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# CCK regulates osteogenic differentiation through TNFα/NF-κB in peri-implantitis

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## Abstract

**Objective:** Peri-implantitis is characterized by peri-implant mucositis and alveolar bone resorption. This study investigated cholecystokinin (*CCK*) expression and the mechanism underlying its involvement in peri-implantitis.

**Methods:** mRNA sequencing was performed using the Gene Expression Omnibus database GSE106090. Human bone marrow mesenchymal stem cells (hBMSCs) were pretreated with various concentrations of CCK (0, 10, 30, or 100 nM) for 1 hour before induction in osteogenic differentiation medium for 2 weeks. Alkaline phosphatase (ALP) activity was determined, and the cells were stained with alizarin red. The expression levels of *TNFα* and the osteogenic markers *ALP*, *RUNX2*, and *OCN* were measured using quantitative real-time PCR. TNFα, phosphorylated P65, and total P65 levels were determined by western blot.

**Results:** Compared with healthy individuals, 262 and 215 genes were up- and down-regulated, respectively, in the periodontal tissues of patients with peri-implantitis. *CCK* expression was significantly upregulated in patients with peri-implantitis. CCK reduced ALP activity, osteogenic differentiation, and levels of the osteogenic markers *ALP*, *RUNX2*, and *OCN*. Moreover, CCK promoted levels of TNFα and phosphorylated P65, which is a marker of activation for the NF-κB inflammatory pathway.

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**Conclusions:** CCK regulates osteogenic differentiation through the TNFα/NF-κB axis in peri-implantitis.

**Keywords**
Cholecystokinin, peri-implantitis, TNFα, NF-κB, osteogenic differentiation, human bone marrow mesenchymal stem cells

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## Introduction

Peri-implantitis is characterized by peri-implant mucositis and alveolar bone resorption and significantly shortens the lifespan of implants.[1] Peri-implantitis is widely considered to be the leading cause of late implant failure in pristine and regenerated bones[2] and has high morbidity and intractability.[3] Ultimately, peri-implantitis is an immune-mediated biological complication that is attributable to bacterial biofilm on an implant's surface.[4] Because the residual microbial biofilm hinders bone fusion, peri-implantitis treatments are often unsuccessful.[5] The pathology of peri-implantitis remains unclear and there is no consensus on its etiology or pathology. Therefore, an in-depth exploration of the molecular mechanisms underlying the pathophysiology of peri-implantitis will help the development of targeted therapies.

Cholecystokinin (CCK) is a peptide hormone that acts on the digestive organs and central nervous system.[6] CCK is a tissue-specific and developmental protein.[7] CCK is also the most abundant brain neuropeptide and participates in related behavioral functions such as memory, cognition, and reward through interactions with the opioid and dopaminergic components of the limbic system.[8] However, its involvement in peri-implantitis has not been elucidated.

CCK is closely associated with the inflammatory factor TNFα,[9] which inhibits the osteogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs).[10,11] hBMSCs possess pluripotent potential and can differentiate into the various cell types required for tissue regeneration including osteoblasts,[12] which are vital for new bone formation.[13] Osteoblasts are primarily derived through the differentiation of hBMSCs in peri-implantitis.[11] As peri-implantitis treatment aims to control infection and prevent bone loss,[14] further studies that explore the mechanisms of osteogenic differentiation and peri-implantitis are warranted.

The NF-κB inflammation pathway has been reported to be involved in the development of peri-implantitis. He *et al.* reported that pyrrolidine dithiocarbamate could inhibit NF-κB expression and reduce LPS-induced inflammatory responses, preventing periodontal inflammation and reducing the occurrence of peri-implantitis.[15] Wu *et al.* demonstrated that melatonin prevented peri-implantitis by inhibiting the TLR4/NF-κB pathway.[1] However, the mechanisms of CCK and NF-κB in peri-implantitis remain unclear.

The newly developed high-throughput experimental methods in biological research have produced large amounts of omics data. Microarray transcriptome analysis is valuable for studying changes in the gene expression profiles of patients with peri-implantitis.[16,17] Herein, we used these methods to study the molecular mechanisms underlying peri-implantitis. We analyzed differentially expressed genes in the

periodontal tissues of patients with peri-implantitis and those of healthy subjects using the Gene Expression Omnibus (GEO) database GSE106090. We found that *CCK* expression was significantly increased in the periodontal tissues of patients with peri-implantitis. Hence, we explored the mechanism of CCK using *in vitro* experiments. Our research may provide a novel strategy for treating peri-implantitis.

## Materials and methods

### *mRNA sequencing*

Data were obtained from the GEO database GSE106090 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE106090). The periodontal tissues of patients with peri-implantitis and healthy subjects were assessed. We downloaded the original expression data from the chip in the GEO database using the R package GEOquery (www.r-project.org) and screened differentially expressed mRNAs using the R package limma. The screening criteria were $|\log FC| \geq 1.5$ and $P \leq 0.05$, and heat maps and scatter plots were constructed. Finally, we selected the top 10 up- and down-regulated genes and constructed a heat map.

### *Clinical samples*

Clinical samples were collected as previously described.[16] This study was approved by the Medical Ethics Committee of the Hospital of Stomatology, Sun Yat-sen University (approval number: KQEC-2022-29-01). Written informed consent was obtained from all patients.

### *Cell treatment and identification of hBMSCs*

hBMSCs (Cellcook, Guangzhou, China) were cultured in 90% high glucose DMEM with 10% fetal bovine serum and were identified *via* immunofluorescence as previously described.[18,19] The following antibodies were used in this study: anti-vimentin (#10366-1-AP, Proteintech, Wuhan, China) and anti-CD44 (#15675-1-AP, Proteintech). The hBMSCs were pretreated with various concentrations of CCK (0, 10, 30, or 100 nM) (R&D Systems, Minneapolis, MN, USA) for 1 hour before being induced for osteogenic differentiation using differentiation medium (Cellcook) for 2 weeks. hBMSCs from passages three to five were used in this study.

### *Quantitative real-time PCR (qPCR)*

Relative expression levels of genes encoding *TNFα* and the osteogenic markers *Runx2*, *ALP*, and *OCN* were determined using qPCR. Briefly, total RNA was extracted using TRIzol and reverse transcribed into cDNAs using a cDNA reverse transcription kit (#CW2569, Beijing ComWin Biotech, Beijing, China). The Ultra SYBR Mixture (#CW2601, Beijing ComWin Biotech) was used to measure relative gene expression on a fluorescence quantitative PCR instrument (QuantStudio1, Thermo Fisher Scientific, Waltham, MA, USA). Using *GAPDH* as an internal reference gene, relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used were as follows: TNFα-F: 5′-GTTCCTCAGCCTCTTCTCCTT-3′, TNFα-R: 5′-CTCTCAGCTCCACGCCATT-3′; Runx2-F: 5′-CAGCACTCCATATCTCTACTAT-3′, Runx2-R: 5′-CTTCCATCAGCGTCAACA-3′; ALP-F: 5′-TGGAAGGAGGCAGAATTG-3′, ALP-R: 5′-ATACAGGATGGCAGTGAAG-3′; OCN-F: 5′-CAGCGAGGTAGTGAAGAG-3′, OCN-R: 5′-GATGTGGTCAGCCAACTC-3′; CRYBA4-F: 5′-GCTTCGAGACTGTGCGATCT-3′, CRYBA4-R: 5′-CCCAGCTTGGATATTCGCCT-3′; CCK-F: 5′-CAGAGAACGGATGGCGAGTC-3′, CCK-R: 5′-GGTCCAGGTTCTGCAGGTT

C-3′; PRR36-F: 5′-GCTCACCCCTGACCA TACAC-3′, PRR36-R: 5′-GTGGGGCTAG GGAAGATTGG-3′; and GAPDH-F: 5′-CATCACCATCTTCCAGGAG-3′, GAPD H-R: 5′-AGGCTGTTGTCATACTTCTC-3′.

### *Western blotting*

Total protein was extracted from cells and tissues using RIPA lysis buffer (#P0013B, Beyotime, Beijing, China). Protein concentrations were quantified using the BCA protein assay kit (#BL521A, Biosharp, Beijing, China). Protein samples were mixed with SDS–PAGE loading buffer (#MB2479, Meilunbio, Dalian, China), separated on a SDS-PAGE gel, and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with primary antibodies against TNFα (26405-1-AP, 1:2000, Proteintech), phosphorylated P65 (p-P65) (AP0475, 1:2000, ABCLONAL, Woburn, MA, USA), total P65 (66535-1-Ig, 1:2000, Proteintech), and GAPDH (10494-1-AP, 1:5000, Proteintech). Next, the membranes were incubated with goat anti-rabbit IgG-HRP (BL003A, 1:5000, Biosharp) or goat anti-mouse IgG-HRP (BL001A, 1:5000, Biosharp) secondary antibodies. ECL color exposure was used to detect immunoreactive bands. An Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE, USA) was used to quantify protein bands, using GAPDH as the internal reference.

### *Quantification of alkaline phosphatase (ALP) activity*

ALP activity was determined 2 weeks after induction. The ALP Assay Kit (C3206, Beyotime) was used to quantify ALP activity, with absorbance measured at 405 nm.

### *Alizarin red staining*

After 2 weeks of induction, the cells were stained with alizarin red. After osteogenic differentiation, the complete osteogenic differentiation medium was removed from the six-well plate, and the cells were rinsed twice with 1 × PBS. Then, 2 mL of 4% neutral formaldehyde was added to each well to fix the cells for 30 min. The neutral formaldehyde solution was removed, and the cells were rinsed twice with 1 × PBS. Next, 1 mL of alizarin red S (pH 4.2; Sigma-Aldrich) was added to stain the cells for 3 to 5 minutes. Subsequently, the alizarin red dye was removed, and the cells were rinsed twice with 1 × PBS. Finally, the effects of osteogenic differentiation were observed under a microscope.

### *Statistical analysis*

All experiments were performed in triplicate. GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. Data are expressed as mean ± standard deviation. The Student's t-test was used to compare two groups, and one-way analysis of variance was used for multigroup comparisons. A P-value $<0.05$ indicated that differences were statistically significant.

## Results

### *Increased CCK expression in the periodontal tissues of patients with peri-implantitis*

First, we performed mRNA sequencing of data in the GEO database GSE106090. Next, we constructed a heat map of the differentially expressed genes in the periodontal tissues of patients with peri-implantitis (peri-implantitis group) and healthy subjects (healthy group) ($n = 6$ per group) (Figure 1a). Then, a scatter plot analysis was performed on the differentially expressed genes. The results showed that 262 genes were upregulated and 215 were downregulated in the periodontal tissues of patients with peri-implantitis

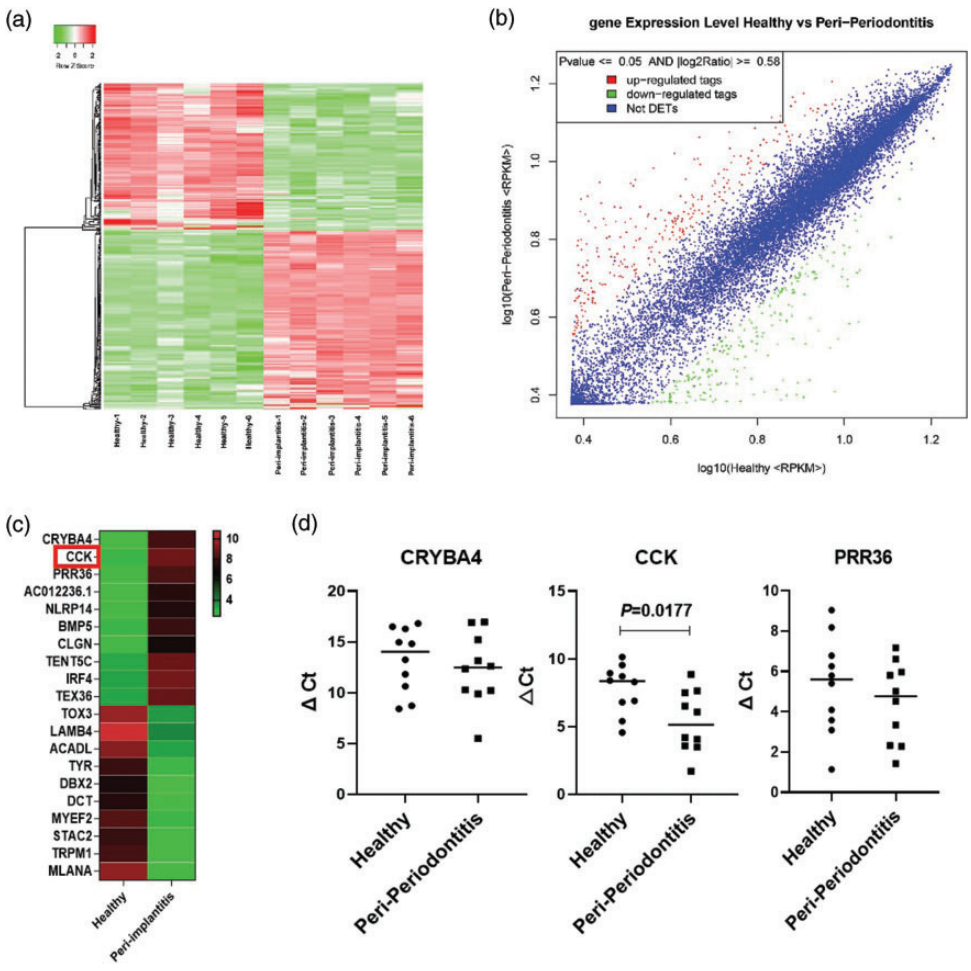


**Figure 1.** *CCK* was upregulated in the periodontal tissues of patients with peri-implantitis. (a) Heat map of differentially expressed genes in the periodontal tissues of healthy subjects (healthy group) and patients with peri-implantitis (peri-implantitis group) from the Gene Expression Omnibus (GEO) database. (b) Scatter plot of differentially expressed genes in the periodontal tissues of the healthy group *vs.* peri-implantitis group from the GEO database. (c) Heat map showing the top 10 upregulated and downregulated genes of the healthy group *vs.* peri-implantitis group and (d) Quantitative real-time PCR was performed to evaluate the expression of *CRYBA4*, *CCK*, and *PRR36* in the tissues of 10 patients.

(Figure 1b). We selected the top 10 up- and down-regulated genes to construct a heat map, which revealed that *CRYBA4*, *CCK*, and *PRR36* were the top three significantly upregulated genes in the peri-implantitis group compared with in the healthy group (Figure 1c and Supplemental Table 1). However, compared with healthy tissues, only *CCK* expression was upregulated in our cohort of 10 clinical peri-implantitis tissues (Figure 1d). Moreover, *CCK* is known to be associated with NF-$\kappa$B activation and inflammation.[20] Therefore, CCK was selected for further analysis.

## *CCK inhibited the osteogenic differentiation of hBMSCs*

We next explored the role of CCK in hBMSCs. First, hBMSCs were identified *via* immunofluorescence. The results revealed that these cells were positive for vimentin and CD44 (Figure 2a). Then, hBMSCs were pretreated with or without CCK (30 nM) for 1 hour before inducing their osteogenic differentiation using differentiation medium for 2 weeks. Increased CCK concentration led to significantly decreased ALP activity (Figure 2b). The results of alizarin red staining showed that with increasing CCK concentrations, cells showed a significantly decreased ability to undergo osteogenic differentiation (Figure 2b). Additionally, hBMSCs were pretreated with or without various concentrations of CCK (0, 10, 30, or 100 nM) for 1 hour before inducing osteogenic differentiation using differentiation medium for 2 weeks. After 2 weeks, the expression levels of osteogenic marker genes including *ALP*, *RUNX2*, and *OCN* were determined by qPCR. With increasing CCK concentrations, the levels of these genes were significantly decreased (Figure 2c). These results indicated that CCK inhibited the osteogenic differentiation of hBMSCs.

## *CCK promoted TNFα expression and activation of the NF-κB inflammatory pathway*

CCK is closely related to TNFα,[9] which inhibits the osteogenic differentiation of hBMSCs.[10,11] Thus, we determined TNFα levels. qPCR analysis showed that with increasing CCK concentrations, TNFα expression was gradually increased

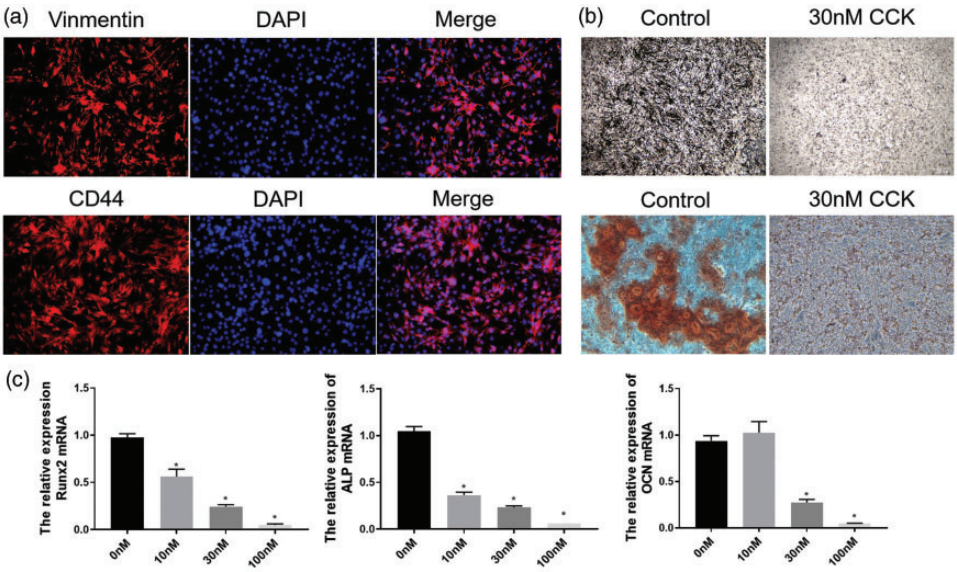


**Figure 2.** CCK inhibited the osteogenic differentiation of hBMSCs. (a) Identification of vimentin and CD44 expression in hBMSCs *via* immunofluorescence; $n = 3$. (b) hBMSCs were pretreated with or without CCK (30 nM) for 1 hour before inducing osteogenic differentiation using differentiation medium for 2 weeks. Then, ALP activity was assessed, and alizarin red staining was performed and (c) hBMSCs were pretreated with various concentrations of CCK (0, 10, 30, or 100 nM) for 1 hour before inducing osteogenic differentiation using differentiation medium for 2 weeks. Then, the expression levels of the osteogenic markers *ALP*, *RUNX2*, and *OCN* were determined by quantitative real-time PCR; *$P < 0.05$; $n = 3$.

(Figure 3a). Additionally, western blotting was performed to determine the levels of TNFα and proteins related to NF-κB signaling. With increasing CCK concentrations, TNFα and p-P65 levels were gradually increased, whereas total P65 levels remained unchanged (Figure 3b). These results suggest that CCK promotes TNFα expression and activation of the NF-κB inflammatory pathway.

## Discussion

Peri-implantitis is an inflammatory disease of the soft and hard tissues surrounding bone implants that is followed by progressive damage to the alveolar bone.[21] Although surgical intervention appears to be effective for treating peri-implantitis, there is no consensus on the best techniques for peri-implantitis.[22] In this study, we used microarray analysis to find that *CCK* expression was upregulated in the periodontal tissues of patients with peri-implantitis; therefore, we explored the mechanism underlying its involvement in peri-implantitis. We found that CCK inhibited the osteogenic differentiation of hBMSCs and promoted TNFα expression and activation of the NF-κB inflammatory pathway. This is the first report to show the role of CCK in peri-implantitis.

High-throughput sequencing has become a powerful tool for genomics, epigenomics, and transcriptomics research that has significantly improved our ability to identify the causes of human diseases.[23,24] In this study of peri-implantitis, microarray transcriptome analysis was used to analyze changes in the gene expression profile of patients with peri-implantitis.[16,17] In this study, we performed mRNA sequencing of the GEO database GSE106090. Compared with the healthy group, 262 genes were upregulated and 215 were downregulated in the periodontal tissues of patients with peri-implantitis. Additionally, *CCK* expression was significantly upregulated in our clinical samples of patients with peri-implantitis. No study has focused on *CCK* in peri-implantitis to date. This study provides insights into the mechanisms underlying peri-implantitis and shows that functional characterization of mRNAs is essential to understanding disease mechanisms.

Stem cell therapy is developing rapidly and has been used to treat various diseases, including bone-related diseases.[25] As the common progenitor of adipocytes and osteoblasts, hBMSCs need to be maintained in a delicate balance of commitment.[26]

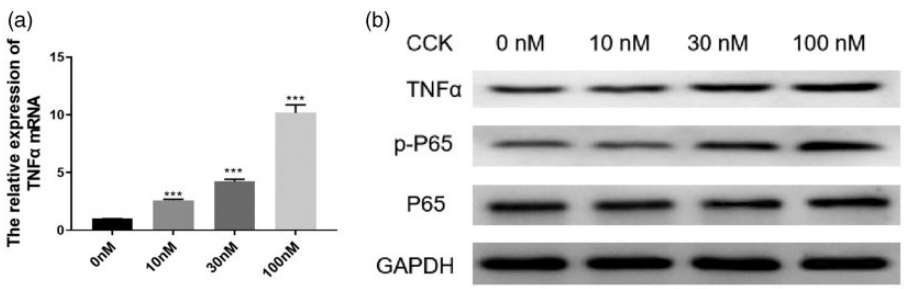


**Figure 3.** CCK promoted TNFα expression and activation of the NF-κB inflammatory pathway. hBMSCs were pretreated with various concentrations of CCK (0, 10, 30, or 100 nM) for 1 hour before induction in the osteogenic differentiation medium for 2 weeks. (a) quantitative real-time PCR was used to evaluate TNFα expression and (b) TNFα, phosphorylated P65 (p-P65), and total P65 levels were determined by western blot; *$P < 0.05$; $n = 3$.

hBMSCs also play an important role in peri-implantitis. Additionally, inflammation and bone loss play essential roles in the occurrence and development of peri-implantitis.[27] Inflammation inhibits osteogenic differentiation, causing peri-implantitis and the loosening of implants. The inflammatory microenvironment of peri-implantitis can also reduce the proliferation and differentiation of mandibular osteoblasts.[28] In this study, we found that CCK reduced ALP activity, osteogenic differentiation, and expression of the osteogenic markers *ALP*, *RUNX2*, and *OCN*. These data indicated that CCK inhibited the osteogenic differentiation of hBMSCs. This is the first study to report the inhibition of osteogenic differentiation of hBMSCs by CCK in peri-implantitis.

CCK is a polypeptide synthesized by various cells, and its immunomodulatory activity has been reported in experimental models of inflammation. According to Saia *et al*., CCK regulates mucosal inflammation and prevents lipopolysaccharide-induced intestinal epithelial barrier dysfunction.[29] Satoh *et al*. reported that PKC-delta and PKC-epsilon regulate NF-κB activation downstream of CCK and TNFα in pancreatic acinar cells.[20] CCK is also closely related to TNFα,[9] which inhibits the osteogenic differentiation of hBMSCs.[10,11] Therefore, we pretreated hBMSCs with various concentrations of CCK before induction and determined TNFα expression. We found that CCK promoted TNFα expression. Additionally, we determined that p-P65 levels, a marker of the NF-κB inflammation pathway, were increased with increasing CCK concentrations, as was activation of the NF-κB pathway. This observation provides a reference for follow-up studies of inflammation in peri-implantitis. However, our findings are preliminary and need further validation because of certain limitations, such as small clinical sample size and relatively small datasets. Thus, more clinical samples should be collected to validate our gene expression results. Microarray screening of genes encoding IRF4, NLRP14, and other inflammation-related proteins should be conducted as well as an analysis of their involvement in inflammation and osteogenic differentiation.

## Conclusion

In this study, we explored the mechanism underlying the involvement of CCK in peri-implantitis. We report, for the first time, that CCK inhibits the osteogenic differentiation of hBMSCs and promotes TNFα expression and activation of the NF-κB inflammatory pathway. This work will contribute toward developing novel treatments for peri-implantitis.

### Author contributions

YX: conception and design of the study and critical revision of the manuscript for important intellectual content; YC and LC: acquisition of data and drafting the manuscript; and KL, ML, YL, XL, and PL: analysis and interpretation of data. All authors approved the final version of the manuscript.

### Declaration of conflicting interests

The authors declare no conflicts of interest.

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### Supplemental material

Supplemental material for this article is available online.

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