Effect of Psoralen on the Intestinal Barrier and Alveolar Bone Loss in Rats With Chronic Periodontitis

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Abstract

Objectives

To study the effects of psoralen on the intestinal barrier and alveolar bone loss (ABL) in rats with chronic periodontitis.

Methods

Fifty-two 8-week-old specific pathogen-free (SPF) male Sprague-Dawley (SD) rats were randomly divided into the following four groups: Control group (Control), Psoralen group of healthy rats (Pso), Periodontitis model group (Model), and Psoralen group of periodontitis rats (Peri+Pso). The alveolar bone resorption of maxillary molars was observed via hematoxylin-eosin staining and micro-computed tomography. The expression level of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) in periodontal tissues were evaluated by immunofluorescence staining. The changes in serum tumour necrosis factor (TNF)-α, interleukin (IL)-10, IL-6, intestinal mucosal occludin and claudin-5 were detected using enzyme-linked immunosorbent assay (ELISA). The level of intestinal mucosal NOD2 was detected using immunohistochemical methods. DNA was extracted from the intestinal contents and the 16s rRNA gene was sequenced using an Illumina Miseq platform.

Results

The expression of NOD2 protein in the intestinal tract of periodontitis rats decreased after intragastric psoralen administration. Psoralen increased the intestinal microbiota diversity of rats. The level of serum pro-inflammatory factor TNF-α decreased and the level of anti-inflammatory factor IL-10 increased. ABL was observed to be significantly decreased in rats treated with psoralen. Psoralen decreased the RANKL/OPG ratio of periodontitis rats.

Conclusions

Psoralen may affect the intestinal immune barrier and ecological barrier, mediate immune response, promote the secretion of anti-inflammatory factor IL-10, and reduce the secretion of the pro-inflammatory factor TNF-α, thus reducing ABL in experimental periodontitis in rats.

1. Introduction

Chronic periodontitis is a chronic inflammatory disease characterised by periodontal tissue destruction, and this tissue destruction is characterised by gingival inflammation, periodontal pocket formation, and alveolar bone resorption[1-2]. Therefore, the treatment of periodontitis can control the inflammatory response, prevent further tissue damage, and promote periodontal tissue regeneration. Periodontitis is primarily treated via mechanical means such as subgingival scaling. In addition, periodontal surgery and drug therapy are also important periodontitis treatment methods. Drug treatment mainly depends on antibiotics, but it is associated with certain challenges, such as drug resistance and oral flora

imbalance[3]. Therefore, it is necessary to study new preparations that have antibacterial and anti-inflammatory effects and can reduce alveolar bone resorption.

The intestinal tract is not only a digestive organ, but also an immune organ. There is an interactive relationship between the intestinal mucosal immune system and the intestinal flora. The immune system can recognise microorganisms and secrete cytokines and antibodies to maintain the homeostasis of the intestinal environment through innate and adaptive immunity. The weakening of the intestinal epithelial barrier leads to the invasion of intestinal pathogenic bacteria and the secretion of harmful metabolites into the host. When various internal and external factors destroy the balance between the intestinal microecosystem and the host, the intestinal microflora can invade the host as an antigen, triggering a series of immune responses, leading to changes in bone mass and structure. A close relationship has been reported between the bones and the immune system, and some scholars have put forward the concept of 'bone immunity' [4]. As a part of the skeletal system, alveolar bone plays an active role in metabolism and remodelling of the skeletal system. Different from other bones in the body, the bone remodelling process of alveolar bone is affected by both the local oral environment and systemic factors. Some studies have found that *Lactobacillus* can affect the metabolism of the alveolar bone by influencing the absorption of minerals of the host. In addition, it can also affect alveolar bone mass by inhibiting the proliferation and differentiation of osteoclasts[5].

Fructus psoraleae is a type of traditional Chinese medicine, which has anti-inflammatory[6], anti-tumour, antibacterial, antifungal, immunomodulatory, oestrogenic, and osteogenic activities[7], and is widely used in the treatment of osteoporosis. Psoralen is an effective component isolated from psoralea. At present, the osteogenic effect of psoralen has been widely studied. *In vitro* experiments have shown that psoralen promotes osteogenic differentiation by increasing alkaline phosphatase (ALP) activity in human periodontal ligament cells (HPDLCs) and upregulating osteogenic genes and proteins (RUNX2, DLX5, and OPG) [8]. It can also inhibit osteoporosis in ovariectomised rats by stimulating bone marrow mesenchymal stem cells to differentiate into osteoblasts[9]. For most traditional Chinese medicine, oral administration is the most convenient route, as its effective components are absorbed, distributed, and metabolised in as well as excreted from the human body. The transformation of intestinal bacteria is the key link that leads to the production of more active compounds in the digestive tract. At present, some plasma pharmacokinetic studies of psoralen have shown that it has rapid oral absorption in the enterohepatic circulation[10]. Based on these findings, we hypothesised that psoralen could improve alveolar bone resorption in periodontitis rats by regulating the intestinal barrier. In this study, a rat model of periodontitis was established and psoralen was administered intragastrically to observe the changes in the intestinal barrier, the levels of serum inflammatory factors in rats with periodontitis, and their effects on alveolar bone loss (ABL) in periodontitis rats.

2. Materials And Methods

2.1 Periodontitis rat model

All animal-related experiments were performed using the protocols approved by our institute (The Ethics Committee of Qingdao Stomatological Hospital Affiliated to Qingdao University, 2019KQYX018). Fifty-two 8-week-old male Sprague-Dawley (SD) rats and weighing 350 ± 20 g were purchased from Shandong Experimental Animal Center. They were caged in specific pathogen-free (SPF) conditions, fed with a normal diet, and subjected to constant temperature and humidity for 12 h each of alternating light/darkness. One week later, adaptive feeding was included in the experiment. The 52 rats were randomly divided into four groups (n=13 each group): 1) Control group (Control); 2) Healthy rats treated with psoralen group (Pso); 3) Periodontitis model group (Model), and 4) Periodontitis rats treated with psoralen group (Peri+Pso). The periodontitis modelling method was conducted as follows: mice were anaesthetised with 10% chloral hydrate via intraperitoneal injection at a dosage of 0.3 mL/100 g body weight, fixed and disinfected, and the gingival tissue of the bilateral maxillary second molars (M2) was separated using a sharp probe. A standard ligating wire (0.25 mm) was passed through the proximal and distal adjacent spaces of the bilateral maxillary M2, ligated around the dental neck, placed in the gingival sulcus as far as possible, and knotted in the mesiobuccal side of M2. The ligation wire was checked daily for shedding, and was re-ligated when the wire detached. Four weeks after ligation, the periodontal condition of the rats was examined. The following changes were checked: whether the second molar gingiva was red and swollen, bled upon probing, had a deep periodontal pocket, had alveolar bone resorption, and had increased tooth mobility. Two rats were randomly selected and sacrificed, and the bilateral maxilla were used for hematoxylin-eosin (HE) staining and micro-computed tomography (CT) scanning. M2 mesial and distal alveolar bone resorption was observed and a large number of inflammatory cells were infiltrated; thus, an experimental rat periodontitis model was established.

2.2 Intragastric administration of psoralen

Psoralen standard (molecular weight, 186.16; purity ≥ 98%) was purchased from Solarbio and was dissolved in normal saline at a final concentration of 4 mg/mL. From the fifth week after the periodontitis model was established, psoralen was administered to the rats according to their body weight at a dose of 8 mg/kg/2 d, while psoralen suspension was administered to the healthy and periodontitis rats at the same time. Samples were obtained after intragastric administration for four weeks as follows. Blood was collected from the heart under abdominal anaesthesia, and the rats were sacrificed by cervical dislocation. The bilateral maxilla and a 2 cm long ileum tissue were immediately removed and fixed in 4% paraformaldehyde for 24 h. The fresh ileum content (approximately 3-5 g) and another 2 cm ileum tissue segment were respectively placed in a 1.5 mL Eppendorf tube aseptically, temporarily stored in an icebox, and then stored at -80 °C until use.

2.3 HE staining and immunofluorescence staining of alveolar bone

The 4% paraformaldehyde-fixed alveolar bone was then decalcified using a 10% EDTA decalcification solution for four weeks. The samples were routinely dehydrated, embedded, and sliced along the proximal and distal direction at a distance of 5 μm. The samples were HE-stained to observe periodontal tissue

inflammation and ABL. Immunofluorescence staining was used to observe the expression level of RANKL and OPG in periodontal tissues.

2.4 Micro-CT analysis of alveolar bone

The maxilla of rats were scanned using a μCT100 microcomputer tomography (SCANCO Medical AG), with a voxel resolution of 15 μm. Mimics-Research 20.0 software was used for three-dimensional reconstruction, and the amount of ABL was evaluated by measuring the average distance from the mesial, central, and distal alveolar crest to the enamel bone boundary of the buccal and palatal side of the second molar[11].

2.5 Analysis of occludin and claudin-5 expression in the ileal mucosa

Fresh ileal tissue (~2 cm) was obtained from each sample, homogenised using a homogeniser, and centrifuged for approximately 20 min (× 1610 *g*). The supernatant was collected carefully, strictly following the instructions of the rat occludin ELISA kit (ELISA, enzyme-linked immunosorbent assay) and rat claudin-5 ELISA kit manufacturers, respectively. A standard curve was established, and the levels of occludin and claudin-5 in the ileal mucosa of each sample were determined.

2.6 NOD2 expression analysis in the ileal mucosa

The expression of NOD2 in the intestinal mucosa was detected using immunohistochemical methods. The tissue sections were dewaxed and the antigens were retrieved. The sections were placed in a 3% hydrogen peroxide solution and incubated at 25 °C for 10 min to block the endogenous peroxidase. Normal goat serum blocking solution was then added and incubated at room temperature for 15 min. Next, 50 μL goat anti-mouse NOD2 polyclonal antibody (Santa Cruz Biotechnology) was added (1:150 dilution) and the samples were incubated overnight at 4 °C, rinsed with phosphate-buffered saline (PBS) thrice, and finally the biotin-labelled secondary antibody was added. After incubation at room temperature for 20 min, the degree of colouration was controlled by viewing under the microscope, and the colour reaction was terminated with distilled water. The samples were then re-stained with haematoxylin for 30-60 s, the sheets were dehydrated until transparent, and a neutral gum seal was added. After scanning each section, six visual fields were randomly selected using Case Viewer 2.4. The positive cells were semi-quantitatively counted using the Image-Pro Plus 6.0 software. The data were expressed as integrated optical density (IOD).

2.7 Sequencing of 16S rRNA and data analysis of intestinal flora in the ileum

After genomic DNA was extracted from the ileal samples, its concentration and purity were detected using 1% agarose gel electrophoresis. The V3 and V4 regions of the bacterial 16s rRNA were amplified using PCR. The upstream and downstream primers used were as follows: 338F: 5'-ACTCCTACGGGAGGCAGCAG-3' and 806R: 5'- GGACTACHVGGGTWTCTAAT-3'. The amplification program was set as follows: 94 °C for 5 min, 94 °C for 30 s, 55 °C for 30 s, 28 cycles of 72 °C for 60 s, and 72 °C

for 7 min. A Miseq library was constructed using the PCR products, and high quality data was analysed using the bioinformatics suite provided by the Illumina Miseq platform.

2.8 Analysis of serum inflammatory factors

The collected blood was centrifuged at 3,000 rpm for 15 min to separate the serum, which was stored at -20 °C until use. A rat IL-6 (IL, interleukin) ELISA kit, rat IL-10 ELISA kit, and rat TNF-α (TNF, tumour necrosis factor) ELISA kit were all purchased from Jiangsu Enzymatic Immunization Company. The levels of IL-6, IL-10 and TNF-α in serum were measured strictly according to the manufacturer's instructions.

2.9 Statistical analysis

Ileal intestinal flora was analysed using R to calculate the alpha diversity and to perform a species composition analysis. Linear discriminant analysis (LDA) effect size (LEfSe) analysis and LDA were used to reduce and evaluate the influence of species with significant differences in data (LDA score), and the threshold was set to 3 to find species that had significantly different abundances between the groups. Other data were analysed using the GraphPad Prism 8 software. The difference between the groups was analysed using single factor analysis of variance. Comparisons between the two groups were conducted using a t-test with two independent samples. When $P < 0.05$, the difference was considered to be statistically significant.

3. Results

3.1 Verification of periodontitis modelling in rats

rats. In HE staining (a-d), a and c mean healthy rats, b and d mean periodontitis rats. In three-dimensional reconstruction of maxillary alveolar bone after Micro-CT scan (e-h), e and g mean healthy rats, f and h mean periodontitis rats. CEJ, cemento-enamel junction; AB, alveolar bone; OC, osteoclast. Scale bars=300μm (a and b); 100μm (c and d).

HE staining showed that the gingival epithelium of rats in the Control group had no pathological changes and the surface of their alveolar bone was smooth. Compared with the Control group, the gingival epithelium of the second molar of the rats in the Model group were found to be thickened, inflammatory cells infiltrated, and the binding epithelium moved away from the enamel bone boundary to the root, thereby resulting in the formation of erosive resorption lacunae on the surface of alveolar bone, and osteoclasts could be observed in the lacuna area (Fig. 1a-d). The three-dimensional reconstruction of the maxillary molars and the alveolar bones of rats after micro-CT scanning showed that the distance from the buccal and palatal alveolar ridge to the enamel bone boundary of the second maxillary molars of rats in the Model and Peri+Pso groups was larger than that of the Control group (Fig. 1e-h).

3.2 Effect of psoralen on ileal mechanical barrier

The protein expression levels of claudin-5 and occludin in the ileal mucosa of rats in each group were detected using ELISA. No considerable differences were found among the four groups (Fig. 2a-b).

3.3 Effect of psoralen on ileal immune barrier

Immunohistochemical sections of rat ileal mucosa showed that NOD2 protein was mainly expressed in the epithelial cells and monocytes. Light yellow granules could be observed in the cytoplasm of a small number of cells in the intestinal mucosa of the Control and Pso groups, and a large amount of dark brown or brown granules could be observed in the cytoplasm of epithelial cells and monocytes in the Model and Peri+Pso groups (Fig. 2d). The NOD2-positive cells were semi-quantitatively counted using the Image-Pro Plus 6.0 software. The data were expressed as IOD. No significant difference was found in the expression of NOD2 protein between the Control and Pso groups. The expression of NOD2 protein in the Model was significantly higher than that in the Control groups ($P < 0.01$). NOD2 expression in the Peri+Pso group was higher than the Control group ($P < 0.05$), but significantly lower than that in the Model group ($P < 0.05$) (Fig. 2c).

3.4 Effect of psoralen on ileal ecological barrier

The alpha diversity of the intestinal flora in rats of each group was analysed. It was found that the Chao1 index of the Pso and Peri+Pso groups was higher than that of the Control, and the Chao1 index of the Pso group was significantly higher than that of the Control ($P < 0.05$) (Fig. 3a). The Shannon index of the Pso and Peri+Pso groups was higher than that of the Control, and the Shannon index of the Peri+Pso group was significantly higher than that of the Model group ($P <0.05$) (Fig. 3b). Compared with the Control and Model groups, psoralen increased the abundance and diversity of intestinal flora in rats to some extent.

By calculating the Firmicutes/Bacteroidetes (F/B) ratios, we found that the F/B ratio in the Model and Pso groups was lower than that in the Control group ($P < 0.001$), and that in Peri+Pso group was lower than that in the Model group ($P < 0.05$) (Fig. 3c). Further LEfSe analysis of intestinal flora showed that there were significant differences between these groups. The relative contents of o_Clostridiales, c_Clostridia, p_Actinobacteria, and o_Bacillales in the Pso group increased, and that of f_Lactobacillaceae, g_Lactobacillus, o_Lactobacillales, and c_Bacilli in the Model group increased. Furthermore, the relative contents of f_Erysipelotrichaceae, g_Allobaculum, f_Clostridiaceae_1, g_Clostridium_sensu_stricto_1, o_Enterobacteriales, f_Enterobacteriaceae, g_Escherichia_Shigella, s_Escherichia_coli, and p_Bacteroidetes in the Peri+Pso group increased (Fig. 4).

3.5 Effect of psoralen on alveolar bone

The distance between the ABC (ABC, alveolar bone crest) and CEJ (CEJ, cemento-enamel junction) in the normal rats was less than 0.25 mm. No significant difference was found in the amount of ABL between the Model and Pso groups, but the amount of ABL in the Model group was significantly higher than that in the Control ($P < 0.001$). The amount of ABL in the Peri+Pso group was significantly higher than that in the Control ($P < 0.001$), but significantly lower than the Model group ($P < 0.01$) (Fig. 5a).

Immunofluorescence staining showed that the expression of OPG increased and the expression of RANKL decreased after administration of psoralen (Fig. 5c). The RANKL/OPG ratio in the Model group was higher than that in the Control group ($P < 0.01$), and the RANKL/OPG ratio decreased after administration of psoralen ($P < 0.01$) (Fig. 5b).

3.6 Effect of psoralen on serum inflammatory factors

No significant difference was found in the serum pro-inflammatory factor IL-6 between the groups (Fig. 6a). The expression level of TNF-α in the Model group was significantly higher than that in the Control ($P < 0.01$), while that in the Peri+Pso group was lower than the Model group ($P < 0.05$) (Fig. 6b). The expression level of the anti-inflammatory factor IL-10 in the serum of rats in the Peri+Pso group was higher than that in Control ($P < 0.05$), and significantly higher than the Model group ($P < 0.01$) (Fig. 6c).

4. Discussion

Psoralen, the main active component of psoralea, has many effects, such as cartilage protection and anti-tumour, immune regulation, and anti-vitiligo activities[12]. Recent studies have also found that psoralen can stimulate osteoblast differentiation by activating BMP (BMP, bone morphogenetic protein) signals[13]. Psoralen can inhibit bone resorption of osteoclasts *in vitro*[14]. Additionally, psoralen can treat arthritis by regulating the balance of Th1/ Th2 cells and inhibiting the expression of TNF-α, IL-6, and IL-1β[8]. Furthermore, it can also increase the bone mass of ovariectomised rats with osteoporosis by stimulating bone marrow mesenchymal stem cells to differentiate into osteoblasts[9, 12].

Chronic periodontitis is a chronic infectious disease, characterised by gingival inflammation, periodontal pocket formation, and alveolar bone resorption. *Porphyromonas gingivalis* is the main dominant bacteria in periodontitis, especially in chronic periodontitis. Recently, it has been found that oral administration of *Pseudomonas gingivalis* caused changes in the composition of intestinal flora, regulated the intestinal immune system, and impaired intestinal barrier function, thereby resulting in systemic inflammation and related pathological changes[15-17]. Among them, the intestinal barrier, as an important factor affecting systemic inflammation, is composed of a mechanical barrier that composed of epithelium and tight junction proteins, an immune barrier composed of immune cells and immune proteins in the intestinal mucosa, and an ecological barrier composed of normal intestinal flora. The three are closely related in function, and together provide intestinal immunity. The function of the intestinal mechanical barrier mainly depends on the transcellular pathway and the paracellular pathway. The basic structure of the paracellular pathway is the tight junction (TJ) composed of the proteins claudin, occludin, and Zo. The occludin and claudin family of proteins together form the transport pathway, which controls epithelial barrier permeability. Epithelial permeability increases when these proteins are overexpressed[18], causing pathogenic bacteria and related metabolites to invade the body. In this study, no significant difference was found in the protein expression of occludin and claudin-5 among the groups, indicating that psoralen had no significant effect on these proteins in the intestinal mechanical barrier.

In the physiological state, the host recognises endogenous or exogenous microorganisms through intestinal NODs, and maintains the gastrointestinal mucosal epithelial barrier by regulating the release of antimicrobial substances and maintaining the homeostasis of intestinal flora. NODs interact with symbiotic bacteria, and symbiotic bacteria promote the expression of NODs. Simultaneously, NOD negative feedback inhibits the proliferation of symbiotic bacteria. NODs play an important role in maintaining the balance between intestinal symbiotic flora and host immune response[19-20]. NOD2 is an innate immune protein in gastrointestinal epithelial cells[21], which can recognise the teichoic acid dipeptide in the bacterial wall and mediate the activation of NF-κB (NF-κB, nuclear factor kappa-B) and the expression of the inflammatory cytokine TNF-α[20, 22], thus initiating the immune response to pathogens[23]. In this study, the expression of NOD2 in the intestinal mucosa of the Model group was found to be higher than that of the Control group, indicating that the intestinal immune barrier was activated. After intragastric administration of psoralen, the expression of NOD2 protein in the Peri+Pso group was significantly lower than that of Model. Psoralen alleviated intestinal injury and decreased the expression of NOD2 to some extent.

Recent studies have shown that bone metabolism such as bone growth and remodeling is closely related to intestinal microflora[24-25]. Intestinal flora can maintain homeostasis of the host immune system, regulate the activity of osteoclasts and osteoblasts, and affect the balance of bone metabolism[26]. In this study, analysis of the alpha index of intestinal flora showed that the Chao1 index of the rats in the Pso group was significantly higher than that in the Control group, and the Shannon index in the the rats in the Peri+Pso group was significantly higher than that in the Model group. The results showed that psoralen increased the abundance and diversity of intestinal flora in rats. Atarashi et al. [27] found that *Clostridium* can promote the number and function of Treg cells, form an environment rich in TGF-β (TGF-β, transforming growth factor-β), and inhibit the formation of osteoclasts. In addition, some studies have found that the *Clostridium leptum* subgroup can promote the production of Treg cells and secrete anti-inflammatory cytokines (such as IL-10) to inhibit self-traumatic inflammation. In this study, LEfSe analysis of intestinal flora in rats also found that the relative contents of o_Clostridiales and c_Clostridia in the Pso group increased, indicating that psoralen can increase the relative content of *Clostridium* in the intestines of healthy rats.

In addition, the F/B ratio was considered to be another significant indicator of microbial structure, as determined by the taxonomic composition[28]. In some studies on human osteoporosis, it was found that the F/B ratio in the osteoporosis group was higher than that in the control group[29-30]. However, we found that the decrease of the number of Firmicutes and the increase of the number of Bacteroidetes in the Pso and Peri+Pso groups led to the decrease in the F/B ratio and the decrease in ABL in the Peri+Pso group. It is suggested that psoralen can reduce the F/B ratio and ABL in periodontitis rats. At the same time, LEfSe analysis of intestinal flora showed that the relative content of p_Bacteroidetes in the Peri+Pso group increased. However, studies on senile osteoporotic rats and humans with low bone mineral density also found a decrease in the F/B ratio[31-32]. This was similar to the increase in bone loss and the decrease in

the F/B ratio in the Model group compared with the Control group in this study. Therefore, the role of the F/B ratio as a significant indicator of bone mineral density needs to be further elucidated.

The aforementioned results indicate that psoralen had a certain effect on the intestinal flora and immune barrier, to verify whether psoralen affected the intestinal tract and alveolar bone resorption. We further statistically analysed M2 ABL in rats, and found significant ABL compared between the Control, Model, and Peri+Pso groups; however, ABL in the Peri+Pso groups was significantly less than that in the Model group. It was suggested that psoralen can reduce alveolar bone resorption in periodontitis rats, and no significant difference was found in ABL between the Control and Pso groups. Combined with the results of NOD2 protein expression analysis in the ileal mucosal immune barrier in rats, psoralen had a significant effect during periodontitis, however, no significant change was observed under normal conditions. We speculate that psoralen mainly plays a role in inflammation and is related to the immune response.

Heredity, hormones, immune system function, nutrition, and lifestyle factors play a decisive role in bone tissue mineralisation. Several mechanisms accelerate bone loss by altering the activity of osteoblasts and osteoclasts. For instance, bone mass loss mediated by oestrogen deficiency is associated with osteoclast differentiation and activity, in part due to the increased production of pro-inflammatory cytokines (TNF- α, IL-1, and IL-6) and the activation of immune cells[33]. At present, some scholars have proposed that intestinal bacteria can lead to the activation of T cells in bone tissue. Inflammatory cytokines IL-6 and TNF-α secreted by T cells can promote gingival inflammation, accelerate periodontal connective tissue destruction, and alveolar bone resorption. TNF-α can induce the production of RANKL and macrophage colony stimulating factor (M-CSF) and downregulate OPG[34-35]. TNF-α can also directly interact with osteoclast precursors and promote their differentiation and maturation. Through the receptor P55, TNF-α can enhance the response of bone marrow macrophages (BMM) to M-CSF and RANKL, and indirectly promote BMM to differentiate into osteoclasts. However, IL-10 can inhibit T lymphocyte synthesis and production of IL-6, TNF-α, and interferons[36-38]. Therefore, we further detected the expression level of RANKL and OPG in periodontal tissues, and the levels of inflammatory cytokines IL-6, IL-10, and TNF-α in serum. This study found that the expression of OPG increased and the expression of RANKL decreased after administration of psoralen. The RANKL/OPG ratio in the Model group was higher than that in the Control group, and decreased after treatment with psoralen. At the same time, we found that there was no significant difference in IL-6 expression between the four groups, however, the content of TNF-α in the Peri+Pso group was significantly lower than that in the Model group. Additionally, the anti-inflammatory factor IL-10 in the Peri+Pso group was significantly higher than that in the Model group. Therefore, psoralen can promote the secretion of IL-10, inhibit the secretion of TNF-α, and slow down the inflammatory reaction. In conclusion, psoralen may affect the intestinal immune barrier and ecological barrier, mediate immune response, promote the secretion of the anti-inflammatory factor IL-10, and reduce the secretion of pro-inflammatory factor TNF-α, thus reduce the expression of RANKL and reduce ABL in experimental periodontitis in rats. These results suggest that psoralen can potentially be used in the treatment of periodontitis.

Declarations

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Code availability: All software application support our published claims and comply with field standards.

Authors' contributions: Hua Liu and Yingjie Xu conceived and designed the study. Hua Liu, Yingjie Xu, Qi Cui, Ning Liu, Fuhang Chu, Beibei Cong, Yingtao Wu performed the experiments. Hua Liu and Yingjie Xu wrote the paper. All authors read and approved the manuscript.

Ethics approval: All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Consent to participate: This manuscript does not contain any studies with human participants performed by any of the authors.

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References

1. Slots J. 2013. Periodontology: past, present, perspectives. *Periodontol 2000* 62:7-19. http://dx.doi.org/10.1111/prd.12011.
2. Di Paola R, Marzocco S, Mazzon E, Dattola F, Rotondo F, Britti D, De Majo M, Genovese T and Cuzzocrea S. 2004. Effect of aminoguanidine in ligature-induced periodontitis in rats. *Journal of Dental Research* 83:343-348. http://dx.doi.org/10.1177/154405910408300414.
3. Palombo EA. 2011. Traditional medicinal plant extracts and natural products with activity against oral bacteria: potential application in the prevention and treatment of oral diseases. *Evid-Based Compl Alt* 2011. http://dx.doi.org/10.1093/ecam/nep067.
4. Arron JR, Choi Y. 2000. Osteoimmunology-bone versus immune system. *Nature* 408:535-536. http://dx.doi.org/10.1038/35046196.
5. Britton RA, Irwin R, Quach D, Schaefer L, Zhang J, Lee T, Parameswaran N and McCabe LR. 2014. Probiotic L. reuteri treatment prevents bone loss in a menopausal ovariectomized mouse model. *Journal of Cellular Physiology* 229:1822-1830. http://dx.doi.org/10.1002/jcp.24636.
6. Chen CH, Hwang TL, Chen LC, Chang TH, Wei CS and Chen JJ. 2017. Isoflavones and anti-inflammatory constituents from the fruits of Psoralea corylifolia. *Phytochemistry* 143:186-193.

http://dx.doi.org/10.1016/j.phytochem.2017.08.004.

7. Chopra B, Dhingra AK and Dhar KL. 2013. Psoralea corylifolia L. (Buguchi)-Folklore to modem evidence: Review. *Fitoterapia* 90:44-56. http://dx.doi.org/10.1016/j.fitote.2013.06.016.
8. Li X, Yu C, Hu Y, Xia X, Liao Y, Zhang J, Chen H, Lu W, Zhou W and Song Z. 2018. New application of psoralen and angelicin on periodontitis with anti-bacterial, anti-inflammatory, and osteogenesis effects. *Front Cell Infect Microbiol* 8:178. http://dx.doi.org/10.3389/fcimb.2018.00178.
9. Yang Z, Huang JH, Liu SF, Zhao YJ, Shen ZY, Wang YJ and Bian Q. 2012. The osteoprotective effect of psoralen in ovariectomy-induced osteoporotic rats via stimulating the osteoblastic differentiation from bone mesenchymal stem cells. *Menopause-the Journal of the North American Menopause Society* 19:1156-1164. http://dx.doi.org/10.1097/gme.0b013e3182507e18.
10. Feng L, Wang L and Jiang XH. 2010. Pharmacokinetics, tissue distribution and excretion of coumarin components from Psoralea corylifolia L. in Rats. *Arch Pharm Res* 33:225-230. http://dx.doi.org/10.1007/s12272-010-0206-5.
11. Jia X, Jia L, Mo L, Yuan S, Zheng X, He J, Chen V, Guo Q, Zheng L, Yuan Q, Xu X and Zhou X. 2019. Berberine ameliorates periodontal bone loss by regulating gut microbiota. *Journal of Dental Research* 98:107-116. http://dx.doi.org/10.1177/0022034518797275.
12. Chen S, Wang Y, Yang Y, Xiang T, Liu J, Zhou H and Wu X. 2017. Psoralen inhibited apoptosis of osteoporotic osteoblasts by modulating IRE1-ASK1-JNK Pathway. *Biomed Res Int* 2017:3524307. http://dx.doi.org/10.1155/2017/3524307.
13. Tang DZ, Yang F, Yang Z, Huang J, Shi Q, Chen D, Wang YJ. 2011. Psoralen stimulates osteoblast differentiation through activation of BMP signaling. *Biochem Bioph Res Co* 405:256-261. http://dx.doi.org/10.1016/j.bbrc.2011.01.021.
14. Wong RW, Rabie AB. 2011. Effect of psoralen on bone formation. *J Orthop Res* 29:158-164. http://dx.doi.org/10.1002/jor.21124.
15. Arimatsu K, Yamada H, Miyazawa H, Minagawa T, Nakajima M, Ryder MI, Gotoh K, Motooka D, Nakamura S, Iida T and Yamazaki K. 2014. Oral pathobiont induces systemic inflammation and metabolic changes associated with alteration of gut microbiota. *Sci Rep-Uk* 4. http://dx.doi.org/10.1038/srep04828.
16. Nakajima M, Arimatsu K, Kato T, Matsuda Y, Minagawa T, Takahashi N, Ohno H and Yamazaki K. 2015. Oral administration of P. gingivalis induces dysbiosis of gut microbiota and impaired barrier function leading to dissemination of enterobacteria to the liver. *Plos One* 10. http://dx.doi.org/10.1371/journal.pone.0134234.
17. Sato K, Takahashi N, Kato T, Matsuda Y, Yokoji M, Yamada M, Nakajima T, Kondo N, Endo N, Yamamoto R, Noiri Y, Ohno H and Yamazaki K. 2017. Aggravation of collagen-induced arthritis by orally administered Porphyromonas gingivalis through modulation of the gut microbiota and gut immune system. *Sci Rep-Uk* 7. http://dx.doi.org/10.1038/s41598-017-07196-7.
18. Anderson JM, Van Itallie CM. 1995. Tight junctions and the molecular basis for regulation of paracellular permeability. *Am J Physiol* 269:G467-475.

http://dx.doi.org/10.1152/ajpgi.1995.269.4.G467.

19. Claes AK, Zhou JY and Philpott DJ. 2015. NOD-like receptors: guardians of intestinal mucosal barriers. *Physiology (Bethesda)* 30:241-250. http://dx.doi.org/10.1152/physiol.00025.2014.
20. Philpott DJ, Sorbara MT, Robertson SJ, Croitoru K and Girardin SE. 2014. NOD proteins: regulators of inflammation in health and disease. *Nat Rev Immunol* 14:9-23. http://dx.doi.org/10.1038/nri3565.
21. Robertson BR, O'Rourke JL, Neilan BA, Vandamme P, On SLW, Fox JG and Lee A. 2005. Mucispirillum schaedleri gen. nov., sp. nov., a spiral-shaped bacterium colonizing the mucus layer of the gastrointestinal tract of laboratory rodents. *Int J Syst Evol Microbiol* 55:1199-1204. http://dx.doi.org/10.1099/ijs.0.63472-0.
22. Caruso R, Warner N, Inohara N and Nunez G. 2014. NOD1 and NOD2: signaling, host defense, and inflammatory disease. *Immunity* 41:898-908. http://dx.doi.org/10.1016/j.immuni.2014.12.010.
23. Barnich N, Aguirre JE, Reinecker HC, Xavier R and Podolsky DK. 2005. Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-kappa B activation in muramyl dipeptide recognition. *J Cell Biol* 170:21-26. http://dx.doi.org/10.1083/jcb.200502153.
24. Yatsonsky Ii D, Pan K, Shendge VB, Liu J and Ebraheim NA. 2019. Linkage of microbiota and osteoporosis: a mini literature review. *World J Orthop* 10:123-127. http://dx.doi.org/10.5312/wjo.v10.i3.123.
25. Rizzoli R. 2019. Nutritional influence on bone: role of gut microbiota. *Aging Clin Exp Res* 31:743-751. http://dx.doi.org/10.1007/s40520-019-01131-8.
26. Ohlsson C, Sjogren K. 2015. Effects of the gut microbiota on bone mass. *Trends Endocrinol Metab* 26:69-74. http://dx.doi.org/10.1016/j.tem.2014.11.004.
27. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov Ii, Umesaki Y, Itoh K and Honda K. 2011. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* 331:337-341. http://dx.doi.org/10.1126/science.1198469.
28. Everard A, Cani PD. 2013. Diabetes, obesity and gut microbiota. *Best Pract Res Clin Gastroenterol* 27:73-83. http://dx.doi.org/10.1016/j.bpg.2013.03.007.
29. Wang J, Wang Y, Gao W, Wang B, Zhao H, Zeng Y, Ji Y and Hao D. 2017. Diversity analysis of gut microbiota in osteoporosis and osteopenia patients. *PeerJ* 5:e3450. http://dx.doi.org/10.7717/peerj.3450.
30. Das M, Cronin O, Keohane DM, Cormac EM, Nugent H, Nugent M, Molloy C, O'Toole PW, Shanahan F, Molloy MG and Jeffery IB. 2019. Gut microbiota alterations associated with reduced bone mineral density in older adults. *Rheumatology (Oxford)* 58:2295-2304. http://dx.doi.org/10.1093/rheumatology/kez302.
31. Li C, Huang Q, Yang R, Dai Y, Zeng Y, Tao L, Li X, Zeng J and Wang Q. 2019. Gut microbiota composition and bone mineral loss-epidemiologic evidence from individuals in Wuhan, China. *Osteoporos Int* 30:1003-1013. http://dx.doi.org/10.1007/s00198-019-04855-5.

32. Ma S, Qin J, Hao Y and Fu L. 2020. Association of gut microbiota composition and function with an aged rat model of senile osteoporosis using 16S rRNA and metagenomic sequencing analysis. *Aging (Albany NY)* 12:10795-10808. http://dx.doi.org/10.18632/aging.103293.
33. Pascale A, Marchesi N, Marelli C, Coppola A, Luzi L, Govoni S, Giustina A and Gazzaruso C. 2018. Microbiota and metabolic diseases. *Endocrine* 61:357-371. http://dx.doi.org/10.1007/s12020-018-1605-5.
34. Jones RM, Luo L, Ardita CS, Richardson AN, Kwon YM, Mercante JW, Alam A, Gates CL, Wu H, Swanson PA, Lambeth JD, Denning PW and Neish AS. 2013. Symbiotic lactobacilli stimulate gut epithelial proliferation via Nox-mediated generation of reactive oxygen species. *EMBO J* 32:3017-3028. http://dx.doi.org/10.1038/emboj.2013.224.
35. Grassi F, Tell G, Robbie-Ryan M, Gao Y, Terauchi M, Yang X, Romanello M, Jones DP, Weitzmann MN and Pacifici R. 2007. Oxidative stress causes bone loss in estrogen-deficient mice through enhanced bone marrow dendritic cell activation. *P Natl Acad Sci USA* 104:15087-15092. http://dx.doi.org/10.1073/pnas.0703610104.
36. Lappin DF, Macleod CP, Kerr A, Mitchell T and Kinane DF. 2001. Anti-inflammatory cytokine IL-10 and T cell cytokine profile in periodontitis granulation tissue. *Clinical and Experimental Immunology* 123:294-300. http://dx.doi.org/10.1046/j.1365-2249.2001.01448.x.
37. Yamazaki K, Tabeta K, Nakajima T, Ohsawa Y, Ueki K, Itoh H and Yoshie H. 2001. Interleukin-10 gene promoter polymorphism in Japanese patients with adult and earlyonset periodontitis. *Journal of Clinical Periodontology* 28:828-832. http://dx.doi.org/10.1034/j.1600-051x.2001.028009828.x.
38. Moore KW, Malefyt RD, Coffman RL and O'Garra A. 2001. Interleukin-10 and the interleukin-10 receptor. *Annual Review of Immunology* 19:683-765. http://dx.doi.org/10.1146/annurev.immunol.19.1.683.

Figures

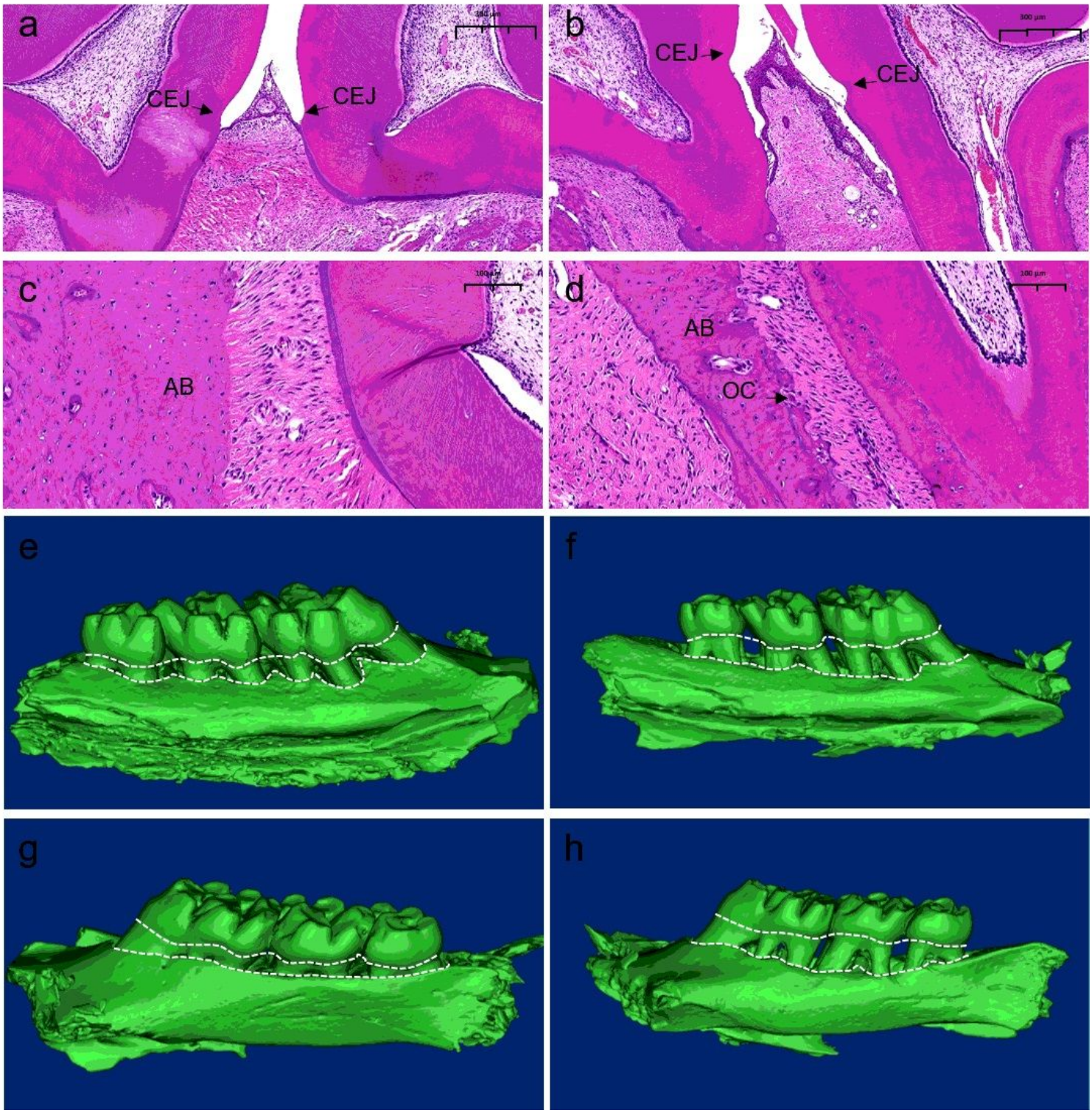

Figure 1

HE staining and Micro-CT three-dimensional reconstruction of healthy rats and periodontitis rats. In HE staining (a-d), a and c mean healthy rats, b and d mean periodontitis rats. In three-dimensional reconstruction of maxillary alveolar bone after Micro-CT scan (e-h), e and g mean healthy rats, f and h mean periodontitis rats. CEJ, cemento-enamel junction; AB, alveolar bone; OC, osteoclast. Scale bars=300μm (a and b); 100μm (c and d).

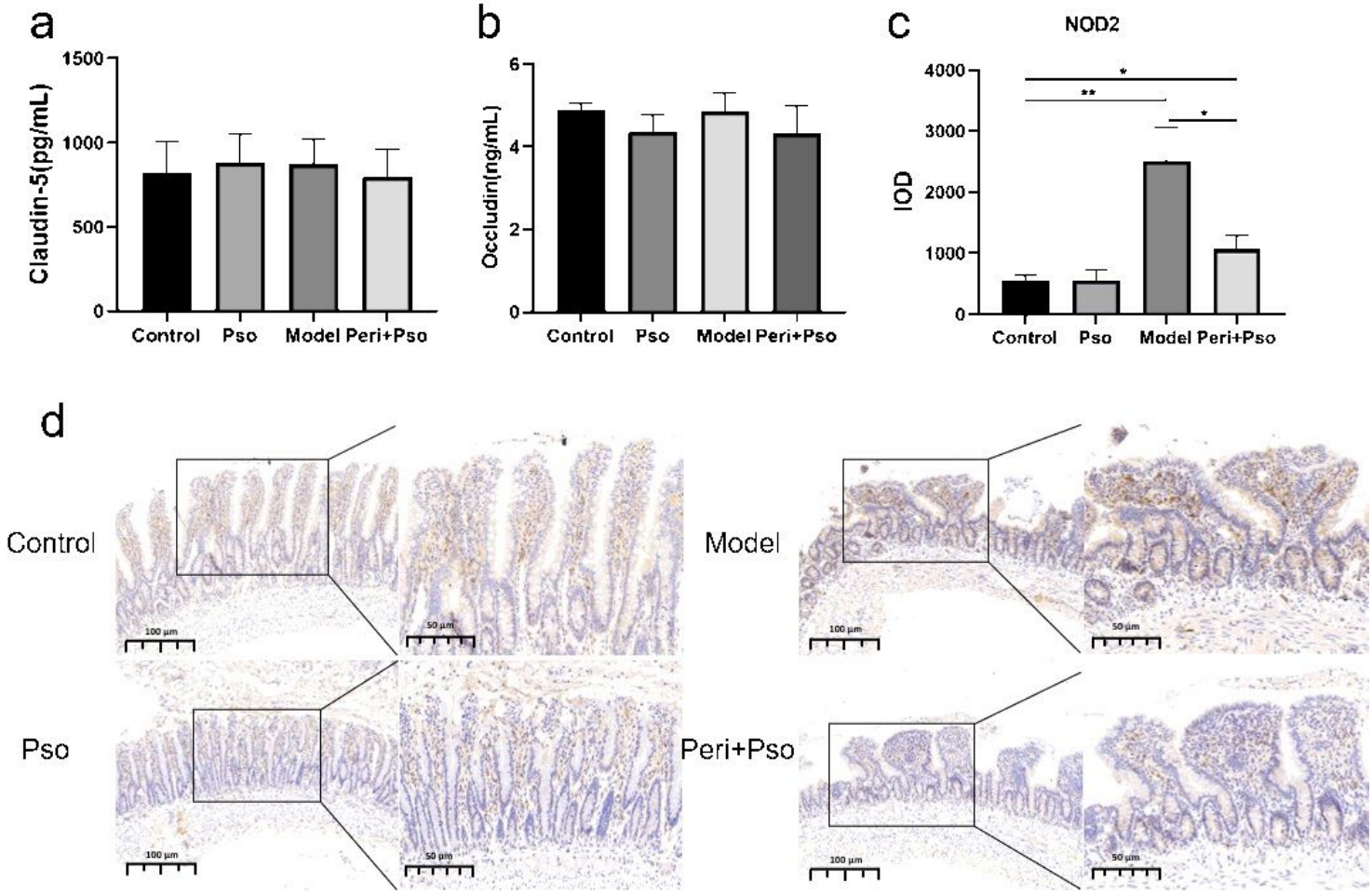

Figure 2

Effect of psoralen on mechanical barrier and immune barrier of ileum. The expression level of Claudin-5 and Occludin in ileal mucosa (a and b). IOD (sum) statistical analysis of positive expression of NOD2 protein in ileum of each group (c). The positive expression of NOD2 protein in ileum tissue (d). The brown granule means positive expression. Bar graphs: Data are presented as mean ± SD. *P < 0.05. **P < 0.01.

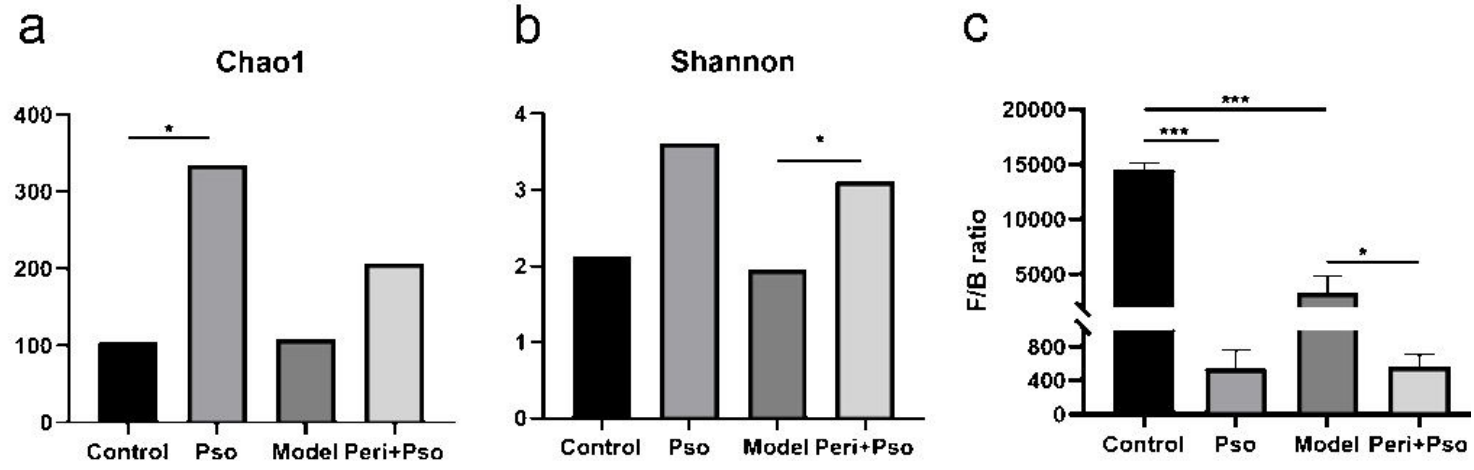

Figure 3

Alpha diversity analysis and F / B ratio analysis of intestinal flora in ileum. Alpha diversity index of ileal intestinal flora (a and b). The change of the Firmicutes/Bacteroidetes (F/B) ratios (c). *P < 0.05. ***P < 0.001.

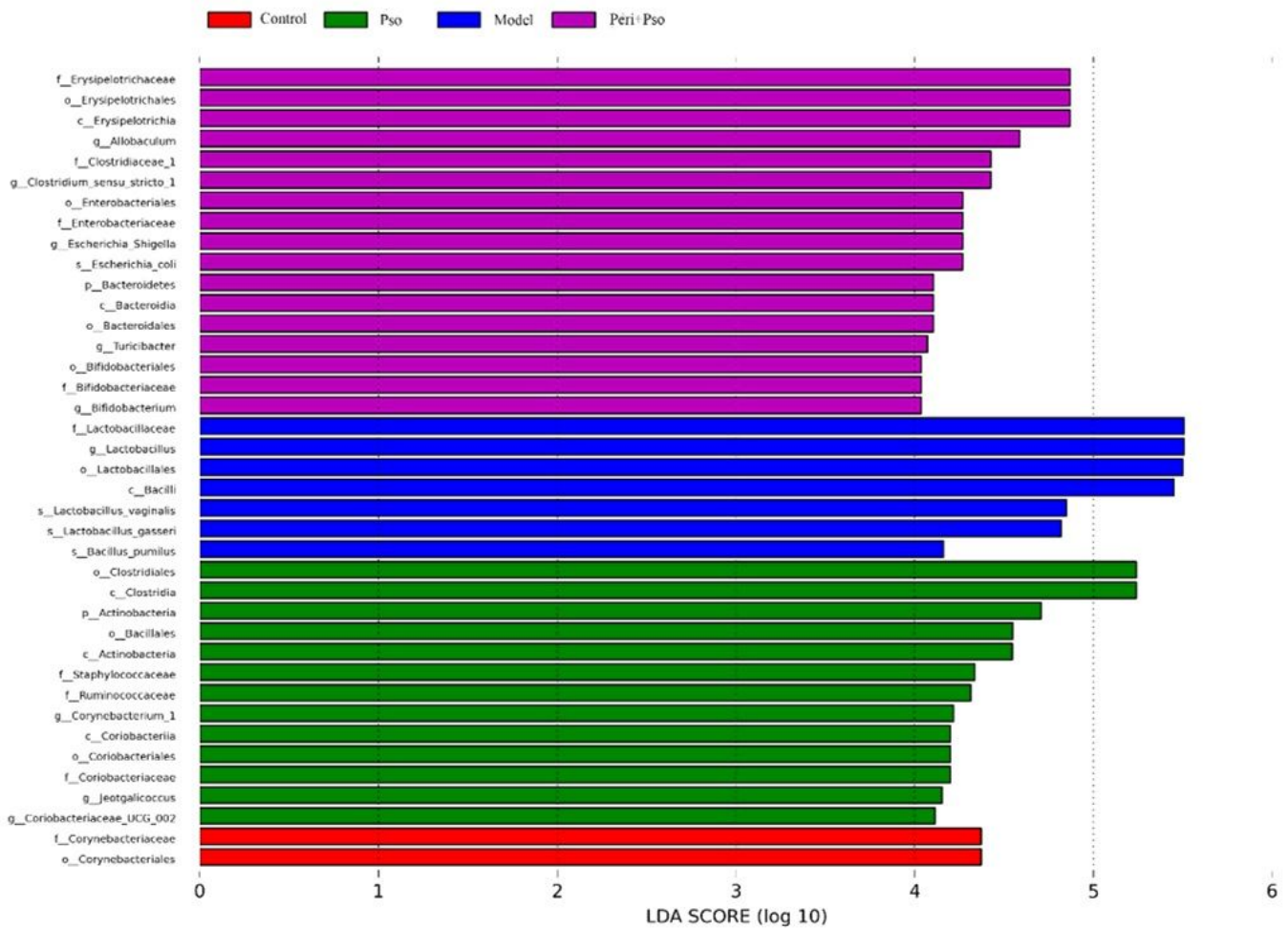

Figure 4

LEfSe analysis of intestinal flora in ileum (p, phylum; c, class; o, order; f, family; g, genus; s, species). Only the taxes having a P < 0.05, LDA value > 2 are shown in the figure.

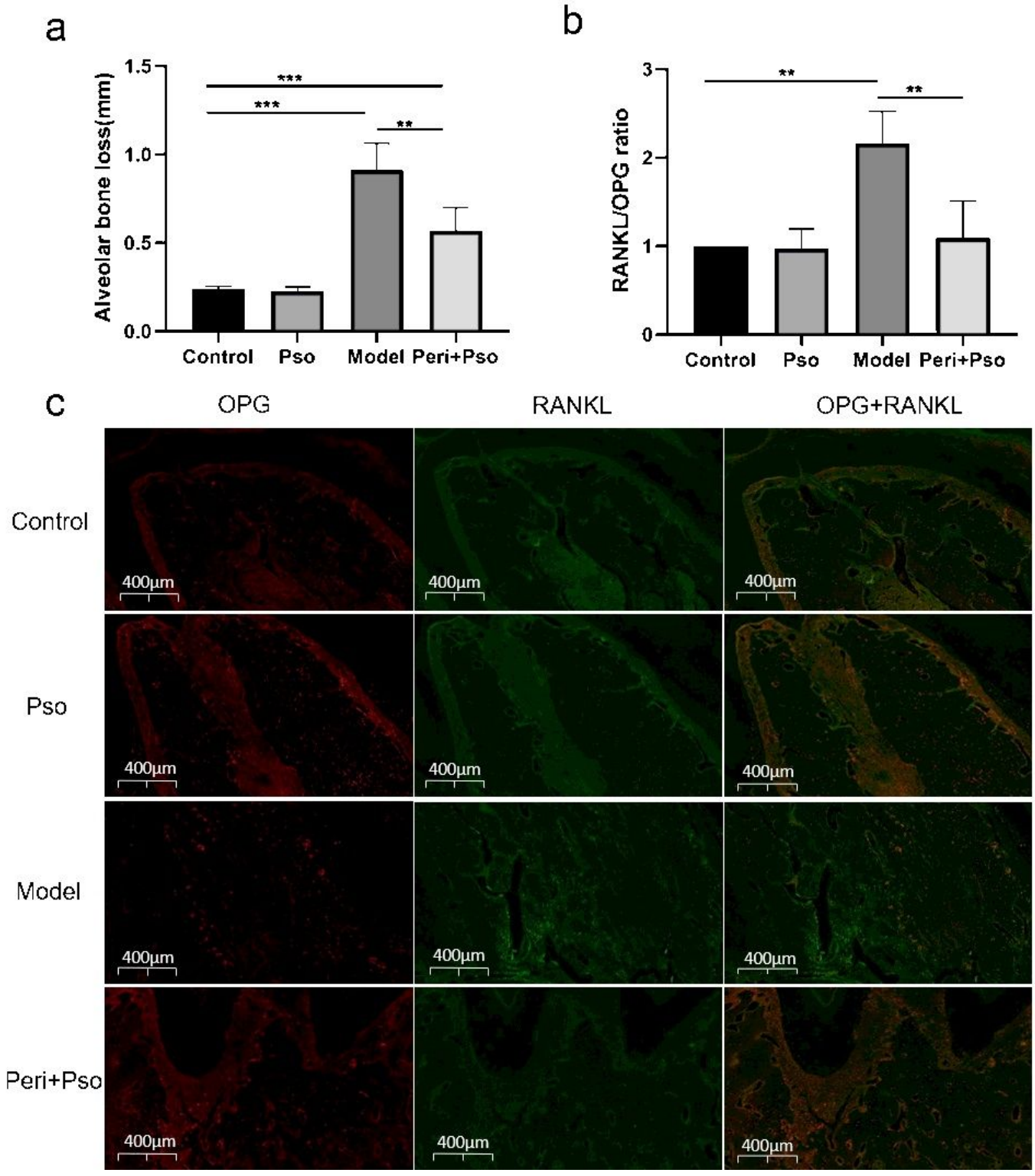

Figure 5

Effect of psoralen on periodontal tissue of rats. Alveolar bone loss of maxillary second molar in rats (a). The changed expression of RANKL/OPG ratio in the periodontal tissue (b). The protein expression of OPG and RANKL was showed by immunofluorescence staining (c). Bar graphs: Data are presented as mean ± SD. **P < 0.01. ***P < 0.001. Scale bars=400μm. Red means OPG and green means RANKL (c).

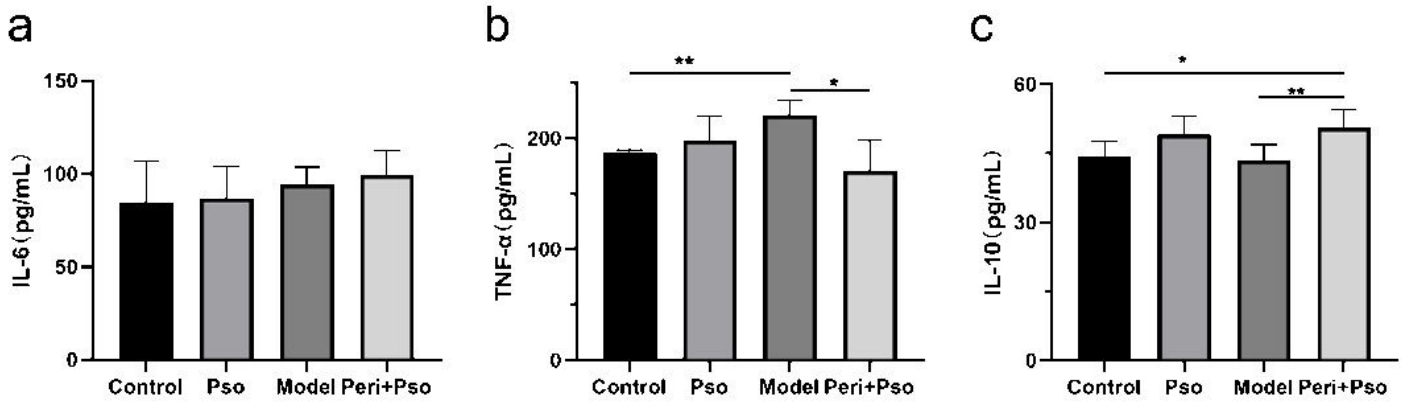

Figure 6

The expression of inflammatory cytokines in serum. Concentration of the secretion of IL-6 from serum by ELISA (a). Concentration of the secretion of TNF-α from serum by ELISA (b). Concentration of the secretion of IL-10 from serum by ELISA (c). Bar graphs: Data are presented as mean ± SD. *P < 0.05. **P < 0.01.