

Original Research Article

Patchouli alcohol suppresses inflammation and autophagy, and reduces osteoclast formation in periodontitis via regulation of ERK and NF- κ B signaling pathways

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Abstract

Purpose: To evaluate the effect of patchouli alcohol (PA) on periodontitis and reveal its possible mechanism.

Methods: Primary human periodontal membrane cells (PDLC) were isolated and treated with lipopolysaccharide (LPS) to establish a periodontitis model. The MTT, flow cytometry and immunofluorescence assay were used to determine the effect of PA on cell viability and apoptosis in PDLC exposed to LPS. The inflammation status and osteoclast formation were measured using enzyme-linked immunosorbent assay (ELISA) and TRAP staining. Immunoblot assays were performed to analyze the effect of LPS on ERK and NF- κ B pathways.

Results: Patchouli alcohol (PA) improved LPS-induced PDLC growth arrest ($p < 0.001$). In addition, PA relieved LPS - induced autophagy of PDLCs ($p < 0.001$). Further data confirmed that PA inhibited LPS-induced inflammation of PDLCs. In addition, PA reduced osteoclast formation, and suppressed inflammation and autophagy, and also reduced osteoclast formation by regulating ERK and NF- κ B pathways.

Conclusion: Patchouli alcohol inhibits inflammation and autophagy, and decreases osteoclast formation in periodontitis via the regulation of ERK and NF- κ B signaling pathways. Thus, this compound has potentials for development for the treatment of periodontitis; however, in vivo studies should first be undertaken.

Keywords: Periodontitis, Patchouli alcohol (PA), Lipopolysaccharide (LPS), Osteoclast formation, ERK and NF- κ B pathways

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INTRODUCTION

Periodontitis is a chronic inflammatory disease characterized by swollen gums, bleeding, absorption of alveolar bone, and loosening of the teeth [1]. It is also the leading cause of tooth loss in adults. Microbial infection is the main cause of

periodontitis, and excessive inflammation can destroy periodontal tissue [2]. The formation of osteoclasts leads to bone loss and stimulates the reabsorption of the alveolar bone, which ultimately leads to tooth loss [3]. Therefore, the inhibition of osteoclast differentiation is the way to treat periodontitis. Secondly, the inhibition of

autophagy relieves symptoms of periodontitis. In the present study, primary human periodontal membrane cells (PDLC) were isolated and treated with *Porphyromonas gingivalis* lipopolysaccharide (LPS) to establish an *in vitro* model of periodontitis.

Chinese medicine has shown promising effect in the treatment of periodontitis [4]. Tanshinone II, A alleviates periodontitis symptoms by inducing osteogenesis of hPDLSC [5]. Extracts of pogostemon cablin have strong antibacterial activity against the periodontitis bacteria. *Patchouli* alcohol (PA), a tricyclic sesquiterpene extracted from *Patchouli*, has anti-inflammatory activity in gastrointestinal cell models [6], and the anti-inflammatory effects of PA in a variety of diseases have been widely revealed.

Patchouli alcohol improves the inflammatory response induced by sodium glucan sulfate (DSS) by activating PXR signal and inhibiting NF- κ B signal, which is a novel idea for the treatment of colitis [7].

Patchouli alcohol hinders the effects of chemotherapeutic agents on the colon by reducing inflammation, protecting the mucosal barrier, and regulating the gut microbiome [8]. In orthopedics, *Patchouli* alcohol inhibits the expression of transcription factors NFATc1 and C-FOS, reduces the formation of osteoclasts, and improves the symptoms of postmenopausal osteoporosis [9]. *Patchouli* alcohol also inhibits excessive autophagy by activating the mTOR signaling pathway, thereby preventing chronic, unpredictable and mild stress-induced depression-like behavior [10]. In addition, PA inhibits influenza A virus by targeting the ERK/MAPK signaling pathway [11]. However, the study of PA in the treatment of periodontitis has rarely been reported, and its mechanism of action is not clear.

This study, therefore, aimed to evaluate the effect of PA on periodontitis and elucidate its probable mechanism of action.

METHODS

Cell culture

The PDLCs were purchased from the BeNa Culture Collection (Beijing, China), and cultured in DMEM/F12 medium with 10 % fetal bovine serum, 100 μ g/mL streptomycin and 100 U/mL penicillin at 37 °C with 5 % CO₂. The PDLCs were stimulated with PA at concentration of 5, 10, 20, 40, 80 and 160 μ M. For LPS treatment, cells were cultured with 10 μ g/mL LPS.

Cell viability

Primary human periodontal membrane cells (PDLCs) were plated into 96-well plates at the density of 1×10^3 cells/well. Cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. After treatment with PA or LPS, 10 % MTT was added to the cells after incubation for 4 h and washing with PBS. Then the absorbance value in each well was quantified with a microplate reader at 490 nm. The absorbance in experimental group were normalized to that of the control group.

Cell apoptosis

For the analysis of apoptotic cell number, cell apoptosis was conducted with the Annexin V/PI apoptosis detection kit after the previous study [12]. The cells were then briefly digested into single cells and re-suspended in reaction buffer containing Annexin V and PI, and incubated for 5 min at room temperature in the dark.

The cell proportion of each fraction of intact cells (Annexin V⁻/PI⁻), early apoptotic cells (Annexin V⁺/PI⁻) and late apoptotic cells (Annexin V⁺/PI⁺) were analyzed by a flow cytometer (BD Biosciences).

Immunoblot assay

Proteins were extracted with RIPA buffer (Beyotime), and protein concentration was measured using the BCA kit. Then, the proteins were separated using 9 % SDS-PAGE, and transferred onto PVDF membranes, followed by blocking with 5 % BSA in TBST buffer. Subsequently, the membranes were incubated with primary antibodies targeting LC-2/III (1:1000, Abcam, Cambridge, UK), p62 (1:1000, Abcam), Beclin1 (1:1000, Abcam), RANKL (1:1000, Abcam), RAcP (1:1000, Abcam), CTSK (1:1000, Abcam), NFATc1 (1:1000, Abcam), c-fos (1:1000, Abcam), p-ERK (1:1000, Abcam), ERK (1:1000, Abcam), p- NF- κ B (1:1000, Abcam), NF- κ B (1:1000, Abcam), and B-actin (1:10000, Abcam) 4 °C overnight. Subsequently, the membranes were incubated with specific secondary antibodies at room temperature for 1 h. The blots were analyzed with ECL kit.

Immunofluorescence

The cells were fixed with formaldehyde, washed with PBS, followed by permeabilization with PBS containing 0.5 % Triton X-100 and staining with primary antibody targeting LC3 (Abcam, Cambridge, UK). Then after rinsing in PBS, the cells were incubated with fluorescent secondary

antibody, further stained with DAPI, mounted with mounting media and photographed with fluorescence microscopy.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total cellular RNA was extracted with TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions, and reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega Corporation). The cDNA was amplified using the primer listed in Table 1.

Table 1: Primer sequence

Gene	Primer sequence
TNF- α	GGTGCCTATGTCTCAGCCTCTT GCCATAGAAGTGTGAGAGGGAG
IL-8	ACTGAGAGTGAT TGAGAGTGGAC AACCTCTGCACCCAGTTTTC
IL-6	AGACAGCCACTCACC TTCTGCCAGTGCCTCTT
GAPDH	AGAAGGCTGGGGCTCATTTG AGGGCCATCCACAGTCTTC

Enzyme-linked immunosorbent assay (ELISA)

Cell supernatants were added to the respective ELISA plate, in order to determine the level of TNF- α , IL-6, and IL-8, in accordance with the manufacturer's guidelines.

The ELISA kits were obtained from Shanghai Xitang Biotechnology Co., Ltd. (Shanghai, China).

Tartrate-resistant acid phosphatase (TRAP) staining for osteoclasts

Cells were fixed in 4 % formalin for 10 min, permeabilized with PBS containing 0.1 % Triton X-100 for 10 min, and incubated for 10 min with a TRAP-staining solution (Sigma-Aldrich, St. Louis, MO, USA).

Tartrate-Resistant Acid Phosphatase positive-multinucleated cells containing more than 3 nuclei were counted as mature osteoclasts.

Statistical analysis

The GraphPad 6.0 was used for statistical analysis. Data are presented as mean \pm SEM. Triplicate determinations were performed for each experiment. One-way ANOVA followed by post-hoc Tukey's test were used for statistical comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

PA promotes cell viability in PDLCs

To evaluate the cell viability exposed to PA in PDLCs, MTT assay was performed. PDLCs were incubated with increasing dose of PA. Cell viability was minimally affected by a low dose of PA, while a high dose of PA (80 and 160 μ M) significantly impaired cell viability. The cells exposed to LPS and PA were subjected to MTT assay, and Lipopolysaccharide treatment led to reduced cell viability of PDLCs. Patchouli alcohol improved cell viability in a dose-dependent manner (Figure 1 A and B). Afterwards, cell apoptosis in PA mediated PDLCs were evaluated using flow cytometry. Lipopolysaccharide treatment induced increased apoptotic cells, while PA treatment improved cell apoptosis (Figure 1 C). Collectively, PA improved cell viability in PDLCs stimulated by LPS.

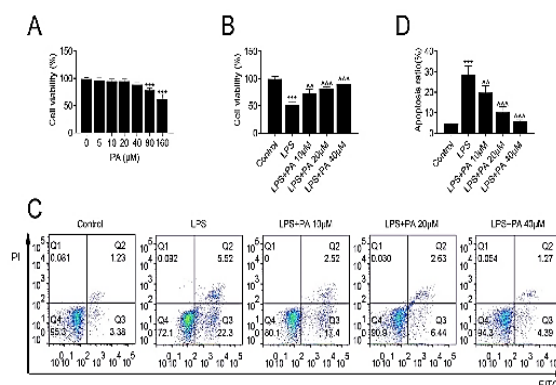


Figure 1: PA promoted cell viability in PDLCs stimulated with LPS, (A) The cell viability of PDLCs in response to increasing dose of PA, (B) The viability of PDLCs in response to LPS and elevated level of PA, (C, and D) PDLCs apoptosis in response to LPS and the elevated level of PA were measured by Flow cytometry. *** $P < 0.001$ vs control, $\wedge p < 0.05$, $\wedge\wedge p < 0.01$, $\wedge\wedge\wedge p < 0.001$ vs LPS

PA relieves cell apoptosis in PDLCs after LPS induction

The effect of PA on cell apoptosis was further confirmed by Immunoblot and Immunofluorescence. Lipopolysaccharides significantly induced the expression of LC3-II, and Beclin1 and downregulated p62. The addition of PA ameliorated the alterations of these proteins (Figure 2 A). Moreover, LPS enhanced the number of LC3 positive cells, but PA decreased LC3 positive cell percentage (Figure 2 B). These data further confirmed the PA reversal of cell apoptosis induced by LPS.

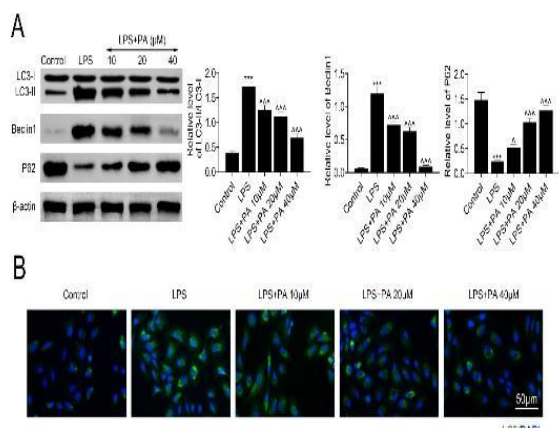


Figure 2: PA relieved cell apoptosis in PDLCs treated with LPS, (A) The expression of LC3II, Beclin1 and p62 in response to LPS and elevated level of PA, (B) Immunofluorescence staining of LC3 in response to LPS and the elevated level of PA. *** $P < 0.001$ vs control, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs LPS

PA inhibited pro-inflammatory cytokines levels in PDLCs

The inflammatory response in PDLCs cells were measured by mRNA and ELISA assay. Lipopolysaccharide stimulation enhanced inflammation as revealed by the increased levels of TNF- α , IL-8 and IL-6 (Figure 3 A and B). *Patchouli* alcohol treatment reduced the levels of TNF- α , IL-8 and IL-6 in PDLCs. Therefore, PA reduced LPS-induced increase in pro-inflammatory cytokine levels in PDLCs.

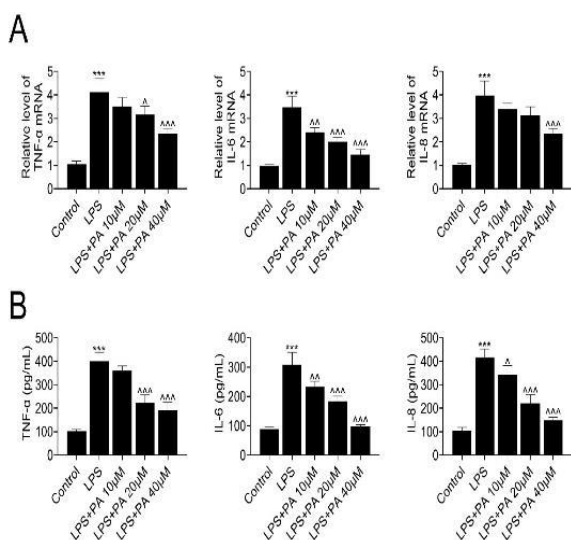


Figure 3: PA down-regulation pro-inflammatory cytokines levels in PDLCs PA decreases pro-inflammatory cytokines levels in HT-22, (A, B) mRNA and levels of TNF- α , IL-8 and IL-6 in each group were determined. *** $p < 0.001$ vs control, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs LPS

PA Inhibited the differentiation of macrophage into osteoclasts

To evaluate the effects of PA on the LPS-induced osteoclast differentiation, RAW 264.7 were stimulated with PA or vehicle. As shown in Figure 4 A, LPS significantly induced TRAP $^{+}$ -osteoclast differentiation. However, PA significantly inhibited the formation of TRAP $^{+}$ -osteoclasts. Consistent with these results, LPS enhanced the levels of RANKL, TRAP, NFATc1, and c-Fos. PA reversed the alterations of these proteins (Figure 4 B). Therefore, PA reduced osteoclast formation in periodontitis.

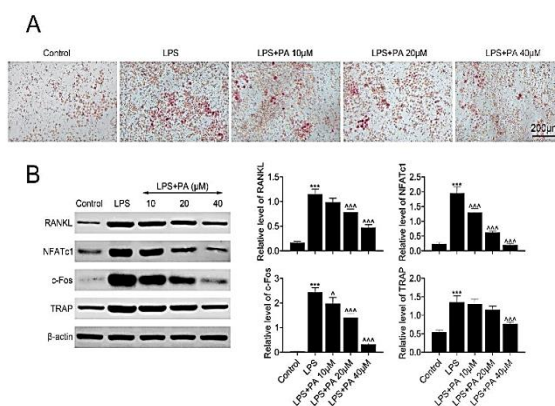


Figure 4: PA Inhibited the Differentiation of macrophage into Osteoclasts, (A) TRAP staining of PDLCs in response to LPS and the elevated level of PA, (B) The expressions of RANKL,TRAP, NFATc1, c-Fos in response to LPS and elevated level of PA. *** $P < 0.001$ vs control, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs LPS

PA inhibited autophagy and inflammation of PDLC

To depict the involved mechanisms underlying the role of PA in autophagy, inflammation and osteoclast formation in periodontitis, the ERK and NF- κ B signaling pathways was analyzed. The enhanced level of p-ERK and p-NF- κ B was found in LPS-induced PDLCs (Figure 5) and PA inhibited the elevation of these proteins. The results indicated that PA inhibits autophagy and inflammation of PDLC by regulating ERK and NF- κ B signaling pathways.

DISCUSSION

Periodontitis is a chronic inflammation of periodontal supporting tissue caused mainly by local factors [13]. If gingivitis is not treated in time, the inflammation may spread from the gingiva deep into the periodontal membrane, alveolar bone and cementum, and develop into periodontitis [14].

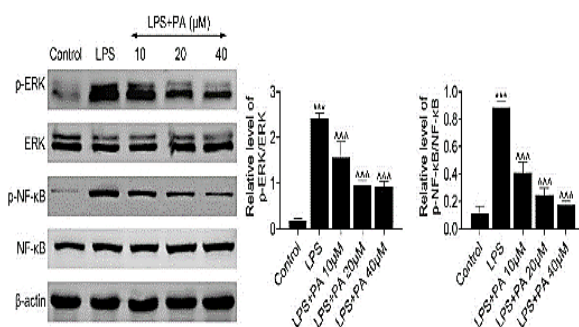


Figure 5: PA inhibited autophagy and inflammation of PDLC by regulating ERK and NF- κ B signaling pathways. Immunoblot assays depicting the expressions of p-ERK and p-NF- κ B in LPS and PA-induced PDLCs. *** $P < 0.001$ vs control, ^ $p < 0.05$, ^^ $p < 0.01$, ^^ $p < 0.001$ vs LPS

When there are symptoms, it is more serious and the teeth is more at risk of damage [15]. Therefore, early therapy and timely treatment are necessary. Patchouli alcohol suppressed inflammation and autophagy, and reduced osteoclast formation in periodontitis. The data showed that Patchouli alcohol could serve as a promising drug for periodontitis treatment.

The inflammatory response in periodontitis is a protective response to pathogen infection. Autophagy inhibits the aggregation of inflammasome by degrading DNA, reactive oxygen species and other endogenous stimuli, and also inhibiting the secretion of IL-1 β and other pro-inflammatory factors by degrading IL-1 β precursors [16]. The virulent factors of periodontal pathogens are involved in the destruction of periodontal tissue, and through the interaction of lipopolysaccharide, peptidoglycan and bacterial DNA carried or released by periodontal pathogens with toll-like receptor (TLR) of host cells, they induce local inflammatory cell infiltration and the release of inflammatory factors, leading to periodontitis [17]. In local periodontal tissues, autophagy also affects inflammatory responses by negatively regulating TLR signals [18]. In this study, PA suppressed inflammation and autophagy, and reduced osteoclast formation in periodontitis, further confirming the importance of autophagy in the progression of periodontitis.

Chinese medicine has shown great effect in the treatment of periodontitis, and PA is a promising anti-inflammatory drug. Through MTT, western blot, ELISA, and IF assays, the data revealed that PA inhibited IPS-induced PDLCs growth and autophagy. Furthermore, through qPCR and TRAP assays, the data revealed that PA suppressed LPS-induced PDLCs osteoclast

formation. Therefore, these findings suggest that PA could serve as a promising drug for periodontitis. The effects of PA on the cell autophagy and inflammation in different diseases have been widely reported. Patchouli alcohol also ameliorated the inflammatory response induced by sodium dextran sulfate (DSS) by activating PXR signal and inhibiting NF- κ B signal. Similarly, the effects of PA on periodontitis through this pathway was also revealed. Patchouli alcohol inhibited the expression of NFATc1 and c-FOS, therefore reducing the formation of osteoclasts [19]. In this study, Patchouli alcohol suppressed LPS-induced PDLCs osteoclast formation. However, the mechanism needs further study. Patchouli alcohol could also inhibit excessive autophagy by activating the mTOR signaling pathway, which was consistent with the study.

Patchouli alcohol inhibited ERK and NF- κ B pathways in LPS-induced PDLCs. The ERK and NF- κ B pathways are widely involved in the regulation of autophagy and inflammation. Both ERK and NF- κ B played a key role in regulating the immune response to infection. Improper regulation of ERK as well as NF- κ B have been associated with inflammation [20]. Several studies confirmed that multiple proteins and drugs suppressed the inflammation by targeting ERK and NF- κ B pathway [20]. The ERK and NF- κ B pathways could serve as promising targets for the treatment of inflammatory diseases, such as periodontitis.

CONCLUSION

Patchouli alcohol inhibits autophagy and inflammation of PDLC, and reduces osteoclast formation in periodontitis by regulating ERK and NF- κ B signaling pathways. Therefore, patchouli alcohol is a promising drug for the management of periodontitis. However, there is a need for *in vivo* and clinical studies on it.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Fei Ye and Changqing Zhang designed the study and carried them out, supervised the data collection, analyzed the data, interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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