Oroxylin A inhibits inflammatory cytokines in periodontitis via HO-1

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Abstract. Periodontal disease is a common infectious disease that can lead to the loss of teeth. Hower how to effectively suppress the inflammation with medication is unclear. The aim of the present study was to investigate the anti-inflammatory effect of Oroxylin A in periodontitis and its potential role through heme oxygenase-1 (HO-1). Primary rat gingival fibroblasts (RGFs) were cultured using the tissue block method and identified by immunofluorescence. Following lipopolysaccharide (LPS) stimulation of RGFs, Oroxylin A was administered at 50, 100, 200 or 400 μg/ml. Reverse transcription-quantitative PCR was used to assess mRNA expression of cyclooxygenase (COX)-2, TNF-α, RANKL and osteoprotegerin (OPG). Western blotting was used to detect protein expression levels of COX -2, TNF-α, RANKL and OPG. Following HO-1 knockdown, the same treatment was performed. The expression of COX-2 in rat gingival tissue was observed by immunohistochemistry. One-way analysis of variance and Student's t test were used for statistical analysis. Oroxylin A downregulated mRNA expression of COX-2, TNF-α, RANKL and OPG in LPS-induced RGFs. With increase of Oroxylin A dose, the expression of HO-1 was gradually upregulated. When HO-1 was knocked down, Oroxylin A did not downregulate the expression of COX-2, TNF-α, RANKL and OPG in LPS-induced RGFs. Immunohistochemical results showed that expression of COX-2 was downregulated by Oroxylin A, and the expression of TNF-α, RANKL and OPG were also downregulated. Oroxylin A decreased expression of inflammatory cytokines in LPS-induced RGFs and had a good inhibitory effect on periodontitis in rats.

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Introduction

Periodontal disease is an infectious bacterial disease occurring in the periodontal support tissue, which can lead to continuous and irreversible destruction of the periodontal support tissue, leading to tooth loss and seriously affecting quality of life (1). Also, periodontitis can lead to destruction of alveolar bone and periodontal ligaments, and is associated with numerous types of systemic disease (2), such as diabetes, heart disease and osteoporosis. However, the treatment of periodontitis is still primarily based on mechanical removal of periodontal pathogenic factors such as plaque and dental calculus, and lifelong periodontal maintenance therapy is required. Treating periodontitis and restoring absorbed periodontal tissue remains a major challenge (3).

Drugs are commonly used as adjuvant therapy for periodontitis (4) to supplement non-surgical treatment of periodontitis. The most commonly used antibiotics, such as metronidazole, minocycline and doxycycline, can enter the mucosa locally (5). These drugs are used in periodontal pockets to inhibit or eliminate periodontal disease-causing microorganisms and regulate inflammatory response of tissue. However, the use of antibiotics can cause immune disorders, or induce bacteria to develop drug resistance, and these adverse effects restrict the use of antibiotics in the treatment of periodontitis (6). Traditional Chinese medicine has shown potential in the treatment of periodontitis (7). Oroxylin A is a flavonoid compound isolated from the root of *Scutellaria baicalensis*. A number of studies have confirmed that Oroxylin A has antioxidant, anti-inflammatory and anti-tumor activity (8-10) In terms of anti-inflammatory effect, Oroxylin A has been shown to inhibit secretion of inflammatory cytokines (11), but the molecular mechanism is unclear.

Heme oxygenase-1 (HO-1) is a key cell protective enzyme. A number of studies have confirmed that HO-1 serves a regulatory role in inflammation subsidence and is considered a potential therapeutic target for numerous drugs to exert anti-inflammatory effects (12-14). Studies have confirmed that

HO-1 serves an important role in the occurrence and development of periodontitis and can regulate certain cytokines, such as cyclooxygenase (COX)-2 and TNF-α, to affect the process of periodontitis (15,16). To the best of our knowledge, there has been no report that Oroxylin A affects inflammation through HO-1. Therefore, it was hypothesized that Oroxylin A may decrease the expression of COX-2 and TNF-α by regulating HO-1, thus alleviating periodontal local inflammation, and at the same time downregulating the RANKL:osteoprotegerin (OPG) ratio, thus inhibiting the absorption of alveolar bone.

Materials and methods

Materials. Penicillin/streptomycin, Minimum Essential Medium-α, trypsin and fetal bovine serum were provided by Biological Industries. Lipopolysaccharide (LPS) and Oroxylin A were purchased from Beijing Solarbio Science & Technology Co., Ltd. RNAiso Plus, TB Green™ Premix Ex Taq™ II and PrimeScript™ RT reagent kit with gDNA Eraser were provided by Takara Bio, Inc. PCR primers were purchased from Sangon Biotech Co., Ltd.

Animals and treatments. The 8-week-old male Wistar rats weighing 250-300 g were purchased from Charles River Laboratories, Inc. A total of 30 rats were housed in the Experimental Animal Center of the Affiliated Hospital of Qingdao University and maintained in a specific-pathogen-free environment at 25˚C, 40% humidity, and a 12-h light/dark cycle. The animals had free access to food and water. Periodontitis was induced by tying silk ligatures around the maxillary second molars and high sugar diet, as previously described (17).

Rats were randomly divided into the following groups (n=10/group): Sham operation (PBS, no periodontitis, no silk ligation), periodontitis (PBS+silk ligation) and treatment group (silk ligation+Oroxylin A). General anesthesia was induced by intraperitoneal injection of pentobarbital sodium (40 mg/kg, 2%). Periodontitis was induced by placing a silk thread between the right maxillary first and second molars. Rats in the treatment group were injected with 8 μl Oroxylin A solution with a final concentration of 0.5 μg/μl at the gingival site every 48 h for 2 weeks, while the sham group and the periodontitis group were injected with the same volume of PBS solution. A total of 4 weeks later, the rats were sacrificed by intraperitoneal injection of sodium pentobarbital (200 mg/kg) followed by rapid decapitation; death was confirmed by loss of respiration and heartbeat. The gingival tissue was collected to extract total RNA and total protein for detecting the expression of COX -2, TNF-α, RANKL and OPG and paraffin sections were prepared. All animal studies were approved by the Research Ethics Committee of the Affiliated Hospital of Qingdao University (Qingdao, China, AHQU-MAL20210326). The expression level of COX-2 was observed by immunohistochemistry. The gingival tissue specimens were formalin-fixed and paraffin-embedded at room temperature (fixative concentration, 4%; duration 12 h). Sections of 3 μm thickness were examined with immunohistochemistry. Antigen retrieval was performed by heating the specimens at 97°C, followed by washing with xylene and gradual rehydration with a descending ethanol series. Sections were blocked with the bovine serum albumin(BSA,3%) for 30 min at room temperature. Immunohistochemistry sections were incubated in 3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity. Primary antibody against COX-2 (1:1000, Abcam, ab151571) was incubated at 37˚C for 1 h. Goat anti mouse IgG conjugated with horseradish peroxidase (1:1000, Abcam, ab6789) used as the secondary antibodies which was incubated at 37˚C for 30 min. Chromogen detection was performed using DAB for 1 min at room temperature. Add sufficient amount of hematoxylin solution to the tissue slices to completely cover and incubate for 1 min at room temperature. Images are acquired at 40× magnification using a light microscope (Olympus, BX43). Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., USA) was used for analysis.

Isolation, culture and identification of primary rat gingival fibroblasts (RGFs). After anesthesia, rat gingival tissue obtained from the attached gingiva of Wistar rats were cut into pieces and cultured in complete Minimum Essential Medium-α containing 10% fetal bovine serum and 1% penicillin/streptomycin, in a humidified incubator of 5% CO_2 at 37˚C (18). When the cells grew out from the edge of gingival tissue, and the fusion rate reached 80%, the cells were digested, centrifuged, and passaged. Third-to-fifth passage cells were used for subsequent analysis. RGFs were identified by immunofluorescence staining with anti-vimentin,anti- fibronectin , anti-cytokeratin antibodies and PBS for control group (19). Cells were divided into the following groups: Control (PBS), LPS (10 pg/ml) and 50, 100, 200 and 400 μg/ml Oroxylin A+LPS (10 pg/ml). All treatments were performed at 37˚C for 24 h. Cells were formalin-fixed at 4˚C (fixative concentration, 4%; 20 min), and blocked with normal goat serum(10%, Absin) for 1 h at 37˚C. Cells were incubated with primary antibodies as follows: Anti-vimentin (1:1000, Abcam, ab8978); anti- fibronectin (1:1,000, Abcam, ab2413), anti-cytokeratin (1:1000, Abcam, ab53280) at 37˚C for 2 h. Goat anti mouse IgG conjugated with horseradish peroxidase (1:1000, Abcam, ab6789) used as the secondary antibodies which was incubated at room temperature for 2h. Nucleus was stained with *DAPI*. Images were captured with fluorescent microscopy (Keyence,BZ-X800). Image-Pro Plus version 6.0 software (Media Cybernetics, Inc.) was used for analysis.

Cell transfection. RGFs were transfected with small interfering (si)RNA (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C. The sequences used were as follows: Rat HO-1 forward, 5'-ACA AGCAGAACCCAGUCUA-3' and reverse, 5'-UAGACUGGG UUCUGCUUGU-3' and negative control (NC) forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACG UGACACGUUCGGAGAATT-3'. RGFs ($5x10^5$ cells/well) were transfected with either HO-1 (20 nM) or NC siRNA (20 nM) using the RNAimax-transfection system (Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection for 36 h, cells were treated with complete medium in the presence of baicalein (50 μM; 37˚C, 5%CO_2) for 12 h (for PCR) or 24 h (for western blotting) (20).

Cell Counting Kit-8 (CCK8) assay. Cell viability was analyzed by CCK8 (Abcam) according to the manufacturer's protocol Cells were seeded and cultured for 3 h at a density of $5x10^3$/well in 100 μl medium into 96-well microplates(37˚C).

Then, the cells were treated with Oroxylin A at 37˚C for 24 h. 10 μl CCK-8 reagent was added to each well and then cultured at 37˚C for 2 h. All experiments were performed in triplicate. The absorbance was analyzed at 450 nm using a microplate reader.

Reverse transcription-quantitative PCR (RT-qPCR). TRIzol (Thermo Fisher Scientific, Inc.)was used to isolate total RNA from RGFs. RT-qPCR was then performed using RT-qPCR kits (SYBR Premix Ex Taq II, DRR820A, Takara), the temperature and duration were according to the manufacturers protocol. RNA was reverse-transcribed into cDNA using a Light Cycler LC480 (Roche Diagnostics). Cycling conditions consisted of initial incubations, followed by 40 cycles of denaturation, annealing, and extension. The thermocycling conditions of the qPCR steps were as follows: activation (temperature: 50°C; duration: 2 min), Dual-Lock DNA polymerase (temperature: 95°C; duration: 2 min), denaturation (temperature: 95°C; duration: 15 sec), and annealing/extension (temperature: 60°C; duration: 1 min). The mRNA expression levels were normalized to the level of GAPDH, a housekeeping gene. The 2-ΔΔCq method was used to determine relative expression of target genes. The primers used were as follows: GAPDH forward, 5'-ACC ACAGTCCATGCCATCAC-3' and reverse, 5'-TCCACCACC CTGTTGCTGTA-3'; TNF-α (GenBank: NM_013091.2) forward, 5'-ATGGGCTCCCTCTCATCAGT-3' and reverse, 5'-AAATGGCAAATCGGCTGACG-3'; COX-2 (GenBank: NM_017232.4) forward, 5'-CTCAGCCATGCAGCAAAT CC-3' and reverse, 5'-GGGTGGGCTTCAGCAGTAAT-3'; RANKL (GenBank: NM_057149.2) forward, 5'-CCTGTA CTTTCGAGCGCAGA-3' and reverse, 5'-AGTCGAGTC CTGCAAACCTG-3'; OPG (GenBank: NM_012870.2) forward, 5'- GAATGTGAGGAAGGGCGCTA-3' and reverse, 5'- CTTCGCACAGGGTGACATCT-3' and HO-1 (GenBank: NM_012580.2) forward, 5'-ATGCCCCACTCT ACTTCCCT-3' and reverse, 5'-TGTGTGGCTGGTGTGTAA GG-3'.

Western blotting. TRIzol (Thermo Fisher Scientific, Inc.) was used to isolate total protein from RGFs. Protein concentrations were estimated via BCA assay. 30 μg total protein was loaded per lane. 12% separation gel and 5% stacking gel were used. Proteins were transferred to PVDF membranes. Membranes were blocked with 5% skimmed milk for 1.5 h at room temperature prior to western blot analysis. Incubate the blot with the primary antibody at 4˚C overnight followed by secondary antibody for 2 h at room temperature. Primary antibodies against COX -2 (ab15191, 1:1000), TNF-α (ab9579,1:1000), OPG (ab73400, 1:1000), RANKL (ab93719, 1:1000) and HO-1 (ab13248, 1:1000) were all purchased from Abcam. Secondary horseradish peroxidase-linked antibodies against rabbit IgG (7074, 1:1,000) and anti-β-actin were from Cell Signaling Technology. Finally, protein bands were observed by ECL (Abcam, ab133406) and quantified using GraphPad Prism (version 8; Dotmatics) and ImageJ 2 (National Institutes of Health).

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 8; Dotmatics). Data are presented as mean ± standard deviation of three independent experimental repeats. One-way analysis of variance followed by Tukey's post hoc test was used to compare groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Isolation, culture and identification of RGFs. After being cultured for 3-7 days, the periodontal tissue fragment adhered to the wall and some cells grew from the edge of the tissue fragment (Fig. 1A). After 2-3 weeks of culture, Cells proliferated toward the center of the tissue fragment where they form a swirl pattern. Cell morphology was mostly fusiform, the nucleus was aggregated in the center and the protrusion formed by the cytoplasm radiated outwards. After 3-4 weeks, the fusion rate of the cells reached 80% (Fig. 1B).

Immunofluorescence results showed positive expression of fibronectin and vimentin antibodies (Fig. 1C and D), while negative expression of keratin antibodies (Fig. 1E). Fig. 1F shows a negative control, and the primary antibody was replaced by PBS without any expression. The outcome of positive expression of fibronectin and vimentin antibodies, while negative expression of keratin antibodies showed that periodontal membrane stem cells were derived from mesenchyma.

Effect of Oroxylin A on expression of inflammation and osteoclast factors of RGFs. Preliminary CCK-8 results showed that Oroxylin A (50, 100, 200M, 400M) has no cytotoxicity (Fig. S1). RT-qPCR showed that compared with the blank control, the mRNA expression levels of COX-2, TNF-α and RANKL in the LPS-stimulated group decreased with the increase of Oroxylin A concentration (Fig. 2A-C). LPS inflammatory stimulation did not significantly increase the expression of OPG, however, preconditioning with Oroxylin A increased the expression of OPG mRNA in LPS-stimulated RGFs in Oroxylin A dose-dependent manner (Fig. 2D). The RANKL/OPG ratio was significantly increased in the LPS group and showed a downward trend with the increase of the dose of Oroxylin A (Fig. 2E).

Western blotting showed that LPS could significantly induce the protein expression levels of COX-2, TNF-α and RANKL and Oroxylin A could downregulate their expression in a concentration-dependent manner (Fig. 2F-I). Oroxylin A could induce OPG protein expression in a concentration-dependent manner (Fig. 2J), while the RANKL/OPG ratio was increased by LPS stimulation and decreased with the dose of Oroxylin A (Fig. 2K).

Regulation of HO-1 expression in non-inflammatory and inflammatory RGFs by Oroxylin A. RT-qPCR results showed that the expression of HO-1 mRNA in normal RGFs was increased following Oroxylin A treatment and the degree of increase was positively associated with the concentration of Oroxylin A (Fig. 3A). The protein expression of HO-1 was increased following by Oroxylin A treatment (Fig. 3B). The expression of HO-1 increased following Oroxylin A treatment in a dose-dependent manner (Fig. 3C)

Following LPS induction, the mRNA and protein expression of HO-1 in RGFs were significantly higher than those in

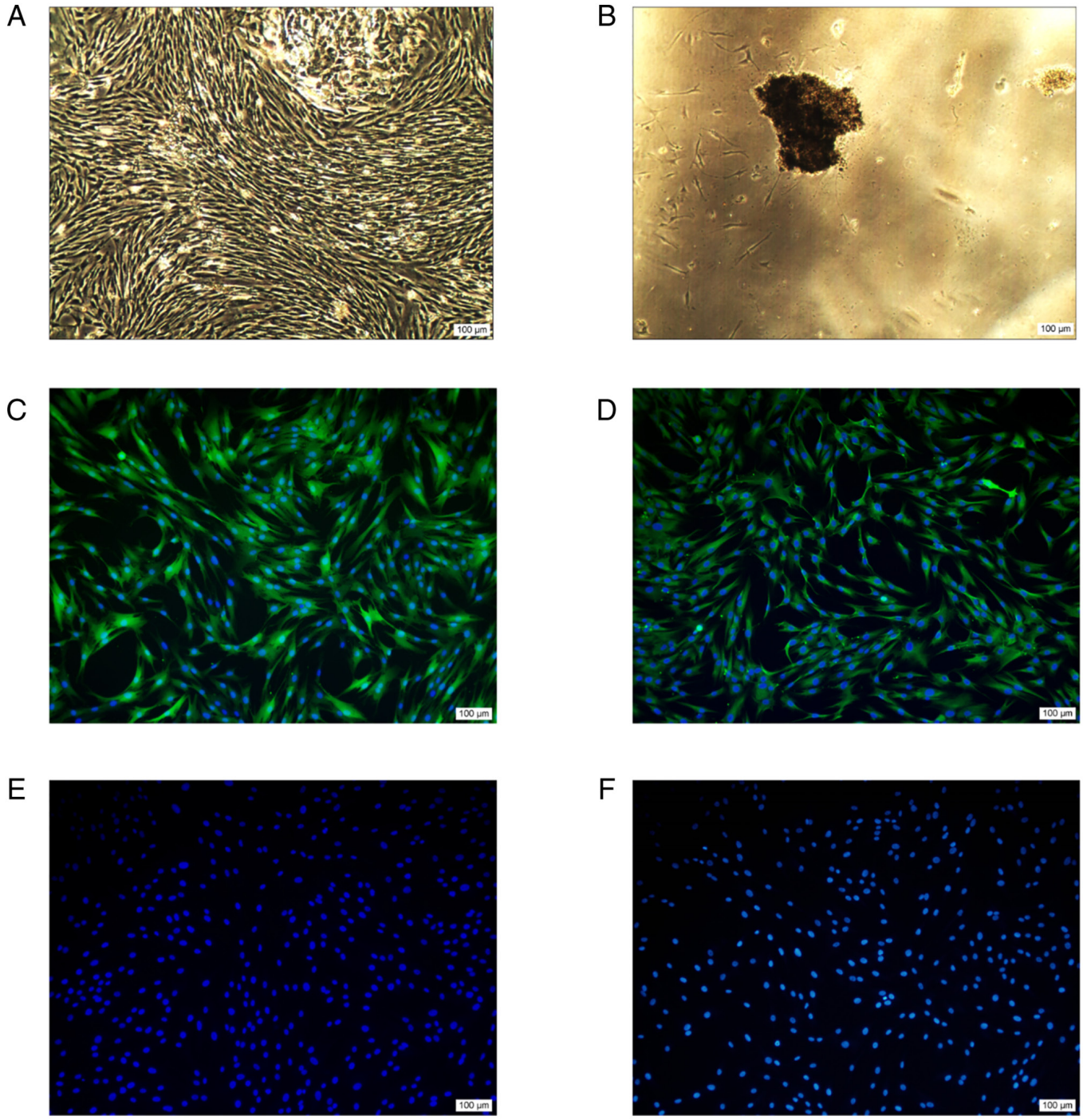

Figure 1. Culture and identification of primary rat gingival fibroblasts. (A) Passaged cells cultured for 7 days, the periodontal tissue fragment adhered to the wall and some cells grew out from the edge of the tissue fragment. (B) Images of passaged cells cultured for 3 weeks. Cells grew rapidly toward the center of the tissue fragment where they form a swirl pattern. Staining of anti-vimentin, (C) anti-fibronectin, (D) anti-cytokeratin antibodies and (E) control group (F) for cell identification.

blank control group without inflammation induction, and HO-1 was upregulated with the increase of Oroxylin A concentration (Fig. 3D-F).

Transfection efficiency of siRNA and effect of HO-1 knockdown in RGFs. Following the transfection of RGFs with fluorescence-labeled siRNA, peripheral cells showed high brightness under microscope (Fig. 4A). The results of fluorescence microscopy showed that the fluorescence was evenly distributed in the cells (Fig. 4B). RT-qPCR and western blotting showed no significant differences in the HO-1 mRNA and protein expression levels of scrambled group and the control group (Fig. 4C-E).

RGFs were induced by LPS inflammation following HO-1 gene silencing, and RT-qPCR and WB showed that the mRNA and protein expression of COX-2, TNF-α and RANKL were significantly increased, and there was no statistical difference in the OPG group (Fig. 5A-K). mRNA and protein expression levels of COX-2, TNF-α, RANKL and OPG as well as the RANKL/OPG ratio were not significantly decreased by different concentrations of Oroxylin A (Fig. 5A-K).

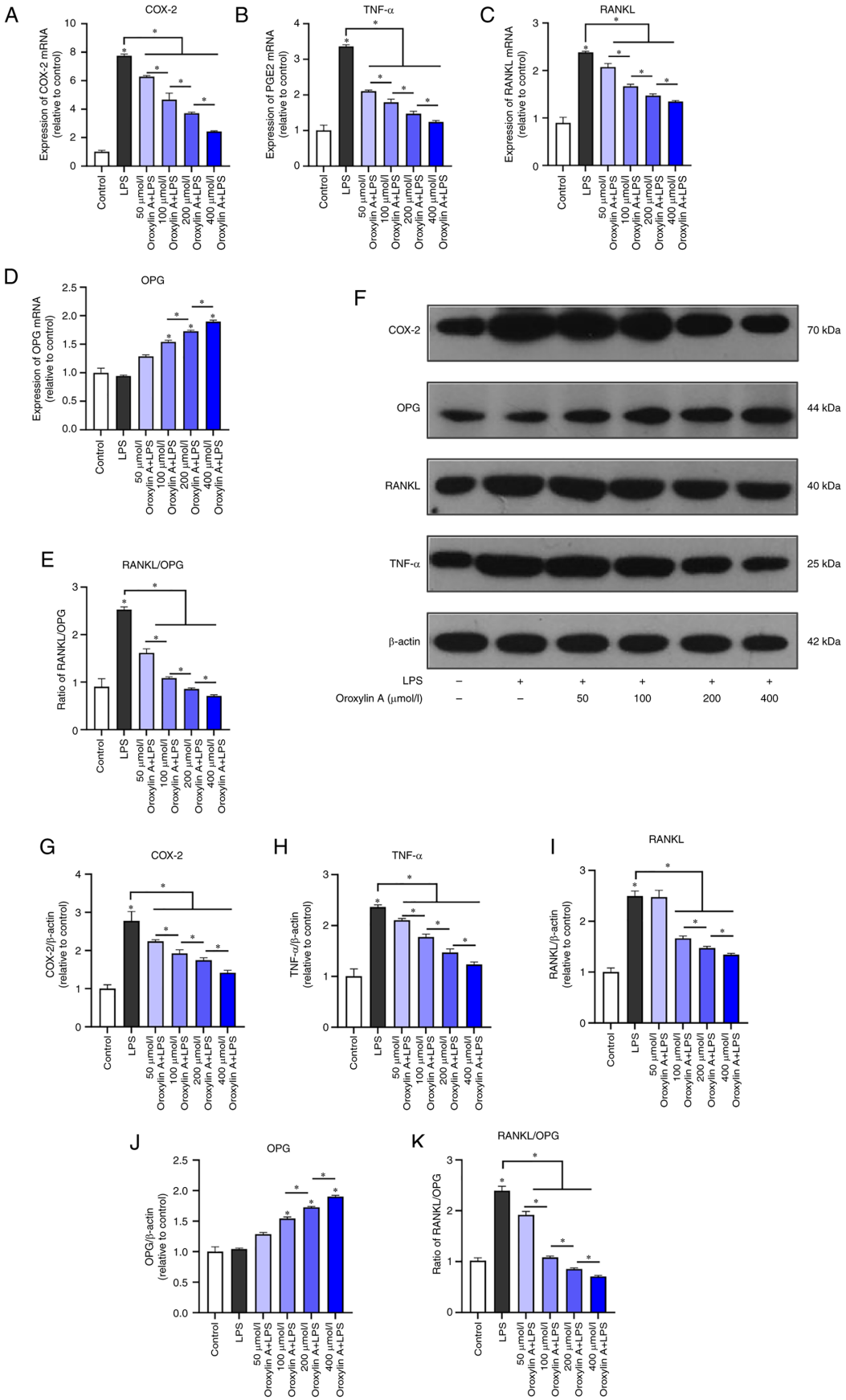

Figure 2. RGFs were infected by LPS and treated with Oroxylin A. Levels of mRNA of (A) COX-2, TNF-α, (B) RANKL, (C) OPG and (D) RANKL/OPG (E) were analyzed by reverse transcription-quantitative polymerase chain reaction. (F) Protein levels of COX-2, TNF-α, RANKL and OPG were analyzed by western blotting. Levels of COX-2, (G) TNF-α, (H) RANKL, (I) OPG and (J) RANKL/OPG (K) between control group, LPS group and different concentration of Oroxylin A groups. The mRNA levels of cytokines in untreated controls were set as 1.0. The blots were stripped and re-probed with β-actin as a loading control. Bars show the levels of cytokines with mean ± SD (n=3). *P<0.05 vs. control. RGF, rat gingival fibroblast; LPS, lipopolysaccharide; COX, cyclooxygenase; OPG, osteoprotegerin.

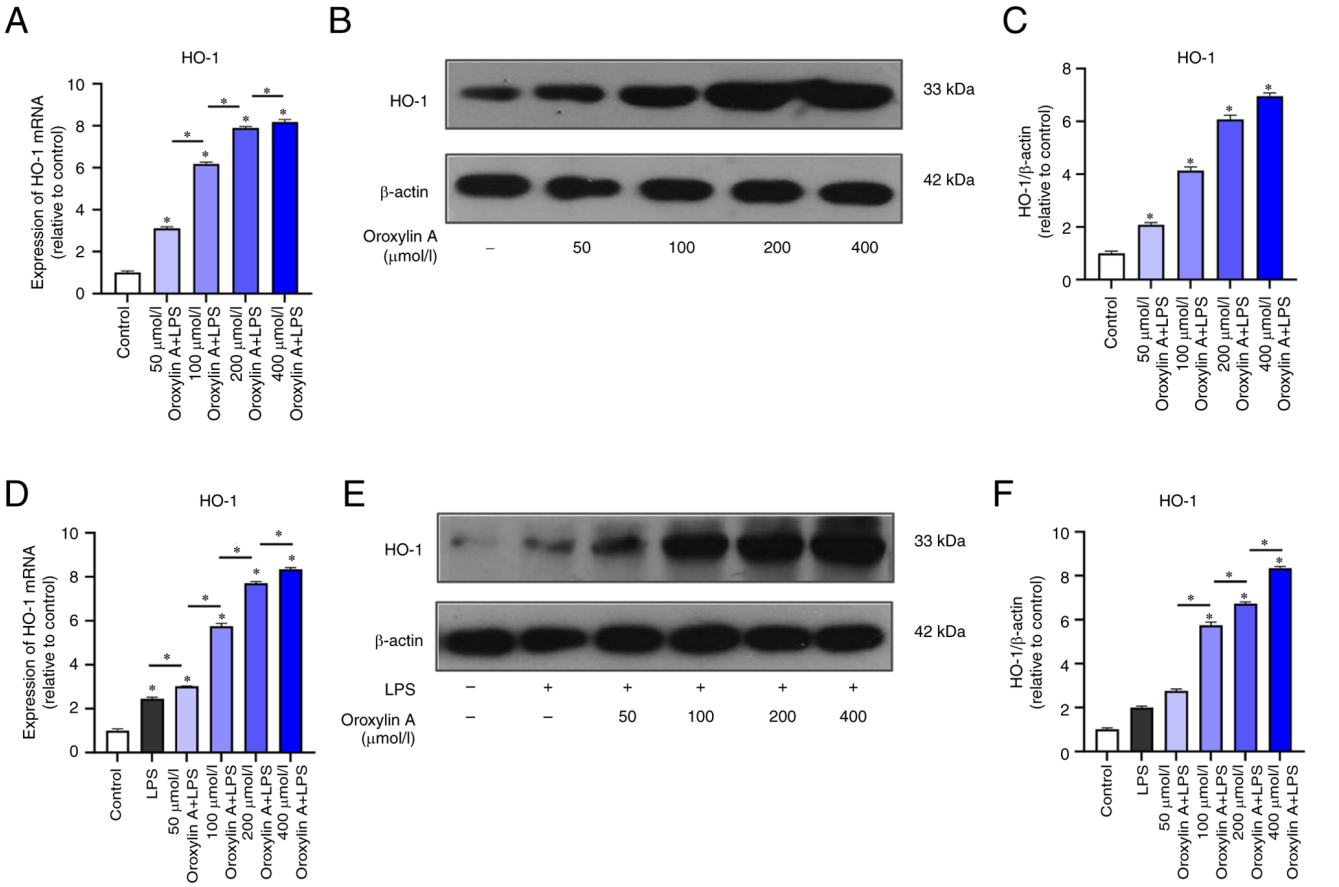

Figure 3. Levels of mRNA and protein of HO-1 in RGFs treated with Oroxylin A. Levels of (A) mRNA and protein (B) of HO-1 in RGFs treated with Oroxylin A. (C) Levels of HO-1 between control group and Oroxylin A groups. (D) Levels of mRNA of HO-1 in RGFs treated with LPS and Oroxylin A. (E) Levels of mRNA of HO-1 in RGFs treated with LPS and Oroxylin A. (F) Levels of HO-1 between control group, LPS group and Oroxylin A groups. RGF, rat gingival fibroblast; LPS, lipopolysaccharide; HO, heme oxygenase. *P<0.05 vs. control.

Effect of Oroxylin A in a rat model of periodontitis. Immunohistochemical results showed that COX-2 was expressed in the periodontal membrane of rats and the positive cells were yellowish brown. Expression of COX-2$^+$ cells in every group was significantly different. The expression of COX-2 in the periodontal tissues of the control and the Oroxylin A treatment group was significantly lower than that of the periodontitis group (Fig. 6A-F) and the expression of COX-2, TNF-α and RANKL/OPG ratio in the periodontal tissue of the Oroxylin A treatment group was slightly higher than that of the control, but the expression level decreased with the increase of the dose (Fig. 6G-L).

Discussion

Traditional Chinese medicine and its extracts have potential in treating periodontitis (21). Oroxylin A is the primary active ingredient in *S. baicalensis*, with anti-inflammatory, antitumor, antioxidant, vascular protection and other pharmacological effects (22,23). Because of these properties, Oroxylin A provides a promising approach for treatment of periodontitis. The purpose of the current study was to investigate the anti-inflammatory effect of Oroxylin A on LPS-stimulated RGFs and the underlying molecular mechanism. In preliminary experiments, *in vitro* CCK-8 assay was used to detect the cytotoxic effect of Oroxylin A on RGFs; there was no significant difference between fibroblasts treated with 0-400 μmol/l Oroxylin A and normal fibroblasts. These results indicate that Oroxylin A has low toxicity on RGFs and has the potential to be applied topically in periodontal tissue.

Techniques commonly employed to identify fibroblasts include morphological observation, immunocytochemical staining, biological characteristic assessment, and molecular biology assay (24,25). Morphological observations via microscopy provide a basic understanding of cell shape and structure but lack specificity. Immunocytochemical staining using antibodies against specific fibroblast markers such as collagen or α-smooth muscle actin can offer higher specificity but may have limitations related to antibody specificity and potential cross-reactivity. Biological characteristic assessment, such as analyzing cell dependency on culture media, proliferation rate and cell cycle progression, could provide functional insights into fibroblast behavior. Similarly, molecular biology techniques such as RT-qPCR or western blotting can confirm the expression of fibroblast-specific genes, offering molecular-level validation (26).

Prostaglandins are synthesized from arachidonic acid by COX (27). The most abundant prostaglandin in the body is TNF-α; COX-2 appears to be the major COX that controls TNF-α synthesis in the inflammatory response (28). Numbers of studies have proved that COX-2 and TNF-α are notably upregulated in the occurrence and development of

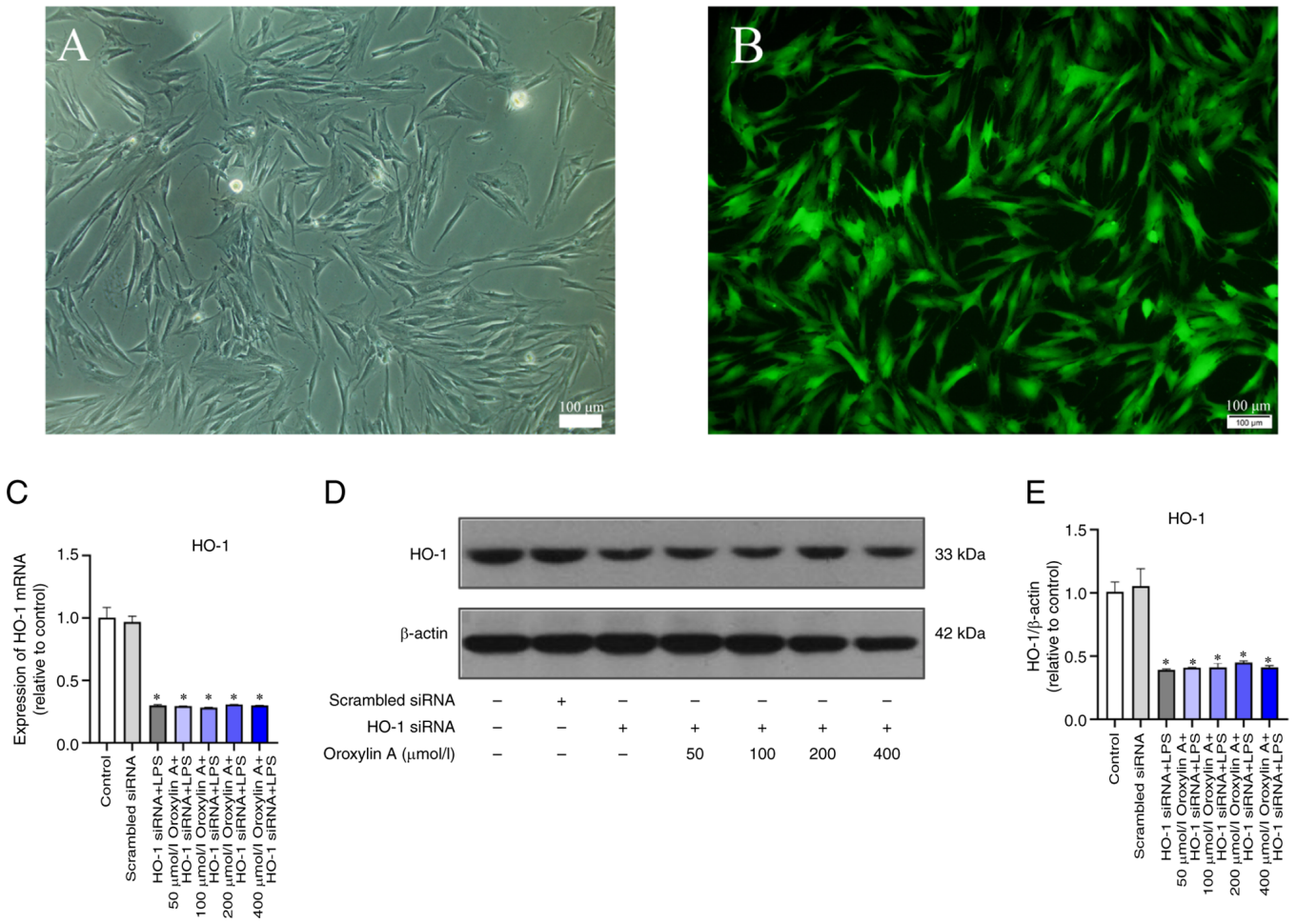

Figure 4. Transfection efficiency. (A) Peripheral cells showed high brightness and good state under microscope. (B) Fluorescence was evenly distributed in the cells. (C) mRNA levels of mRNA of HO-1 were analyzed by reverse transcription-quantitative PCR. (D) Protein levels of HO-1 were analyzed by western blotting. (E) Levels of HO-1 between control group, scrambled siRNA group, LPS group and different concentration of Oroxylin A groups. HO, heme oxygenase; *P<0.05 vs. control.

periodontitis and are involved in the damage of the periodontal tissue, leading to gingival inflammation and alveolar bone resorption (29-31). Therefore, in the present study, COX-2 and TNF-α were selected to detect the effects of Oroxylin A on these cytokines. According to RT-qPCR and western blotting results, Oroxylin A can inhibit production of COX-2 and TNF-α in LPS-induced RGFs suggesting that Oroxylin A may serve a role in regulating the immune response related to periodontal disease.

RANKL is the master regulator of osteoclast differentiation and function, serving an integral role in osteoclast formation (32,33). OPG, a decoy receptor of RANKL, is a key bone protection factor that serves a core role in bone homeostasis (34). A large number of studies have confirmed that upregulation of RANKL, but also the downregulation or degradation of OPG, are related to the loss of periodontal bone, and the RANKL/OPG ratio reflects the level of bone resorption (32,35,36). The present study revealed that Oroxylin A regulated the RANKL/OPG ratio on the mRNA and protein levels and participated in the process of osteogenic repair in periodontitis. Oroxylin A can affect the outcome of periodontitis in both decreasing inflammation and promoting osteogenesis.

HO-1 expression is upregulated in the bone, which may be an important antioxidant defense mechanism (37,38). The present study showed that HO-1 increased with Oroxylin A dose. Therefore, it was hypothesized that Oroxylin A exerted osteogenic and anti-inflammatory properties by upregulating the expression of HO-1. The expression of HO-1 was knocked down, and the mRNA and protein levels of COX-2, TNF-α, RANKL and OPG were detected. Oroxylin A did not decrease expression of these cytokines upregulated by LPS stimulation. This indicates that Oroxylin A relies on HO-1 to play its role and downregulates the expression of inflammatory factors in LPS-stimulated RGFs.

Rankl regulates osteoclasts, serving a key role in bone metabolism (39). Here, a rat model of ligation-induced periodontitis was constructed and Oroxylin A was used to intervene. Oroxylin A could protect the periodontal tissues from damage of COX-2 and TNF-α and decrease the RANKL/OPG ratio at the same time. Ligation promotes plaque accumulation, leading to periodontitis with a pathogenesis similar to human periodontitis (40). Immunohistochemistry showed that Oroxylin A decreased the expression of COX-2 in periodontitis rats. Western blotting showed that COX-2, TNF-α and RANKL/OPG ratio

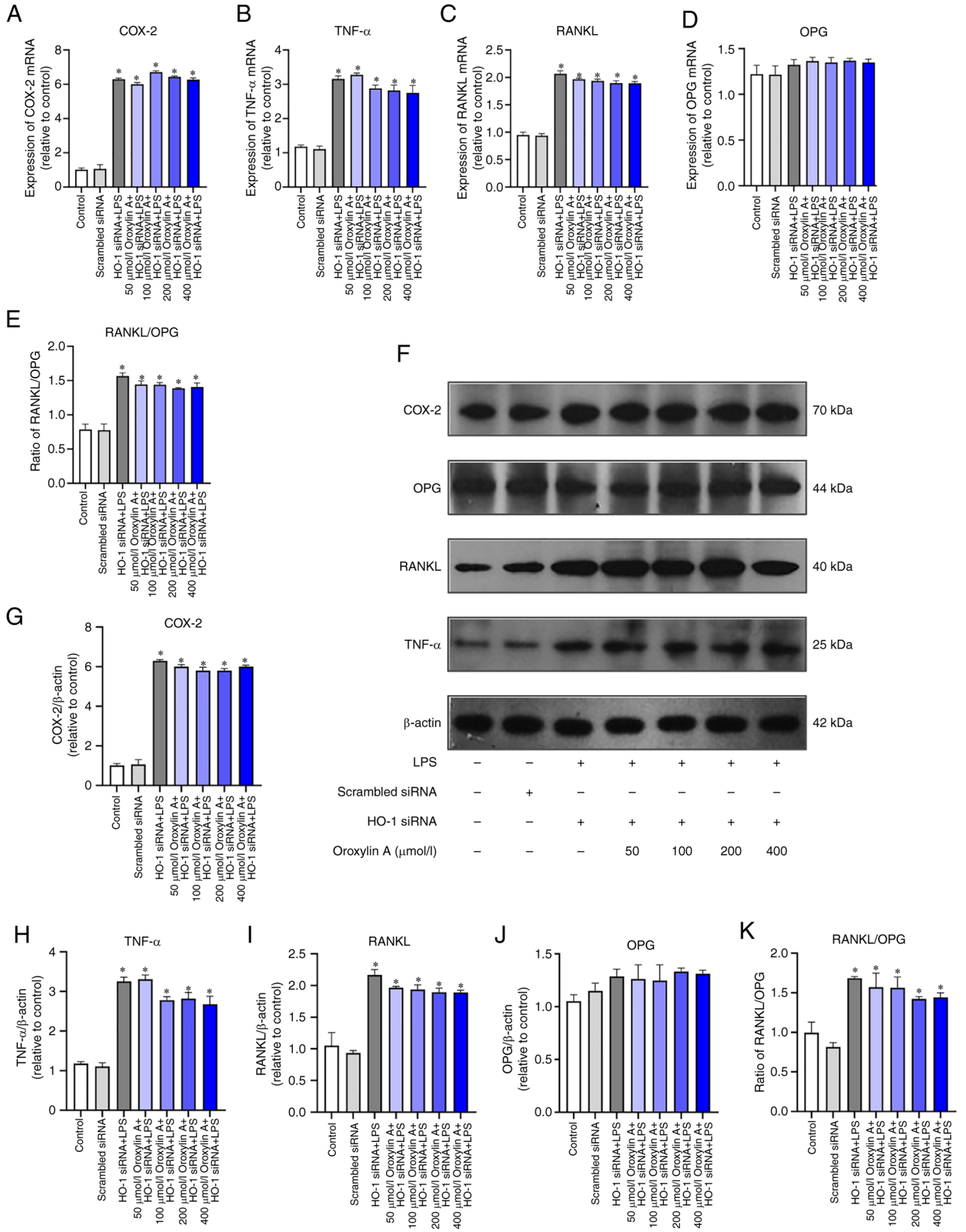

Figure 5. RGFs (HO-1^{-}) infected with LPS and treated with Oroxylin A. Levels of mRNA of COX-2, (A) TNF-α, (B) RANKL, (C) OPG (D) and RANKL/OPG (E) were analyzed by reverse transcription-quantitative PCR. (F) Protein levels of COX-2, TNF-α, RANKL and OPG were analyzed by western blotting. Levels of COX-2, (G) TNF-α, (H) RANKL, (I) OPG and (J) RANKL/OPG (K) between control group, scrambled siRNA group, LPS group and different concentration of Oroxylin A groups. The mRNA levels of cytokines in untreated controls were set as 1.0. The blots were stripped and re-probed with β-actin as a loading control. *P<0.05 vs. control. RGF, rat gingival fibroblast; HO, heme oxygenase; COX, cyclooxygenase; OPG, osteoprotegerin; LPS, lipopolysaccharide; si, small interfering.

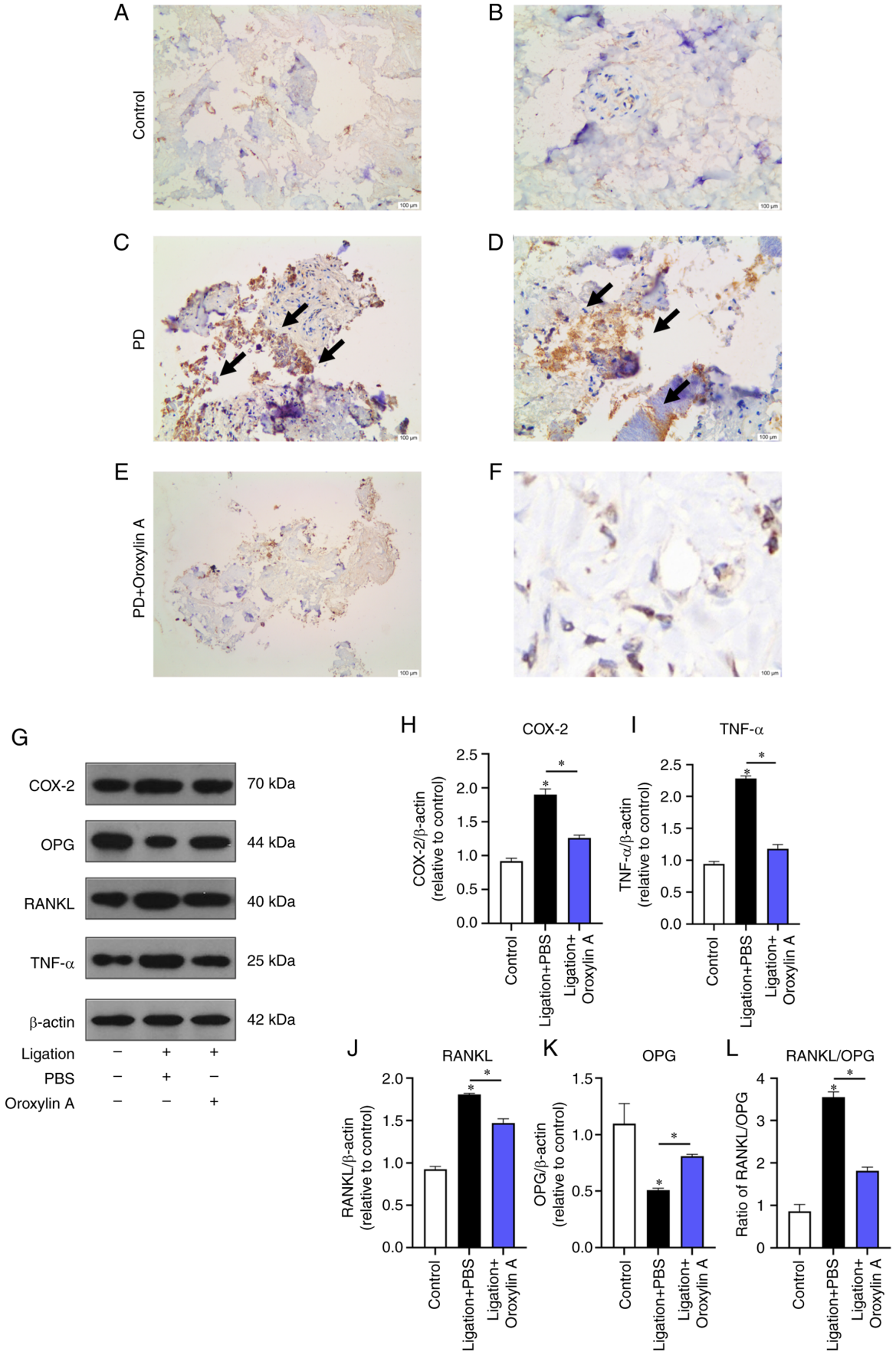

Figure 6. Immunohistochemical expression of COX-2 and the protein expression of COX-2, TNF-α and RANKL/OPG ratio in the control group, the ligation group and the Oroxylin A treatment group. Immunohistochemical results of the expression of COX-2 in control group, (A) in control group, (B) in PD group, (C) in PD group, (D) in PD +Oroxylin A group, (E) in PD+ Oroxylin A group (F). (G) Protein levels of COX-2, OPG, RANKL and TNF-α in gums. Levels of COX-2, (H) TNF-α, (I) RANKL(J), OPG and (K) RANKL/OPG (L) between three groups of PBS, ligation +PBS, ligation +PBS +Oroxylin A. COX, cyclooxygenase; OPG, osteoprotegerin. *P<0.05 vs. control.

were all downregulated in the periodontal tissue of rats with periodontitis. A number of studies have confirmed that Oroxylin A inhibits cellular inflammation, as well as osteoarthritis, respiratory inflammation and skin tumors (9,41,42). Oroxylin A was here applied to the treatment of periodontitis, providing novel potential options for the treatment of periodontitis in the future.

The present study investigated the effect of Oroxylin A on HO-1 expression to verify whether Oroxylin A inhibits inflammatory cytokines in periodontitis via HO-1. Oroxylin A did not induce expression of inflammatory cytokines in the case of HO-1 knockdown, demonstrating that Oroxylin A inhibiting inflammatory cytokines in periodontitis via HO-1. In conclusion, Oroxylin A decreased expression of inflammatory cytokines in LPS-induced RGFs and has a good inhibitory effect on periodontitis in rats, providing new drug options for the treatment of periodontitis.

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Availability of data and materials

The data generated in the present study are included in the figures and tables of this article.

Authors' contributions

TW, ZW and CJ performed experiments and wrote the article. YZ and LT analyzed and interpreted data and wrote the manuscript. JF and XX conceived and designed the study and revised the manuscript. All authors have read and approved the final manuscript. TW and JF confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee for the Welfare of Experimental Animals of the Affiliated Hospital of Qingdao University (approval no. AHQU-MAL 20210326).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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