman patients.

Disclosure of conflict of interest

None.

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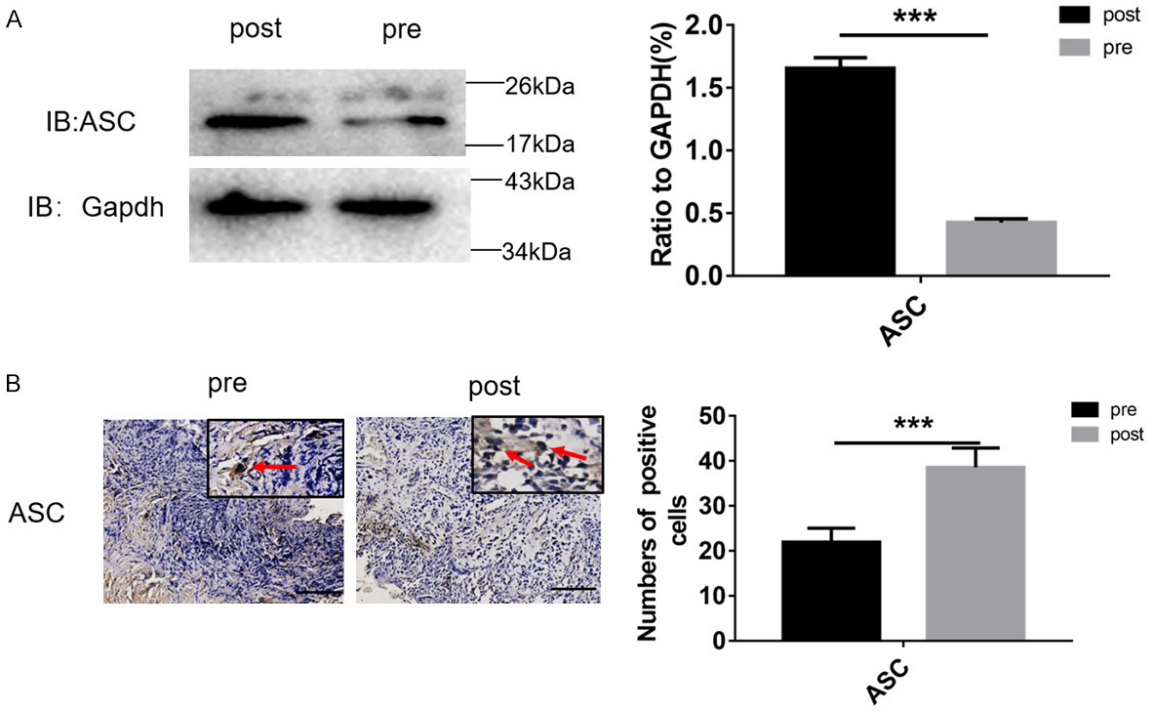

Figure S1. The expression levels of ASC in periapical tissues of periapical periodontitis in postmenopausal and premenopausal patients. A. Representative immunoblotting images of ASC as indicated (left panel), and the quantification analysis of ASC based on the immunoblotting results (right panel). B. Representative images of immunohistochemical staining using ASC antibody as indicated (left panel), and the quantification analysis of the ASC immunoactive positive cells in infected periapical tissues of postmenopausal and premenopausal patients. Scale bar, 100 μm. Data are presented as mean ± SEM. ***P<0.001.

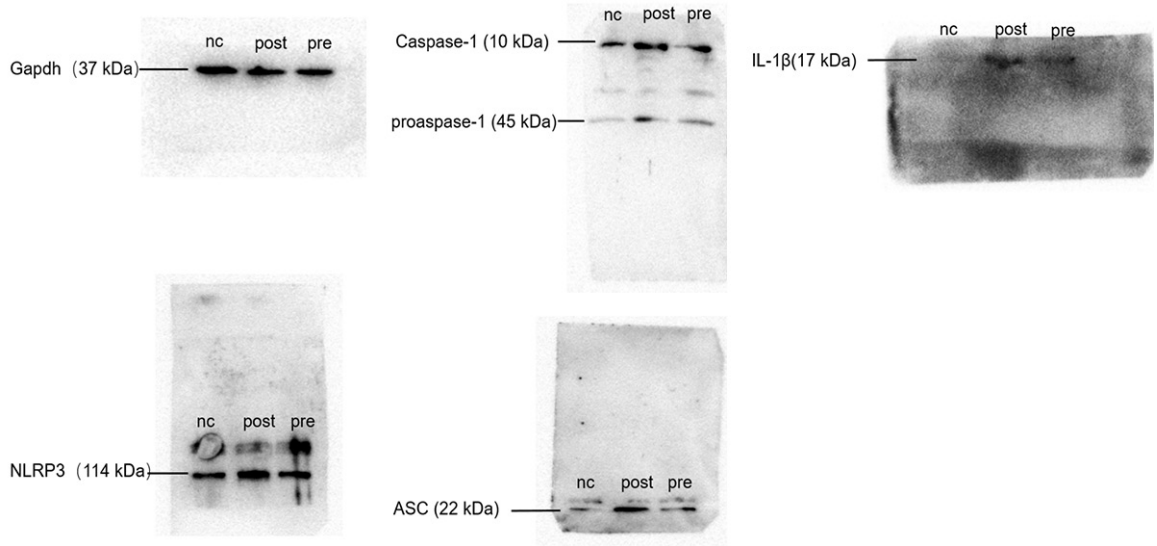

Figure S2. Photographs of the original western blot membranes showing expression levels of NLRP3, procaspase-1, caspase-1, IL-1β, and ASC in **Figures 1A** and S1. nc: healthy patients; post: postmenopausal patients; pre: premenopausal patients.