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Original Article

TNF-α promotes expression of inflammatory factors by upregulating nicotinamide adenine dinucleotide phosphate oxidase-2 expression in human gingival fibroblasts

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Tumor necrosis factor-α (TNF-α)

Abstract *Background/purpose:* Periodontitis is a chronic infectious disease. The oxidative stress environment can cause or exacerbate the inflammation in periodontitis. Nicotinamide adenine dinucleotide phosphate oxidase (NOX) may be the most important source of reactive oxygen species (ROS) in periodontal tissues. The pathological mechanism of periodontitis may be related to the increased ROS caused by enhanced NOX activity. The purpose was to investigate the effect of tumor necrosis factor (TNF-α) on inflammatory cytokines and ROS, and the role of NOX-2 in human gingival fibroblasts (HGFs).
Materials and methods: HGFs were cultured and divided into the normal control group (NC group) and the inflammatory model group (TNF-α group) induced by 10 ng/ml TNF-α. Thereafter, NOX-2 siRNA was used to knock down NOX-2 gene expression. Quantitative real-time PCR was applied to detect *IL-6*, *MCP-1*, and *NOX*-2 mRNA levels. The levels of IL-6 and MCP-1 protein were examined by ELISA. The level of NOX-2 was evaluated by Western blot. ROS expression was measured by the fluorescence microplate.
Results: The mRNA and protein expression levels of *IL-6*, *MCP-1*, and *NOX-2* were significantly increased, and the expression of ROS was significantly elevated in response to 10 ng/ml TNF-α.

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Compared with the si-NC group, the mRNA and protein expression levels of *IL-6* and *MCP-1* were significantly down-regulated and ROS expression was significantly decreased in the si-NOX2 group stimulated by 10 ng/ml TNF-α.
Conclusion: TNF-α promotes the expression of NOX-2 in human gingival fibroblasts and enhances the expression of inflammatory factors and ROS in human gingival fibroblasts through the upregulation of NOX-2 partly.
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Introduction

Periodontitis is a chronic infectious disease caused by plaque microorganisms. The interaction between the plaque microorganisms and the host determines the development and process of the disease, leading to the inflammatory destruction of periodontal tissue.[1] The main clinical manifestations of periodontitis include gingival redness, attachment loss, formation of the periodontal pocket, alveolar bone resorption, and tooth loosening and displacement. It is the leading cause of tooth loss in adults.[2] In recent years, increasing evidence has shown that the oxidative stress environment can cause or exacerbate the inflammatory response of periodontitis.[3]

Reactive oxygen species (ROS) are considered a "double-edged sword" that exerts its effects by fighting against microorganisms. Under physiological conditions, ROS resist and kill invading pathogenic microorganisms.[4] ROS play an important role in cell signaling, gene regulation, and antimicrobial defense.[5] However, excessive ROS production can lead to increased oxidant load with unchanged or reduced antioxidant capacity, resulting in a state of oxidative stress in the tissues. It can cause pathological changes that damage the periodontal supporting tissues of the host and eventually lead to tooth loss with the destruction of periodontal tissue structure.[6,7] There has been a longstanding interest in the relationship between ROS and periodontitis.[8,9]

Nicotinamide adenine dinucleotide phosphate oxidase (NOX) may be the most important source of ROS in periodontal tissues.[10] NOX is the terminal component of the respiratory chain and plays a role in electron transfer, obtaining a single electron from the cytoplasmic nicotinamide adenine dinucleotide phosphate (NADPH), transferring it through the plasma membrane to external oxygen molecules, and leading to the production of ROS.[11] It has been suggested that the pathological mechanism of periodontitis may be related to the increased production of ROS caused by enhanced NOX activity. Therefore, it is crucial to inhibit NOX activity and adequately regulate the production of ROS in periodontitis.

Seven isoforms of NOX proteins exist in human organs, namely NOX 1–5, DUOX-1, and DUOX-2.[12] The NOX protein family is a multimeric complex (excluding NOX-5) that includes transmembrane subunits (such as NOX-2, $p22^{phox}$) and cytoplasmic subunits (such as $p47^{phox}$, NOXO1, $p67^{phox}$, NOXA1 $p40^{phox}$, Rac).[10] In the resting state, the cytoplasmic and transmembrane subunits are separated. In the activated state, the cytoplasmic subunits undergo phosphorylation and translocation to the transmembrane subunits, forming a polymeric complex that functions.[13] NOX-2 is expressed in the gingival tissue of chronic periodontitis.[14] For example, in NOX-2, $p47^{phox}$, $p40^{phox}$, and $p67^{phox}$ exist as a complex in the cytoplasm in the resting state. In the activated state, the $p47^{phox}$ subunit is phosphorylated and then this complex as a whole moves toward the plasma membrane and binds to Cytb558 on the membrane, forming a NOX polymer complex.[15]

Gingival fibroblasts (GFs) have the ability to proliferate and self-renew rapidly.[16] The maintenance of normal physiological functions such as proliferation and differentiation of GFs is an important mechanism for self-healing after periodontal tissue injury.[17] In this study, we investigated the effects of TNF-α on inflammatory cytokines and ROS in human gingival fibroblasts and the role of NOX-2 through *in vitro* experiments.

Materials and methods

Primary cell culture

Human gingival fibroblasts (HGFs) were extracted from healthy gingival tissues. Gingival tissues were obtained from healthy periodontal individuals requiring crown lengthening surgery in the Department of Periodontology, Peking University School and Hospital of Stomatology, Beijing, China. The Biomedical Ethics Committee of Peking University School and Hospital of Stomatology approved this study (IRB00001052-08010), and informed consent forms were signed prior to the surgery. Gingival tissues were immediately immersed in Dulbecco Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with penicillin (200 U/ml)—streptomycin (200 U/ml) (Gibco). After removal of the epithelium, collected gingival specimens were minced into small pieces (0.5–1 mm^3 in size), and then plated onto a cell culture dish in DMEM medium containing 10% Foetal Bovine Serum (FBS; Gibco) and penicillin (100 U/ml)—streptomycin (100 U/ml). The cells were incubated at 37 °C in a 5% CO_2, 95% humidified atmosphere. The following experiments were conducted with 3–6 passages of HGFs. HGFs were divided into two groups: the normal control group (NC group) and the inflammatory cell group (TNF-α group). TNF-α (PeproTech, Cranbury, NJ,

USA) was added to the TNF-α group when the cell density reached 80%–90% (final concentration of 10 ng/ml). Cells were continued to be cultured for 48 h, and samples were collected.

Small interfering RNA transfection

NOX-2-specific small interfering RNA (siRNA) and non-targeting control siRNA were synthesized by RiboBio (Guangzhou, China). 1×10^6 HGFs were seeded into 6-well plates (Corning Inc., Rochester, NY, USA) for 24 h, and the siRNAs were transfected into HGFs grown to 70% using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The sequence of NOX2 siRNA was 5′-CCGGAGGTCTTACTTTGAA-3′.

Quantitative real-time polymerase chain reaction

Cells were collected and Trizol reagent (Ambion, Austin, TX, USA) was added to extract total RNA from cells. Total RNA content was detected by spectrophotometer. RNA was reversely transcribed into cDNA using reverse transcription kit (TOYOBO, Osaka, Japan) and cDNA was used as the template for real-time fluorescence quantitative PCR detection (TOYOBO). Using GAPDH as internal reference, mRNA relative expression levels of IL-6, MCP-1, and NOX family (including NOX-1, NOX-2, NOX-3, NOX-4, and NOX-5) were calculated by the $2^{-\triangle\triangle Ct}$ method. The primers were synthesized by Sangon Biotech (Shanghai, China) as follows: 5′-CGACAGTCAGCCGCATCTT-3′ (forward primer) and 5′-CCAATACGACCAAATCCGTTG-3′ (reverse primer) for the human GAPDH gene, 5′-GTGAGGAACAAGCCAGAGC-3′ (forward primer) and 5′-TACATTTGCCGAAGAGCC-3′ (reverse primer) for the human IL-6 gene, 5′-CAGCCAGATGCAATCAATGCC-3′ (forward primer) and 5′-TGGAATCCTGAACCCACTTCT-3′ (reverse primer) for the human MCP-1 gene, 5′-GGAATTAGGCAAAGTGGGTTTT-3′ (forward primer) and 5′-CAGTG GCCTTGTCAAAGTTTAA-3′ (reverse primer) for the human NOX-1 gene, 5′- AAGATGCGTGGAAACTACCTAA-3′ (forward primer) and 5′-CAGTGGCCTTGTCAAAGTTTAA-3′ (reverse primer) for the human NOX-2 gene, 5′-AACATCACCTTCTGTAGAGACC-3′ (forward primer) and 5′-CTTCTTGTTG AAATCGCCAGAA-3′ (reverse primer) for the human NOX-3 gene, 5′-TCACAGCCTCTACATATGCAAT-3′ (forward primer) and 5′-CAGCAGCATGTAGAAGACAAAG-3′ (reverse primer) for the human NOX-4 gene, 5′-TGAGCAGAAAGACACTATCTGG-3′ (forward primer) and 5′-CTGATGCCTTGAAGGACTCATA-3′ (reverse primer) for the human NOX-5 gene.

Western blot analysis

Cells were collected and added with RIPA lysate (Solarbio, Beijing, China) and PIC protease inhibitor (Solarbio). Ultrasonic cracking and centrifugal collection of supernatant. The BCA protein detection kit (Thermo Fisher Scientific, Waltham, MA, USA) measured the protein concentration of the sample. The samples were separated by gel electrophoresis with sodium dodecyl sulfate polyacrylamide (SDS-PAGE; Dakewe Biotech, Shenzhen, China), after which the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Thermo Fisher Scientific). 5% skim milk (BD, Franklin Lakes, NJ, USA) was enclosed for 1 h and the membrane was hybridized with NOX-2 monoclonal antibody (Abcam, Cambridge, UK) or GAPDH monoclonal antibody (Cell Signaling Technology, Beverly, MA, USA). TBST was washed and incubated with horseradish peroxidase labeled secondary antibodies (Cell Signaling Technology). ECL reagent (Solarbio) was used for chemiluminescence. The protein band strength was quantitatively analyzed by Image J software, and the GAPDH protein strength was used as an internal reference for standardized analysis.

Enzyme-linked immunosorbent assay

Cell supernatant of cultured HGFs was collected, centrifuged, and stored at −80 °C. Content of IL-6 and MCP-1 in supernatant was detected by Enzyme-linked immunosorbent assay (ELISA) according to the instructions of the human IL-6 and MCP-1 ELISA kit (Meimian, Jiangsu, China).

Reactive oxygen species ROS

ROS levels were detected using a chemiluminescent probe, DCFH-DA reagent (Beyotime Biotechnology, Shanghai, China). Cells were stimulated with 10 ng/ml TNF-α for 24 and 48 h. Then serum-free DMEM medium containing DCFH-DA was added (volume ratio 1:1000) and cells were incubated at 37 °C for 20 min. After trypsin digestion, cells were collected by centrifugation after suspension, washed with PBS 2–3 times, and cell precipitates were collected for fluorescence detection. The collected cells were made into cell suspension with PBS. The excitation wavelength was 488 nm while the emission wavelength was 525 nm.

Statistical analysis

Excel 2019, SPSS 25.0, and Prism 8.0 statistical analysis software were used to analyze the experimental data. Each experiment was repeated more than 3 times. The unpaired T test was used to compare the difference between the experimental and control groups. $^*P < 0.05$ was considered statistically significant.

Results

TNF-α promoted expression of IL-6 and MCP-1 in human gingival fibroblasts

We firstly constructed an inflammatory cell model using TNF-α of 10 ng/ml. RT-qPCR was used to detect mRNA expression level of IL-6 and MCP-1 and our results showed that the expression level of IL-6 and MCP-1 in TNF-α group was significantly increased (Fig. 1A and B). Concentrations of IL-6 and MCP-1 in supernatant were detected by ELISA and the results demonstrated that TNF-α significantly upregulated expression of IL-6 and MCP-1 protein in the supernatant (Fig. 1C and D).

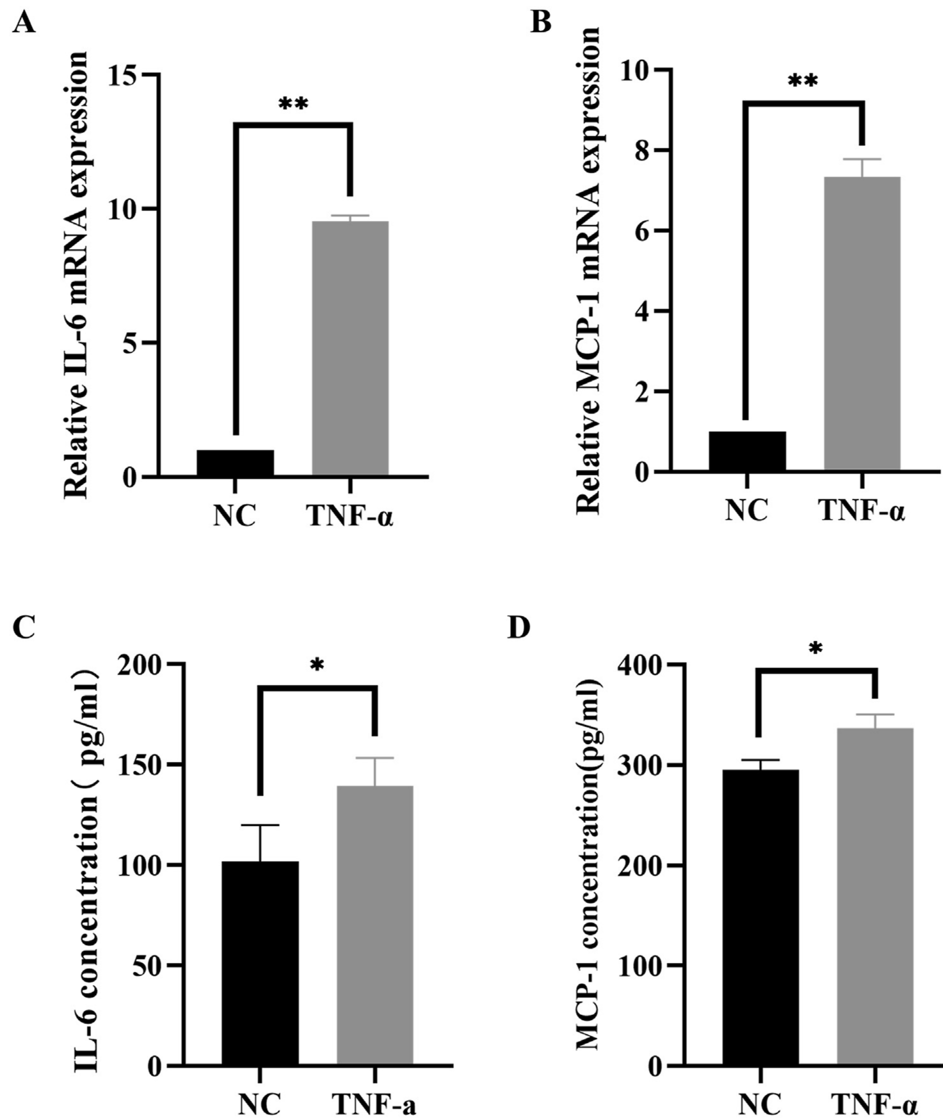

Figure 1 Gene and protein expression levels in HGFs. (A) The relative mRNA expression of IL-6 was detected by RT-qPCR. (B) The relative mRNA expression of MCP-1 was detected by RT-qPCR. (C) Concentration of IL-6 in supernatant was measured by ELISA. (D) Concentration of MCP-1 in supernatant was measured by ELISA. *: $P < 0.05$; **: $P < 0.01$.

Expression of ROS was upregulated in human gingival fibroblasts with TNF-α stimulation

In order to elucidate the association between TNF-α and oxidative stress, we determined ROS levels in HGFs with TNF-α stimulation. Our results found that intracellular ROS levels markedly increased after incubation with 10 ng/ml TNF-α in HGFs for 48 h (Fig. 2A and B).

TNF-αpromoted the expression of NOX-2 in human gingival fibroblasts

To determine the role of NOX in HGFs, we examined the mRNA expression of the five NOX family members in response to 10 ng/ml TNF-α. Among these NOX family mRNA, NOX-2 expression was significantly increased (Fig. 3B) and NOX-4 expression was significantly decreased (Fig. 3D) in the TNF-α group of HGFs, while incubation of HGF with TNF-α had no significant effect on the mRNA expression of NOX-1, NOX-3, and NOX-5 (Fig. 3A, C, and E). The results of Western blot analysis demonstrated that the protein expression of NOX-2 was also significantly elevated in TNF-α group compared with the NC group in HGFs (Fig. 3F and G).

NOX-2 gene silencing reduced the expression level of IL-6 and MCP-1

NOX-2 siRNA (si-NOX2) and negative control siRNA (si-NC) were transfected with HGFs, followed by stimulation with 10 ng/ml TNF-α for 48 h. The mRNA expression level of IL-6 and MCP-1 was detected by RT-qPCR, and the protein level of IL-6 and MCP-1 in the cell culture supernatant were measured by ELISA. After knockdown of NOX-2, the mRNA expression levels (Fig. 4A and B) and protein secretion

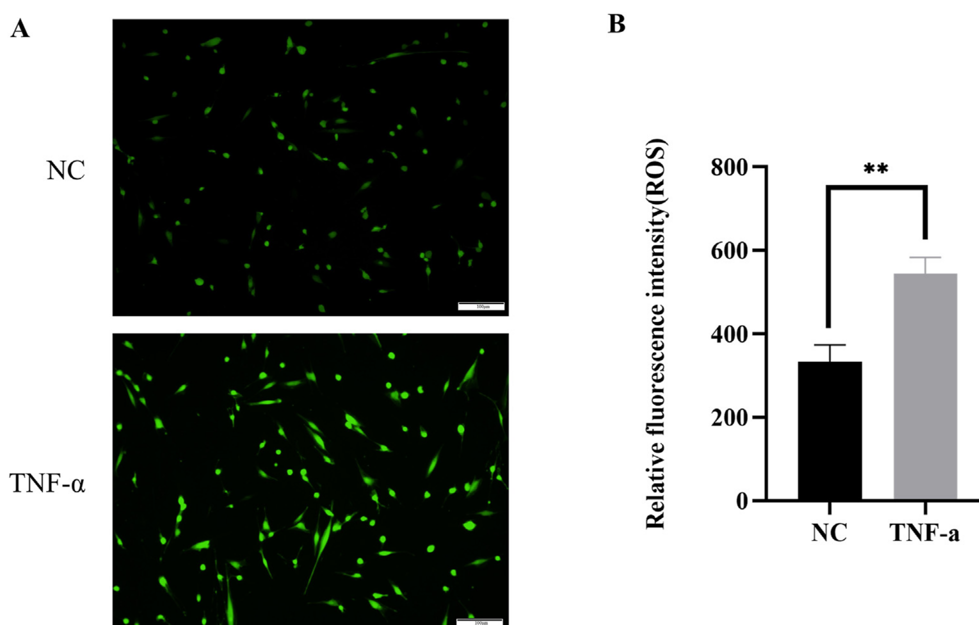

Figure 2 Production level of ROS in HGFs. (A) The intracellular ROS (green) with or without 10 ng/ml TNF-α stimulation for 48 h. Bar = 100 μm. (B) The intracellular ROS levels that HGFs produced as measured by fluorescence microplate. **: $P < 0.01$. Abbreviations: ROS, reactive oxygen species.

levels (Fig. 4C and D) of IL-6 and MCP-1 were significantly down-regulated in response of TNF-α.

NOX-2 gene silencing decreases the expression of ROS

The results of fluorescence microplate revealed that NOX-2 downregulation significantly decreased the level of ROS production in the si-NOX2 group compared with the si-NC group (Fig. 5A and B).

Discussion

Periodontitis is a chronic infectious disease with progressive loss of periodontal tissues caused by plaque microorganisms, eventually leading to tooth loosening and abscission.[2] In 2010, periodontitis was the sixth most common disease worldwide, with a prevalence of approximately 10.8%, affecting 743 million people worldwide.[18] TNF-α is an important pro-inflammatory cytokine that plays an important role in the attachment loss of periodontal tissues and the progression of periodontal diseases.[19] 10 ng/ml TNF-α is often used to induce inflammatory cell models.[16,20] Therefore, in this study, 10 ng/ml TNF-α was used to induce an inflammation model of human gingival fibroblasts.

IL-6 is an inflammatory cytokine involved in bone resorption.[21] MCP-1 is a chemokine secreted by various cell types involved in a variety of biological functions such as recruitment of inflammatory cells, wound healing, inhibition of stem cells, and maintenance of effector immune responses.[22,23] Changes in IL-6 and MCP-1 levels can reflect the severity of periodontal inflammation.[24] Therefore, this study investigated the expression levels of inflammatory cytokines IL-6 and MCP-1 in inflammatory models of human gingival fibroblasts. The results showed that the expression levels of genes and proteins of IL-6 and MCP-1 were significantly increased after 10 ng/ml TNF-α stimulation in human gingival fibroblasts. This proves that TNF-α upregulates the expression levels of IL-6 and MCP-1 in human gingival fibroblasts.[25]

In recent years, many studies have shown that the oxidative stress environment is an important factor in the occurrence and progression of a large variety of chronic inflammatory diseases, such as periodontitis,[5] type 2 diabetes,[26] atherosclerosis,[27] rheumatoid arthritis,[28] and inflammatory lung disease.[29] A large number of case-control and longitudinal studies comparing the levels of different antioxidant markers in the saliva of healthy and periodontitis subjects have shown that the levels of oxidative stress biomarkers (such as 8-OHdG and MDA) in saliva of periodontitis patients are significantly increased compared with healthy individuals.[30–32] After non-surgical treatment for periodontitis, the levels of antioxidants in saliva of periodontitis patients were significantly increased and were associated with the improvement of periodontal clinical index after treatment.[33] These results indicate a direct correlation between increased oxidative stress or ROS levels and the development of periodontitis or clinical improvement of periodontitis after treatment.[30,33]

As considered a "double-edged sword", ROS play an important role in combating microorganisms under physiological conditions, while cause cytotoxicity to host cells when overactivated.[34] The relationship between ROS and periodontitis has long been a concern.[8,9] In recent years, NOX has been considered to be the main source of ROS in periodontal tissues. In this study, the 10 ng/ml TNF-α induced inflammation cell model showed that TNF-α can promote the production of ROS.[35] Similarly, compared with

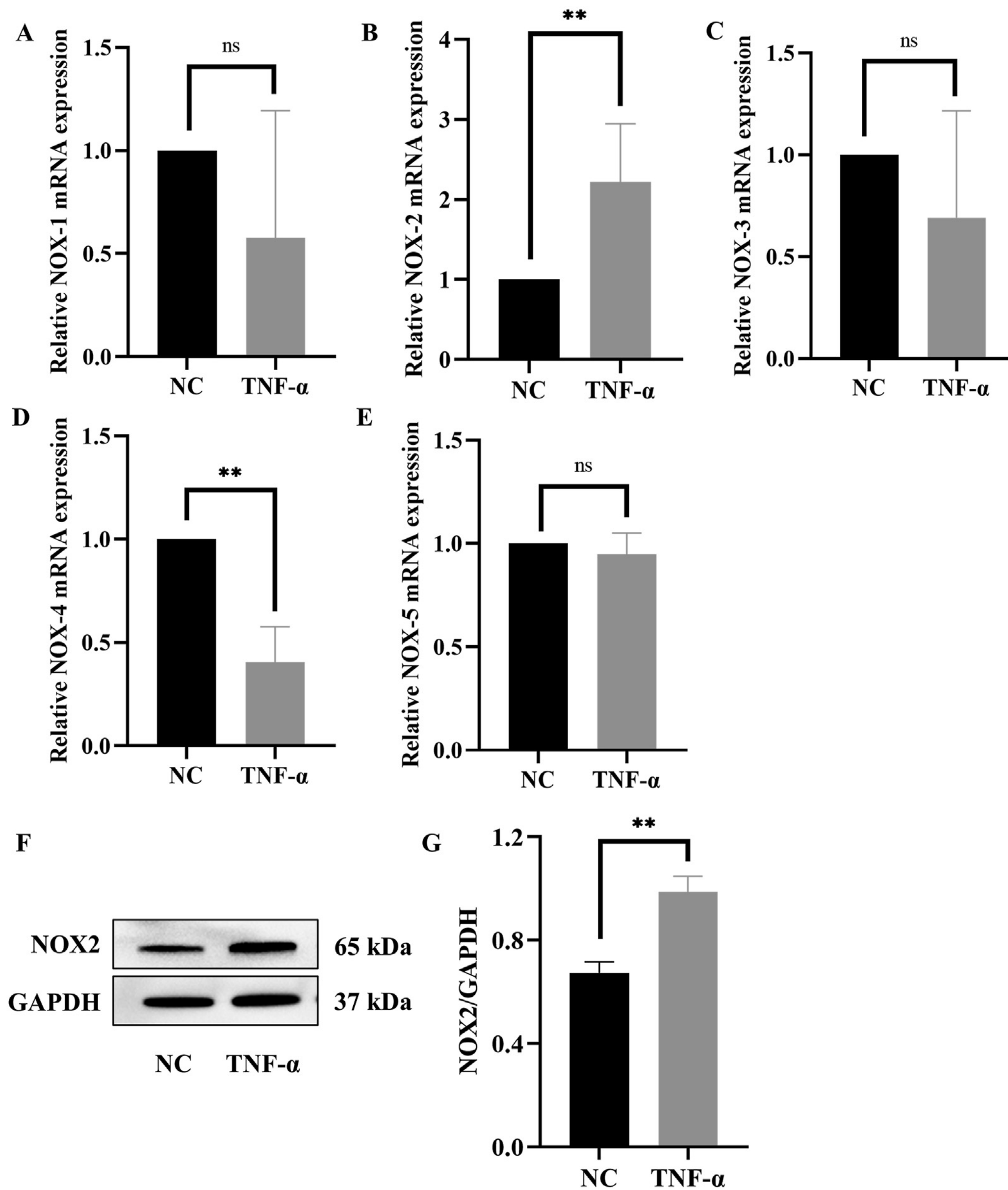

Figure 3 Gene and protein expression levels of the NOX family in HGFs. (A—E) The relative mRNA expression of NOX-1, NOX-2, NOX-3, NOX-4, NOX-5 was detected by RT-qPCR. (F) The protein expression of NOX-2 and GAPDH in HGFs. (G) The relative protein level of NOX-2 compared with GAPDH in HGFs. *: $P < 0.05$; **: $P < 0.01$; ns: not significant.

the control group, the expression levels of the NOX-2 gene and protein in the inflammatory cell model group were significantly increased. This proves that TNF-α can upregulate the expression level of NOX-2 in human gingival fibroblasts. Lin et al. reported that TNF-α significantly promoted the activation of NOX and the production of ROS (including O^{2-} and H_2O_2), using TNF-α to induce human alveolar epithelial cells.[36] This is consistent with our findings.

ROS in periodontal tissue is believed to be mainly produced by NOX.[35] At present, there is limited research on the impact of NOX-2 on inflammatory cytokines IL-6 and MCP-1. Some studies have shown that in the mouse model of periodontitis induced by *Aggregatibacter actinomycetemcomitans* (*Aa*), NOX-2 gene knockout mice have significantly reduced alveolar bone volume, increased inflammatory cell infiltration and the number of osteoclasts in periodontal tissues compared with the wild-type control group.[37] It has been reported that the level of IL-6 protein in central nervous system tissue was lower in NOX-2 knockout mice with experimental autoimmune encephalomyelitis.[38] In experimental atrial fibrillation mice, mRNA expressions of IL-6 and MCP-1 in atrial tissue of NOX-2 overexpressed mice showed no difference compared with wild-type control group.[39] These results suggest that NOX-2 may play an important role in tissue inflammation. Therefore, this study investigated the regulatory mechanism of TNF-α in cellular inflammatory response after NOX-2 gene silencing in human gingival fibroblasts. The results showed that after TNF-α stimulation of human gingival fibroblasts,

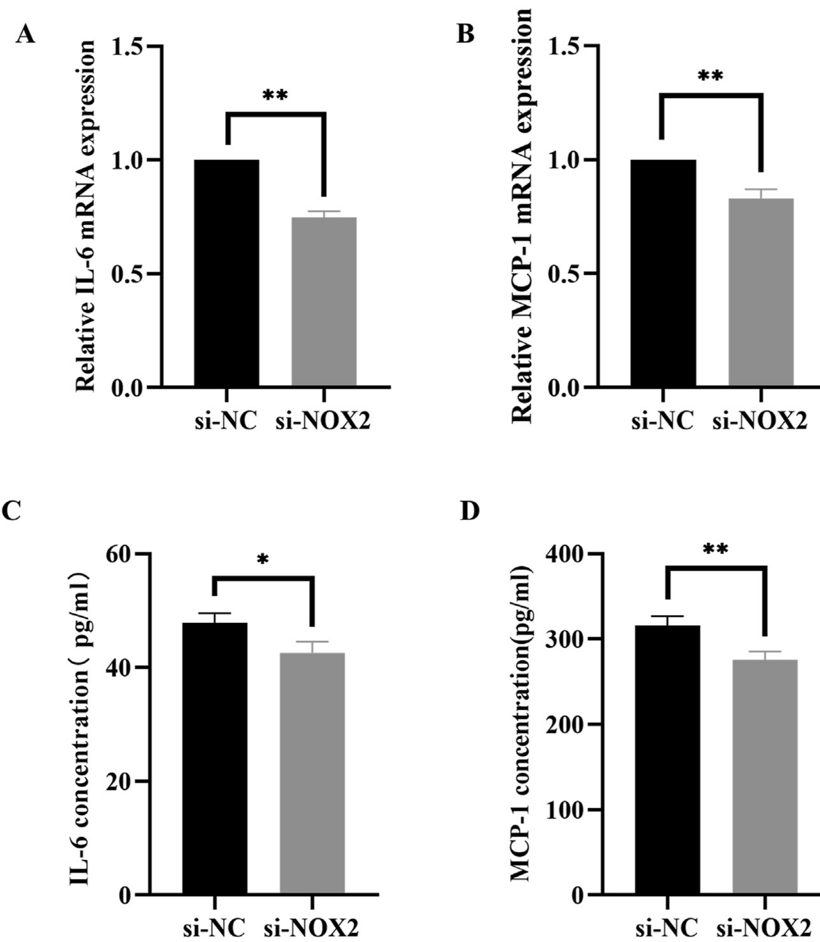

Figure 4 Changes in IL-6, MCP-1 expression level in HGFs after knockdown of NOX-2. (A) The relative mRNA expression of IL-6 was detected by RT-qPCR. (B) The relative mRNA expression of MCP-1 was detected by RT-qPCR. (C) Concentration of IL-6 in supernatant was measured by ELISA. (D) Concentration of MCP-1 in supernatant was measured by ELISA. *: $P < 0.05$; **: $P < 0.01$. si-NC: negative control siRNA group; si-NOX2: NOX-2 siRNA group.

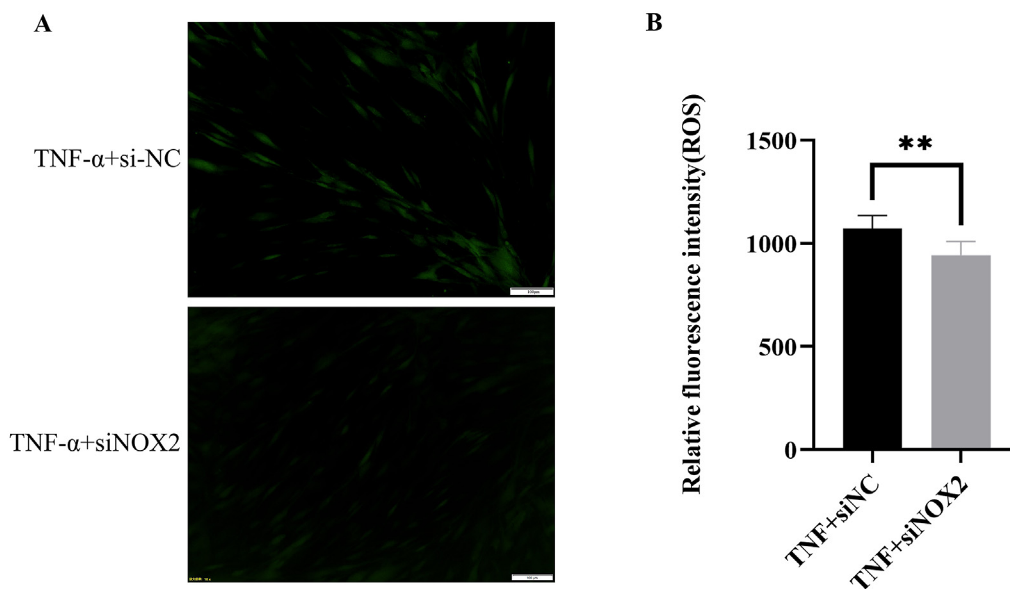

Figure 5 Changes in ROS expression level in HGFs after knockdown of NOX-2. (A) The intracellular ROS levels with 10 ng/ml TNF-α stimulation for 48 h. Bar = 100 μm. (B) The intracellular ROS levels that HGFs produced as measured by fluorescence microplate. **: $P < 0.01$. Abbreviations: ROS, reactive oxygen species.

the expressions of IL-6 and MCP-1 genes and proteins were significantly down-regulated in the NOX-2 gene silencing group, while the production of ROS was significantly decreased.

In conclusion, TNF-α can significantly improve the expression levels of IL-6 and MCP-1, promote the expressions of NOX-2 and ROS, and reverse the increase in the expression levels of IL-6 and MCP-1 after NOX-2 gene silencing in human gingival fibroblasts. These results suggest that TNF-α increases the expression of IL-6, MCP-1, and ROS in HGFs by upregulating the expression of NOX-2 partly.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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