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Nootkatone mitigates periodontal inflammation and reduces alveolar bone loss *via* Nrf2/HO-1 and NF-κB pathways in rat model of periodontitis

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ABSTRACT

Introduction. Periodontitis (PD) is a chronic inflammatory disease leading to alveolar bone loss. This study investigated the effect of nootkatone and regulatory mechanism in reducing periodontal inflammation and alveolar bone loss in a rat model.

Material and methods. Twenty male Sprague-Dawley rats were divided into control, periodontitis, and nootkatone-treated groups (45 or 90 mg/kg). Ligature induction method was adopted to establish the PD model. After 21 days, rats received daily gavage of either saline or nootkatone for 10 days. Alveolar bone loss was assessed using micro-CT. Histological analyses included hematoxylin and eosin (H&E), tartrate-resistant acid phosphatase (TRAP), and Masson's trichrome stainings. Immunohistochemistry for heme oxygenase 1 (HO-1) and nuclear factor erythroid-2 related factor 2 (Nrf2) were performed in periodontal tissues. Content of inflammatory cytokines IL-1β, IL-6, and TNF-α in gingival tissues around ligature were assessed using ELISA kits. Malondialdehyde (MDA) level and superoxide dismutase (SOD) activity were analyzed and Western blot for NF-κB expression in gingival tissues were performed.

Results. Nootkatone significantly reduced the distance from cementoenamel junction to alveolar bone crest (CEJ-ABC), enhanced bone mineral density (BMD), bone volume (BV), and BV/total volume (TV) ratio in ligature-induced rats. Higher dose of nootkatone (90 mg/kg) did not show more significant therapeutic effect than lower dose (45 mg/kg). Histological staining showed decreased osteoclasts' number and improved bone architecture in the nootkatone group. Content of IL-1β, IL-6, and TNF-α and inflammatory cell infiltration level in gingival tissues around the ligature were decreased in the nootkatone-treatment rats. Nootkatone increased Nrf2 and HO-1 protein expression and decreased NF-κB protein level, suppressing MDA levels and enhancing SOD activity.

Conclusions. In a rat model, nootkatone effectively mitigates periodontal inflammation and alveolar bone loss through the Nrf2/HO-1 and NF-κB pathways. These findings suggest nootkatone as a promising therapeutic agent for the treatment of periodontitis.

Keywords: nootkatone; periodontitis; alveolar bone loss; cytokines; oxidative stress; NF-κB

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INTRODUCTION

Periodontitis (PD) is a chronic inflammatory disease affecting the supporting structures of teeth, leading to alveolar bone resorption and even tooth loss [1, 2]. This condition is primarily caused by microbial biofilms, which trigger an inflammatory response resulting in the destruction of periodontal tissues, including the alveolar bone [2, 3]. Effective management of periodontitis remains a significant clinical challenge, highlighting the need for new therapeutic agents that can reduce inflammation, promote tissue regeneration, and restore periodontal health [4].

The pathogenesis of periodontitis involves mediators like tumor necrosis factor-alpha (TNF-α), interleukins (IL-1β and IL-6), and nuclear factor kappa B (NF-κB), which are crucial in driving the inflammatory response and subsequent tissue destruction [5–9]. Natural compounds have shown promise in modulating inflammatory pathways and promoting tissue regeneration in periodontal disease models [10]. Studies have shown that curcumin can reduce in experimental periodontitis the production of pro-inflammatory cytokines IL-1β and TNF-α through the inhibition of the NF-κB pathway and decrease the infiltration of inflammatory cells [11, 12]. Additionally, curcumin has shown potential bone-protective effects by inhibiting the expression of TNF-α and IL-6 in pulp exposure-induced apical periodontitis in rat, making it a promising candidate for periodontal therapy [13]. Another natural compound, resveratrol, found in grapes and berries, could attenuate periodontal tissue destruction by reducing oxidative stress and modulating inflammatory responses in periodontitis [14]. Epigallocatechin-3-gallate could protect against periodontitis in rat model by inhibiting inflammation, oxidative stress and thus reducing the alveolar bone loss [15].

Similar to resveratrol, nootkatone, a sesquiterpenoid present in grapefruit and Alaskan yellow cedar [12], has also been studied in model diseases due to its anti-inflammatory and antioxidant properties [16–18]. Previously, nootkatone showed anti-inflammatory and neuroprotective effect in murine model of Parkinson's disease [19]. Nootkatone was discovered to alleviate liver injury and neurotoxicity induced by melamine in rats and inhibit oxidative stress and inflammation acting *via* NF-kβ pathway [17, 20]. Nootkatone also showed its anti-inflammation and protective effect against cartilage degeneration in mouse by inhibiting NF-kβ signaling pathway [21].

Given the anti-inflammatory and antioxidant effects of nootkatone, we hypothesize that nootkatone treatment could alleviate periodontal inflammation, reduce osteoclast activity, and enhance alveolar bone regeneration. The aim of this study is to evaluate the possible therapeutic potential of nootkatone and its regulatory mechanism in a rat model of ligature-induced periodontitis.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (n = 20), weighing 250 ± ± 10 grams and aged 8 weeks, were selected for the experiments (VitalRiver Biotech, Beijing, China). These rats were kept in a controlled environment with a 12-hour light/dark cycle and were provided with unlimited access to food and water. The animal experiments were approved by the Animal Ethics Committee of Tianjin Key Laboratory of Food Biotechnology (TKLFB-2023YJS-20).

Induction of periodontitis

To create a model of periodontitis, a 3-0 silk ligature was applied in the gingival areas around the second molars (M2) on the mandibles (n = 15) , as described in [22], with the unligated rats as a control (n = 5). After a period of 21 days, the rats were given either 0.9% NaCl (PD group, n = 5) or nootkatone (Acmec, Shenzen, Guangdong, China) at doses of 45 (n = 5) or 90 mg/kg (n = 5) *via* oral gavage daily for 10 days. Nookatone was dissolved in corn oil at 4.5 and 9 mg/mL, respectively, and 2.5 mL was administered each time.

Micro-CT analysis

Following the treatments, the animals were euthanized through inhalation of isoflurane, followed by decapitation, and their mandibles were harvested and fixed in 4% paraformaldehyde for 24 h. Micro-computed tomography (micro-CT, SKYSCAN 1276, Bruker, Beijing, China) was used to scan the mandibles as described by Borges *et al.* [23]. The images were analyzed to measure the cement-enamel junction to alveolar bone crest (CEJ-ABC) distance and various bone parameters, including bone mineral density (BMD), bone volume (BV), total volume (TV) and the ratio of BV/TV.

Histological procedures

The harvested mandibles were decalcified using 10% EDTA for 28 days, then embedded in paraffin. Serial sections of 5 μm thickness were prepared and subjected to hematoxylin and eosin (H&E) staining to examine tissue morphology and inflammatory cells' infiltration, tartrate-resistant acid phosphatase (TRAP) staining to identify osteoclasts, and Masson's trichrome staining to assess collagen fibers and bone architecture [24, 25]. We examined five sections from five mandibles in each group (n = 5). The specimen after staining were scanned on a digital Case Viewer (3D Histech, Budapest, Hungary). Inflammatory cells including lymphocytes, neutrophils and mononuclear macrophages in the gingival areas between the first molar (M1) and second molar (M2) were counted on the H&E sections by using Image J software (NIH, Bethesda, MD, USA). Representative images of H&E staining were captured at the magnifications of 5× and 40×. TRAP-positive cells in the area between M1 and M2 in each section were counted at magnification 20× with the use of Case Viewer Software (3D Histech). Representative images of TRAP staining were captured at magnification 40×.

Immunohistochemistry

Immunohistochemical staining was conducted on paraffin sections to evaluate heme oxygenase 1 (HO-1) and nuclear factor erythroid-2 related factor 2 (Nrf2) expression. Sections were deparaffinized, rehydrated, and underwent antigen retrieval in citrate buffer (pH 6, 10 mM) at 121°C for 10 min. Endogenous peroxidase activity was blocked

using 3% hydrogen peroxide solution. Sections were then incubated overnight at 4°C with primary antibodies: anti-Heme Oxygenase 1 rabbit pAb (GB11549-100; 1:600, ServiceBio, Wuhan, Hubei, China) and anti-NRF2 rabbit pAb (GB113808-100; 1:600, ServiceBio). Afterwards, the sections were treated with HRP-conjugated goat anti-rabbit antibody (GB23303; 1:500, ServiceBio) for 50 min at room temperature, and signals were visualized using a DAB kit (G1212-200T, ServiceBio). Hematoxylin was used for counterstaining. The integrated optical density (IOD) values of HO-1 and Nrf2 was analyzed with the use of Image J (NIH).

Enzyme-linked immunosorbent assay (ELISA)

Gingival tissues around second molars on the mandibles were homogenized on ice in phosphate-buffered saline (PBS) and centrifuged at 5000 *g* for 10 min at 4°C to collect supernatants. The concentrations of IL-1β, IL-6, and TNF-α in the supernatants were measured using ELISA kits (E-HSEL-R0002, E-HSEL-R0004, E-EL-R2856; Elabscience, Wuhan, Hubei, China) according to the manufacturer protocols. Absorbance readings were taken at 450 nm using a microplate reader (Thermo Fisher, Waltham, MA, USA).

Determination of MDA levels and SOD activity

Malondialdehyde (MDA) level and superoxide dismutase (SOD) activity in gingival tissue supernatants were quantified using commercial kits (Elabsicence). MDA levels were determined *via* reaction with thiobarbituric acid [26], while SOD activity was measured through a colorimetric assay that monitors the inhibition of nitro blue tetrazolium reduction as *per* the manufacturer's protocol.

Western blot analysis

Proteins were extracted from gingival tissues using RIPA lysis buffer with added protease and phosphatase inhibitors (Beyotime, Shanghai, China). Protein samples were subjected to SDS-PAGE, then transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with anti-NF-κB rabbit pAb (GB11997-100; 1:800, ServiceBio) and anti-glyceraldehyde-3-phosphate dehydrogenase antibody (anti-GAPDH) rabbit pAb (GB15004-100; 1:5000, ServiceBio). After washing, membranes were incubated with HRP-conjugated goat anti-rabbit antibody (GB23303; 1:10000, ServiceBio). Protein bands were visualized using an ECL detection kit (ServiceBio), and the relative protein expression levels against GAPDH were analyzed using ImageJ software (imagej.net).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Inc., San Diego, CA, USA). Group comparisons were made using one-way ANOVA followed by Tukey's *post-hoc* test for multiple comparisons. A P-value of less than 0.05 was considered statistically significant.

RESULTS

Nootkatone alleviates the alveolar bone resorption in rats with ligature-induced periodontitis

As described above, a periodontitis rat model was established using the ligature-induced methods around the second molar (M2) of the left mandible, with the uninduced counterpart as a control (Ctrl). After 21 days of ligature inducement, the rats were treated with 0.9% NaCl or nootkatone (45 or 90 mg/kg) by gavage for 10 days. The CEJ-ABC distance of M2 on the lingual side was analyzed and results showed that ligature induction increased the CEJ-ABC distance significantly, signifying the successful establishment of the PD rat model (Fig. 1A–C). The oral administration of nootkatone decreased the CEJ--ABC distance, yet the larger dose (90 mg/kg) exerted similar effect as the 45 mg/kg dose (Fig. 1A–C). The micro-CT analysis also revealed that the bone parameters BMD, BV and BV/TV were decreased in PD group whereas treatment with nootkatone restored these parameters to control values (Fig. 1D, E, G). No significant difference in TV values among the groups were found in this study (Fig. 1F). Furthermore, the results showed that the larger dose (90 mg/kg) didn't show better effect than 45 mg/kg (Fig. 1D, E, G). These results initially demonstrated that nootkatone could alleviate the alveolar bone resorption in PD rats and larger dose (90 mg/kg) didn't show better effect than 45 mg/kg.

Nootkatone decreased number of osteoclast cells and promoted the new bone formation

The TRAP staining was used to analyze the periodontal tissues in each group for the counts of osteoclast cells. Results presented an increase in the number of osteoclast cells in alveolar bone area in ligature-induced PD group compared to the Ctrl (Fig. 2A, B, D). The treatment with nootkatone (45 mg/kg) for 10 days significantly reduced number of osteoclasts and no significant difference was found between the nootkatone and Ctrl groups in the number of TRAP-positive cells (Fig. 2C, D). Masson staining displayed intact epithelium and regular junctional epithelium in the Ctrl group (Fig. 2E), absence of interdental papilla, migration of the junctional epithelium in the PD group (Fig. 2F), and its restoration in the nootkatone group (Fig. 2G). The maturity of the bone was high in the Ctrl group (Fig. 2E). In PD group, Masson staining showed more collagen fibers, correlated with low calcification of the alveolar bone and Haversian structure was still in the stage of initial formation (Fig. 2F).

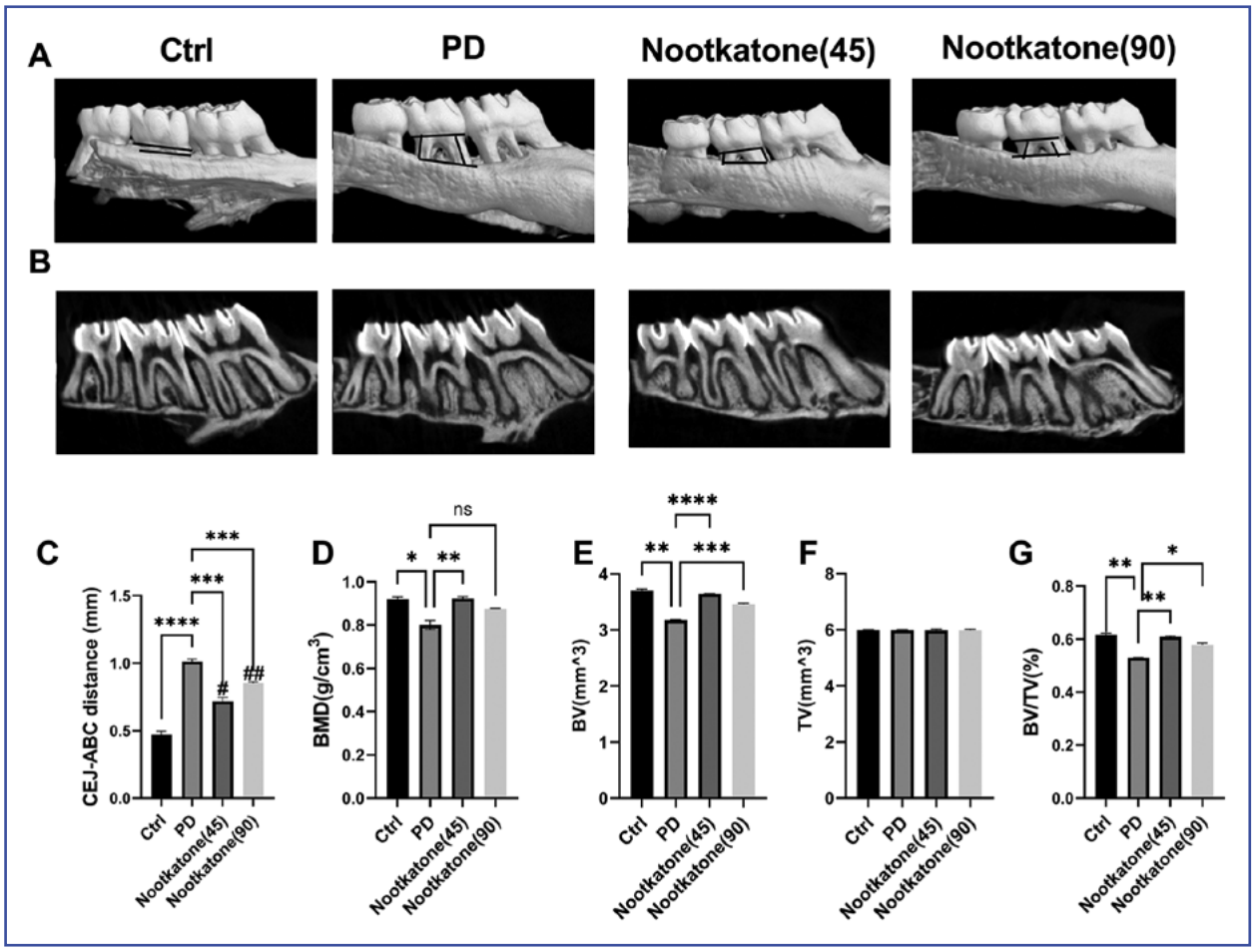

Figure 1. Nootkatone alleviates the alveolar bone resorption in mandibles with ligature-induced periodontitis (PD) in rat. PD rat models were established using the ligature-induced methods around the M2 of the left mandible, with the uninduced counterpart as a Ctrl. After 21 days of ligature inducement, the rats were treated with 0.9% NaCl or nootkatone (45 or 90 mg/kg) by gavage for 10 days. Micro CT method was applied to evaluate the PD-induced changes of the bone. **A.** Representative images of the molars after 3D reconstruction of the mandibles in each group. **B.** Representative scanning images of the molars that are used to analyze the CEJ–ABC distance. **C.** CEJ-ABC distance. **D.** BMD. **E.** BV. **F.** TV. **G.** The ratio of BV/TV. In histograms the bars represent means and the whiskers standard errors. $^{*}P < 0.03$; $^{**}P < 0.002$; $^{***}P < 0.0002$; $^{****}P < 0.0001$; $^{\#}P < 0.03$; $^{\#\#}P < 0.002$ in comparisons with the Ctrl group. Abbreviations: BMD — bone mineral density; BV — bone volume; CEJ–ABC — cementoenamel junction to alveolar bone crest; CT — computed tomography; Ctrl — control; M2 — second molar; ns — not significant; TV — total volume.

Nootkatone inhibited the inflammation in ligature-induced periodontitis rats

Consistent with the results of Masson staining, H&E staining also demonstrated the loss of interdental papilla and apical migration of junctional epithelium in PD group, which was restored in the nootkatone group (Fig. 3A–C). On the other hand, compared to the control rats, the number of inflammatory cells was significantly increased in the PD group and was reduced by nootkatone treatment, whereas no significant difference was found between the nootkatone and Ctrl groups (Fig. 3D). The levels of inflammatory cytokines, IL-1β, IL-6 and TNF-α, were examined in gingival tissues using ELISA. The concentrations of these proteins were elevated in PD group (*versus* Ctrl) and were decreased in the nootkatone group. Still, the IL-1β, IL-6 and TNF-α concentrations were significantly higher in nootkatone-treated rats than control ones (Fig. 3E, F, H).

Nootkatone alleviated the periodontitis in rats through oxidative stress related-Nrf2/HO-1/NF-kβ pathway

Immunohistochemistry results showed that HO-1immunoreactivity (-Ir) was lower in PD group in comparison with the control group, whereas nootkatone treatment increased HO-1-Ir to the level higher in nootkatone group than the Ctrl group (Fig. 4A, C). Nrf2-Ir was lower in the PD group; however, the nootkatone treatment significantly increased Nrf2-Ir as compared to the control group (Fig. 4B, D). In addition, western blot analysis showed that NF-κβ expression was enhanced in the PD group and reduced in the nootkatone

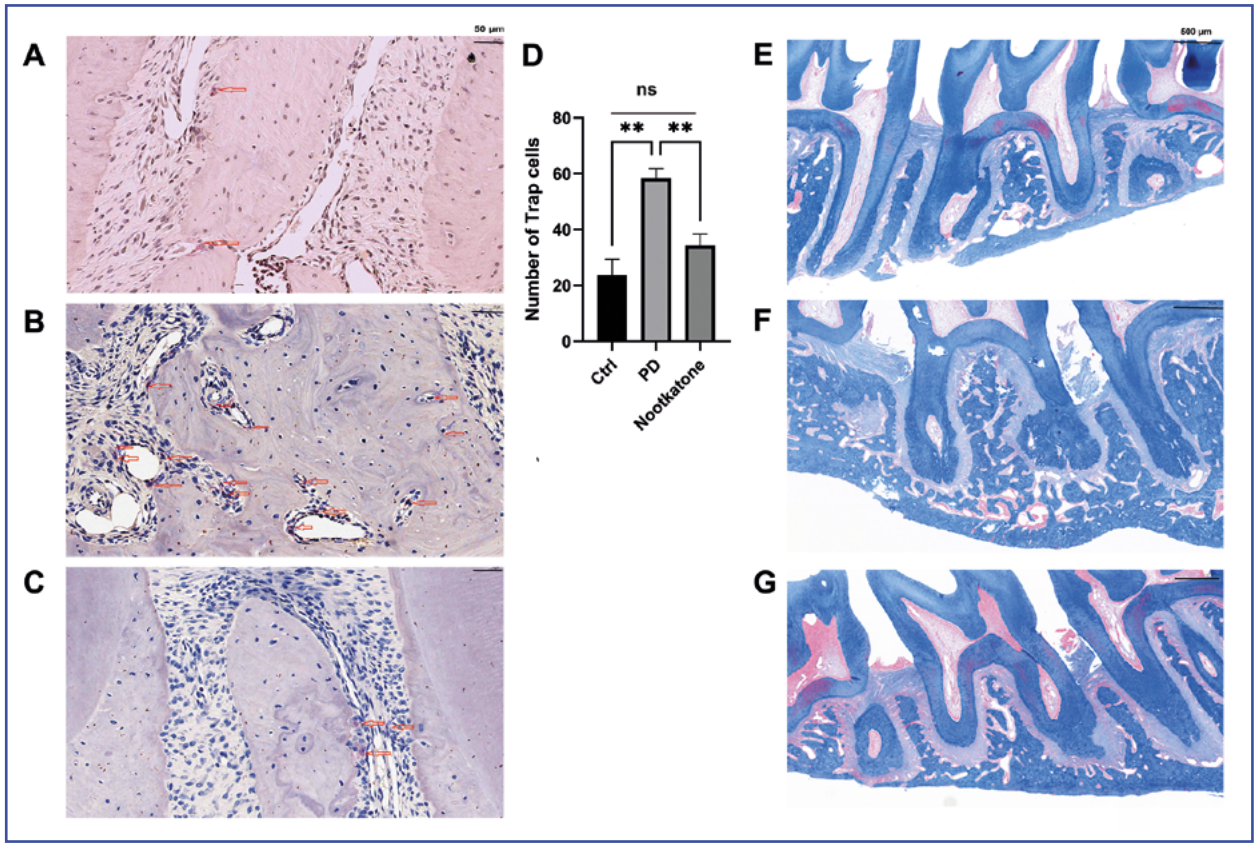

Figure 2. Nootkatone promoted the new bone formation and decreased number of osteoclasts identified by TRAP staining. The periodontal tissues were examined using the TRAP and Masson staining methods. **A–C.** TRAP staining in Ctrl, PD and PD + nootkatone (45 mg/kg) groups, respectively. The red arrows marked the osteoclasts. **D.** TRAP-positive cells in each group were counted as described in Methods. **E–G.** Masson staining in Ctrl, PD and PD + nootkatone (45 mg/kg) groups, respectively. $^{**}P < 0.002$. Abbreviations: Ctrl — control; ns — not significant; PD — periodontitis; TRAP — tartrate-resistant acid phosphatase.

group (Fig. 4E). The MDA content was enhanced in PD group but was decreased in the nootkatone group (Fig. 4F). SOD activity was inhibited in the PD group but it was recovered in the nootkatone group (Fig. 4G). In addition, no significant differences were found in the levels of MDA and SOD activity, as well as NF-κβ protein expression between the nootkatone and control groups (Fig. 4E–G). These results indicated that the oxidative stress parameters activated in ligature-induced periodontitis rats were reversed by the nootkatone treatment.

DISCUSSION

In this study, we showed that nootkatone treatment leads to a reduction in alveolar bone loss compared to the periodontitis group using micro-CT analysis. Histological staining showed decreased osteoclasts' number and improved gingival tissue structure in the periodontitis rats after nootkatone treatment. These findings suggest that nootkatone could alleviate periodontitis. Previous studies showed that nootkatone could protect against oxidative stress in murine models of liver injury, neurotoxicity, and nephrotoxicity [17, 20, 27]. In liver injury murine model, nootkatone was confirmed to suppress oxidative stress through Nrf2/HO-1/NF-kβ pathway [28]. In myocardial injury model, nootkatone also inhibited oxidative stress *via* NF-kβ route [29]. Previous research showed that herbal medicine could regulate Nrf2/HO-1 to protect human or mouse vascular endothelial cells from oxidative stress induced by H_2O_2, IL-1, TNF-κ, and other factors [30]. Herbal medicine was also discovered to inhibit neuroinflammation and depression *via* oxidative stress inhibition through Nrf2/HO-1 signaling [31]. For instance, Daidzein, a naturally occurring dietary isoflavone, alleviated the neuropathic pain neuroinflammation induced in mice by paclitaxel, inhibiting oxidative stress by activating Nrf2/HO-1 pathway [32]. Nrf2 is a transcription factor that regulates the expression of antioxidant proteins protecting against oxidative damage triggered by injury and inflammation [33]. HO-1, a downstream target of Nrf2, has been shown to exert anti-inflammatory and cytoprotective effects [34]. Sinensetin, a polymethoxylated flavone alleviated ligature-induced periodontitis in rat by upregulating HO-1 and

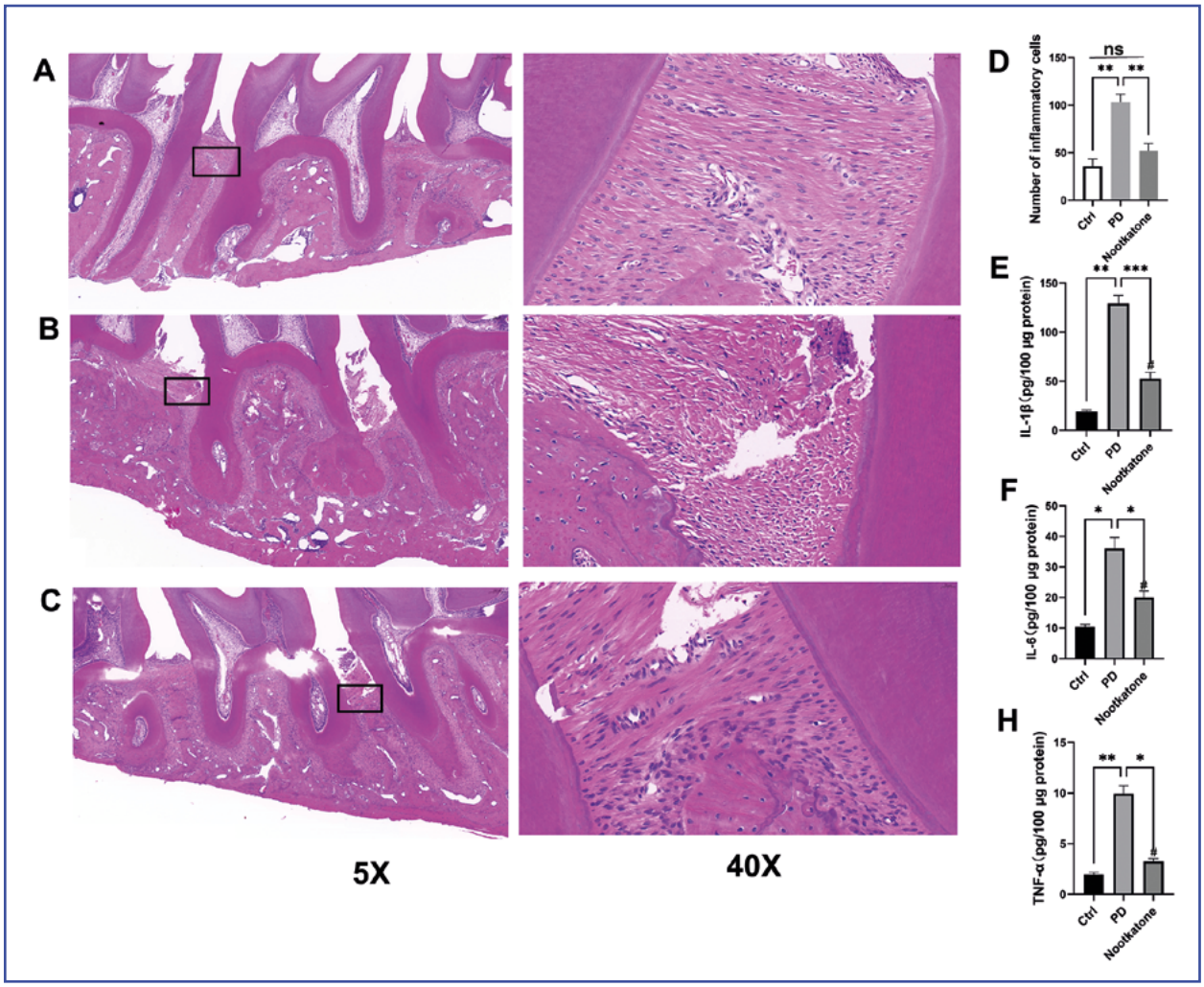

Figure 3. Nootkatone inhibited the infiltration of inflammatory cells and concentrations of proinflammatory cytokines in ligature-induced periodontitis (PD) in rat. H&E staining was performed on sections of mandibles from control and PD-induced rats. **A.** Respective H&E-stained section in the Ctrl group. The black boxed area in the gingiva was augmented in the right panel at 40×. **B.** Ligature-induced PD group. **C.** PD and nootkatone (45 mg/kg) group. **D.** Number of inflammatory cells in gingival area was determined as described in Methods. **E–G.** ELISA kits were used to analyze the protein levels of IL-1β, IL-6 and TNF-α in the gingival tissues. $^{*}P < 0.03$; $^{**}P < 0.002$; $^{***}P < 0.0002$; $^{\#}P < 0.03$; $^{\#\#}P < 0.002$ in comparisons with the Ctrl group. Abbreviations: Ctrl — control; H&E — haematoxylin and eosin; ns — not significant; PD — periodontitis.

suppressing oxidative stress [35]. In our study, administration of nootkatone to rats with periodontitis increased the immunoreactivity of Nrf2 and HO-1 in periodontal tissues, suggesting that nootkatone enhances the antioxidant defense mechanisms, reducing oxidative stress as shown by decreased MDA level and enhanced SOD activity. Oxidative stress is one of key factors in the pathogenesis of periodontitis in rat and human [36]. The Nrf2/HO-1 pathway plays a crucial role in mitigating oxidative stress by upregulating the expression of antioxidant enzymes such as SOD, thereby neutralizing ROS and protecting periodontal tissues from oxidative damage [37]. In the *in vitro* and *in vivo* models of periodontitis, excessive ROS can activate the NF-κB, further exacerbating the inflammatory response and contributing to the destruction of periodontal tissues [38].

Pro-inflammatory cytokines IL-1β, IL-6 and TNF-α are widely reported in inflammation-related diseases, including periodontitis and rheumatoid arthritis [39, 40]. Periodontitis, triggered by pathogen microbes, is characterized by destructive inflammatory immune response and resultant connective tissue damage and alveolar bone loss [41]. In periodontitis patients, pro-inflammatory cytokines IL-1β, IL-6 and TNF-α are biomarkers of the disease severity [42, 43]. Treatment with antagonists of IL-1 and TNF-α showed significant therapeutic effect in a non-human primate model of periodontitis induced by ligature impregnated with *Porphyromonas gingivalis* [44]. In ligature-induced

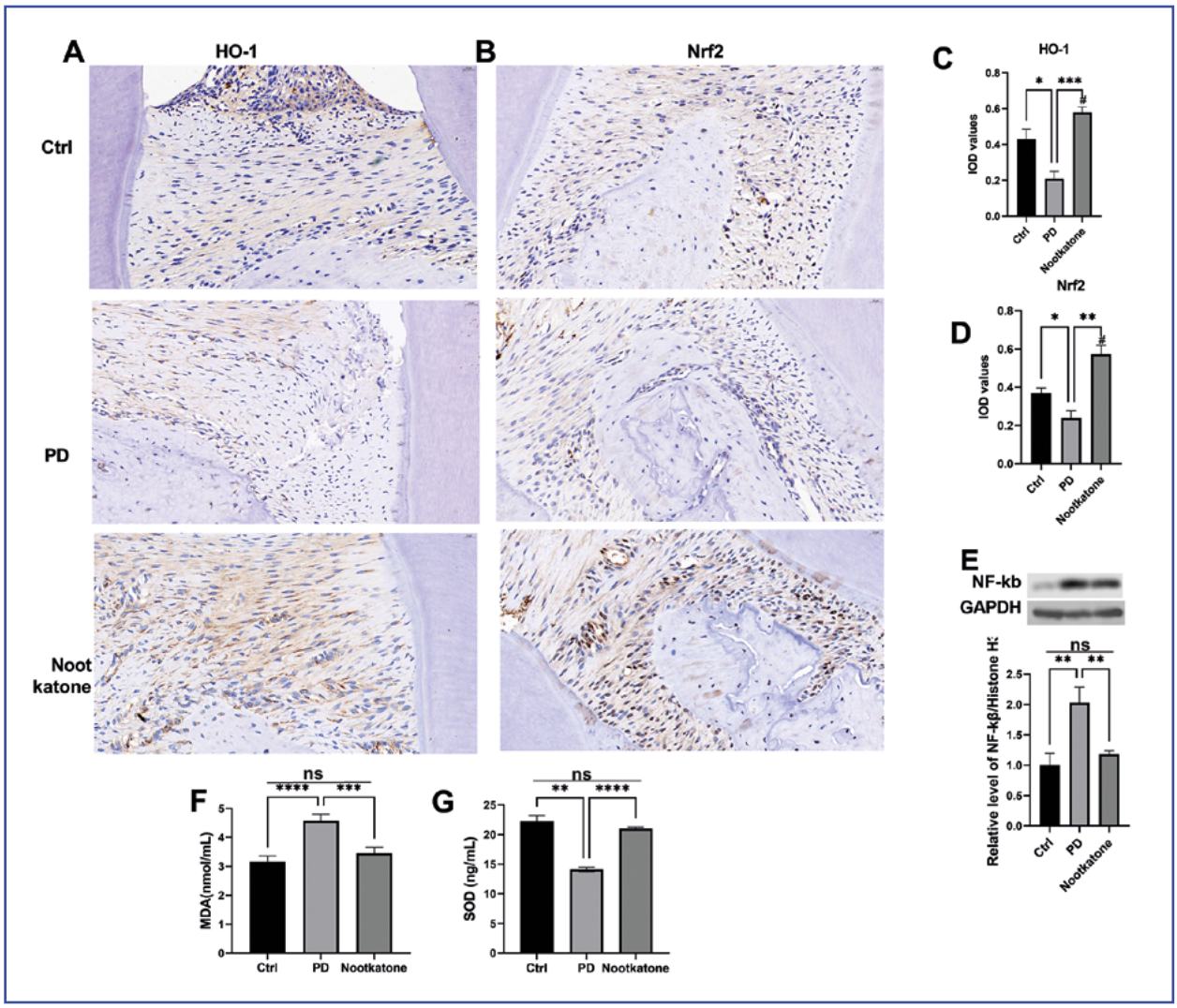

Figure 4. Nootkatone alleviated the periodontitis in rat acting on Nrf2/HO-1/NF-κB signaling pathway. **A–D.** Immunohistochemical method was applied to analyze the presence of HO-1 and Nrf2 proteins in periodontal tissues. Image J software was used to analyze the IOD values of HO-1 and Nrf2 in each group. **E.** Western blot was performed to measure the protein levels of NF-kβ with normalization to GAPDH. **F, G.** MDA and SOD levels were measured as described in Methods. $^{*}P < 0.03$. $^{**}P < 0.002$, $^{***}P < 0.0002$; $^{****}P < 0.0001$; $^{\#}P < 0.03$; $^{\#\#}P < 0.002$ in comparisons with the Ctrl group. Abbreviations: GAPDH —glyceraldehyde-3-phosphate dehydrogenase antibody; IOD — integrated optical density; MDA — malondialdehyde; ns — not significant; SOD — superoxide dismutase.

periodontitis rats, blocking IL-6 inhibited inflammatory cells' infiltration, and reduced attachment damage and bone loss [41]. Our findings showed that nootkatone decreased the levels of IL-κB, IL-6 and TNF-α in gingival tissue of rats with induced periodontitis, which likely contributes to reduced alveolar bone loss.

The therapeutic potential of natural compounds in the therapy of periodontitis has been increasingly recognized due to their safety profile and efficacy in modulating biological pathways involved in inflammation and tissue regeneration. Curcumin, for instance, has been extensively studied for its anti-inflammatory and bone-protective effects in periodontitis models [45]. Resveratrol has also shown promise in reducing oxidative stress and modulating inflammatory responses in periodontal disease [46, 47]. Our study adds to this growing body of evidence by highlighting nootkatone as a novel natural compound with therapeutic benefits for periodontitis. The limitation of this study exists in the limited exploration of complex molecular mechanisms related to nootkatone action and on the specific type of mechanically induced PD. Animal models induced by placement of *P. gingivalis*-infected silk ligatures, have been widely used in validating therapeutic effects of new material or compound in periodontitis [48, 49].

In summary, this study identifies nootkatone as a promising candidate for the possible adjunctive treatment of periodontitis if future research could show its effectiveness in other models of PD.

Article information and declarations

Data availability statement

All data are incorporated into the article.

Ethics statement

The animal experiments were approved by the approved by the Animal Ethics Committee of Tianjin Key Laboratory of Food Biotechnology (TKLFB-2023YJS-20).

Author contributions

Ye Yin: Conception and design, research preparation, paper writing. ZeYu Ma: data analysis, experiment execution. Peiliang Shi: data collection, diagramming.

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Conflict of interest

The authors declare that there is no conflict of interest.

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