Original Article

BRAZILIN MEDIATES THE HUMAN PERIODONTAL LIGAMENT STEM CELLS FOR PERIODONTITIS TREATMENT THROUGH THE YAP SIGNALING PATHWAY

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Abstract

Objective: To explore the effects of *Brazilin* on the Human periodontal ligament stem cells (hPDLSCs) mediating periodontal tissue regeneration in periodontitis and the Yes-associated protein (YAP) pathway for periodontitis. **Methods**: The anti-inflammatory effects and osteogenic effects of *Brazilin* in the inflammatory microenvironment were mainly evaluated by polymerase chain reaction (PCR), western blot (WB), staining, and the blocking and deletion of the YAP pathway *in vitro* by verteporfin (VP) and shRNA to evaluated the effect on the YAP pathway. A rat periodontitis model was established and the therapeutic effects were evaluated by Micro-CT, hematoxylin and eosin (HE) staining, Masson staining, and immunohistochemistry. **Results**: *Brazilin* effectively down-regulated the expression of inflammatory factors, showed deeper staining, and showed up-regulation of related osteogenic factors, and inhibition of the YAP pathway is followed by inhibition of the osteogenic effects of *Brazilin*. *Brazilin* was effective in reducing inflammation and inhibiting alveolar bone loss in the rat periodontitis model. **Conclusion**: *Brazilin* can promote periodontal tissue regeneration in periodontitis through the YAP pathway, providing new ideas for the treatment of clinical periodontitis.

Keywords: *Brazilin*, human periodontal ligament stem cells, periodontitis, Yes-associated protein.

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Introduction

Periodontitis is an inflammatory and destructive disease of periodontal support tissue that ranks first in the causes of loss of teeth in adults (Peres *et al.*, 2019). Moreover, accumulating data links periodontal disease to systemic diseases, including adverse pregnancy outcomes, cardiovascular disease, cancer, and diabetes (Choi and Hyun, 2020; Heikkilä *et al.*, 2018). Therefore, preventing or treating periodontal disease is important for improving oral health and averting systemic diseases.

Traditional treatment methods for periodontitis cannot achieve the ideal regeneration of periodontal tissue defects. Human periodontal ligament stem cells (hPDLSCs) possess characteristics of multipotential differentiation and immunomodulation, and hPDLSCs-mediated periodontal tissue regeneration is now regarded as a promising method for periodontitis treatment (Yan *et al.*, 2018). However, previous studies demonstrated that hPDLSCs differentiation and periodontal tissue regeneration ability declined in the inflammatory microenvironment (Zhao *et al.*, 2020). Therefore, how to regulate the inflammatory response and promote periodontal regeneration is an urgent problem to be solved.

The knowledge that periodontitis is a chronic inflammatory disease provides us with the option of using anti-inflammatory agents as therapeutic strategies. Preventing periodontitis by using nonsteroidal anti-inflammatory drugs has been indicated to be effective. However, nonsteroidal anti-inflammatory drugs cause adverse effects, including gastrointestinal effects, renal effects, hemostatic effects, and hypersensitivity reactions, as well as other symptoms including headaches, dizziness, and vertigo (Preshaw, 2018). Natural compounds have been used for the pre-

vention and treatment of periodontitis, receiving extensive attention and research. They are considered more suitable for long-term use than chemical synthetic drugs due to their fewer adverse reactions (Elburki *et al.*, 2014). *Caesalpinia sappan L* (*C. sappan*), commonly known as Sumu in China, is a famous herbal medicine. Its heartwood has been traditionally used for various diseases, such as trauma and stasis pain, attributed to its effects of, anti-inflammation, dissipating stasis, and easing pain (Nirmal *et al.*, 2015). *Brazilin* is a major homoisoflavone isolated from *C. sappan* that is used as marker components for the quality control of *C. sappan* in the current version of the China Pharmacopoeia (14-yaodian). In traditional Chinese medicine, *Brazilin* is used for the treatment of increased blood circulation, promotes menstruation, and exhibits analgesic and anti-inflammatory potentials (Li *et al.*, 2017), however, whether *Brazilin* can reverse the adverse effects of inflammation on hPDLSCs is unknown.

Yes-associated protein (YAP) is a transcriptional coactivator that is regulated by the Hippo signaling pathway, which plays an important role in promoting tissue regeneration and maintaining the self-renewal of stem cells (Jia *et al.*, 2019). Pan proved that bone marrow stem cells (BMSCs) of YAP knockout mice owned lower osteogenic differentiation ability (Pan *et al.*, 2018). More importantly, the YAP pathway is closely related to hPDLSCs and can be involved in proliferation and apoptosis to regulate its biological behavior (Jia *et al.*, 2018). Regulating YAP expression and thus hPDLSCs to promote periodontitis may be an effective approach. A study has shown that flavonoid drugs bind to and inhibit the expression of YAP (Li *et al.*, 2019), suggesting that YAP may be the pathway by which *Brazilin* regulates hPDLSCs.

Taken together, the aim of this paper is to explore the effects of *Brazilin* on the osteogenic differentiation of hPDLSCs under inflammatory conditions and to further explore its therapeutic effect *in vivo* by establishing a rat periodontitis model. Whether YAP plays a role in this process is the focus of this study.

Materials and Methods

Isolation and Culture of hPDLSCs

The study protocol was approved by the Medical Ethics Committee of the Affiliated Stomatology Hospital of Kunming Medical University, China (KYKQ2021MEC025). All participating patients signed a written informed consent form. Briefly, periodontal tissues were gently separated from the middle third of the molar root and digested in a solution of 4 mg/mL type II dispase (Sigma, St Louis, MO, USA) and 3 mg/mL type I collagenase (Gibco, Grand Island, NE, USA) for 30 min at 37 °C. The obtained cells were cultured in a basic medium (α-MEM supplemented with 10 % FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin) and incubated at 37 °C in 5 % CO_2.

Identification of hPDLSCs

The characteristics of hPDLSCs were examined by detecting the colony formation efficiency, differentiation ability, and phenotypic molecular markers of mesenchymal stem cells. The cells (500) were cultured, and the aggregation of more than 50 cells was identified as a colony. The osteogenic induction medium consisted of a basic medium containing 50 μg/mL vitamin C (Sigma, St Louis, MO, USA) and 10 mM β-glycerophosphate (Solarbio, Beijing, China). The adipogenic induction medium consisted of a basic medium containing 2 μM dexamethasone, 0.2 mM indomethacin, 0.01 mg/mL insulin, and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma, St Louis, MO, USA). For chondrogenic differentiation, 6×10^5 cells were centrifuged and cultured in a chondrogenic differentiation medium (Cyagen, Santa Clara, CA, USA). Mineral nodules, lipid droplets, and chondrogenic cells pellets were detected by Alizarin Red Staining (ARS), Oil Red O staining, and alcian blue, respectively. Mesenchymal markers, including CD29, CD44, CD34, and CD45 (BD Biosciences, Milpitas, CA, USA), were detected by flow cytometry.

Cells Viability and Proliferation Assay

Brazilin (molecular weight 286.29, purity ≥98 %, purchased from (Solarbio, Beijing, China) was dissolved in dimethyl sulfoxide (DMSO, the final concentration of DMSO ≤0.1 % v/v). DMSO was used as the vehicle control in the experiments. hPDLSCs were seeded in 96-well plates at a density of 2×10^3 cells/well. After 24 hours, hPDLSCs were then cultured in a basic culture medium with various concentrations of *Brazilin* (2.5, 5, 10, 20, 40, and 80 μM) for 1, 3, 5, and 7 d. Furthermore, to investigate the effect of 5 μM *Brazilin* on hPDLSC proliferation in the lipopolysaccharide (LPS)-induced inflammatory microenvironment, hPDLSCs were treated with 10 μg/mL LPS (InvivoGen, San Diego, CA, USA) and 5 μM *Brazilin* for 1, 3, 5, and 7 d. A cell-counting kit-8 (CCK8, Meilune, Dalian, China) solution was added, and the absorbance was detected.

Alkaline Phosphatase (ALP) Staining and Alizarin Red Staining (ARS) Assay

Cells were counted and seeded into 6-well plates at a density of 2×10^5 per well. When the cells growth fused to 90 %, each group was replaced with an osteogenic induction medium, different concentrations of *Brazilin* were added to each group for intervention, and a complete change of fluid was carried out every 3 days. The culture was terminated on day 14, BCIP/NBT ALP staining kit (Beyotime, Shanghai, China) and alizarin red staining kit (Cyagen, Santa Clara, CA, USA) was performed according to the instructions. To investigate the osteogenic properties in the inflammatory microenvironment, 10 μg/mL LPS and (or) 5 μM *Brazilin* were co-cultured in the medium, followed by the staining

described above. Observe under an inverted microscope and photographs were taken.

Real-Time Polymerase Chain Reaction (RT-PCR)

To examine the effects of *Brazilin* on the mRNA expression of inflammatory mediators, hPDLSCs were treated with 10 μg/mL LPS for 24 h, followed by treatment with 2.5, 5, and 10 μM *Brazilin* for 24 h (coincubation). To examine the effects of *Brazilin* on the mRNA expression of osteogenic markers, an osteogenic induction medium with various *Brazilin* concentrations (2.5, 5, and 10 μM) was used to culture hPDLSCs for 7 and 14 d. Furthermore, the impacts of 5 μM *Brazilin* on osteogenic markers in inflammatory microenvironments were investigated. An osteogenic induction medium was used with 5 μM *Brazilin* and 10 μg/mL LPS to culture hPDLSCs for 7 and 14 d.

The RNA of treated cells was isolated using the Eastep® Super Total RNA Extraction Kit (Promega, Fitchburg, WI, USA) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara, Kyoto Prefecture, Japan) at 37 °C for 15 min and inactivated at 85 °C for 5 s. RT–PCR was performed using SYBR qPCR Master mix (Vazyme, Nanjing, China). During PCR, a dissociation curve was constructed in the range of 60 °C to 95 °C, and the cycling parameters of PCR were followed: 1 cycle for 30 s at 95 °C, 40 cycles for 10 s at 95 °C, and 30 s at 60 °C. Gene expression was presented using $2^{-\Delta\Delta Ct}$ values, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control.

Western Blot (WB) Analysis

To examine the anti-inflammatory effects of *Brazilin* in LPS-induced hPDLSCs, WB analysis was performed to quantify the protein expression levels of inflammatory mediators. Cells were lysed using RIPA buffer containing 1 % protease inhibitor (Solarbio, Beijing, China). BCA protein assay kit (Beyotime, Shanghai, China). Protein samples (20 μg/lane) were loaded on a 12 % SDS-polyacrylamide gel, separated, and then transferred to polyvinylidene difluoride (PVDF) membranes (Biosharp, Hefei, China), which were blocked with QuickBlock™ Blocking Buffer (Beyotime, Shanghai, China) for 1 hour at room temperature. Then, the membrane was further incubated overnight at 4 °C with primary antibodies against GAPDH, Interleukin-8 (IL-8), Matrix Metalloproteinases 1 (MMP1), and Matrix Metalloproteinases 3 (MMP3), all were purchased from Proteintech, Wuhan, China. The membrane was then incubated with an HRP-conjugated secondary antibody (Proteintech, Wuhan, China) for 1 h. The proteins were visualized using an imaging system.

Animal Experiments

All experimental procedures involving animals were reviewed and approved by the Ethics Committee of Kunming Medical University. A total of 30 6-week-old male Wistar rats (SD rats) with weighing 200 g on average were kept for 1 week under standard laboratory conditions to adapt to the environment. All studies involving animals were strictly adhered to the ARRIVE guidelines and the National Research Council's Guide for the Care and Use of Laboratory Animals. Periodontitis was induced with ligation silk over the second molar of the maxilla for a month. Then, the right maxillaries (N = 5) were scanned by Micro-CT to evaluate the completion of the experimental periodontitis model. The study contains 5 groups: (1) control, (2) periodontitis group (initially ligated rat only), (3) solvent group (initially ligated rat with solvent), (4) low *Brazilin* group (initially ligated rat with 5 μM *Brazilin*), and (5) high *Brazilin* group (initially ligated mice with 10 μM *Brazilin*). After a month, the maxillaries were fixed for Micro-CT evaluation, histological staining, Masson's trichrome staining, and immunohistochemical (IHC) staining.

Micro-CT and Analysis

The maxillary bone samples of the SD rats were trimmed and placed into a 4 % paraformaldehyde fixative solution for 24 h. The next day, the samples were collected and prepared for micro-CT scanning with a Skyscan 1176 scanner (Bruker, Karlsruhe, Germany). The scanning parameters were as follows: a rotation angle of 360°, a tube voltage of 70 kV, a tube current of 353 μA, an X-ray exposure time of 404 ms, and a scanning layer thickness of 18 μm. For the assessment of alveolar bone loss, the distance between the cementoenamel junction and the alveolar bone crest (CEJ-ABC) for the distal buccal root of the first molars was measured in 3D images viewed from the buccal side. The measurements were repeated three times per site, and mean distances in millimeters were obtained.

Hematoxylin and Eosin (HE) Staining and Masson's Trichrome Staining

The right maxillaries were collected and fixed in 4 % paraformaldehyde for 48 h and then placed into 10 % EDTA decalcifying solution for 1 month. The samples were dehydrated with a series of graded ethanol solutions and embedded in paraffin wax. The embedded maxillaries were cut in a buccal-lingual direction to acquire transverse sections (5 μm thick). The prepared sections were stained with HE and Masson's trichrome staining.

Immunohistochemical (IHC) Staining

After deparaffinized by xylene and hydration using graded alcohol, antigen repair was conducted using citrate antigen retrieval solution (P0081, Beyotime, Shanghai, China) at 100 °C for 15 min. Sections were treated with H_2O_2 for 30 min, then blocked for 1 h (β-catenin) or 2 h. Diluted primary antibodies (β-catenin:1/100, ab32572 and RUNX2:1/400, ab76956, Abcam, Cambridge, UK) were

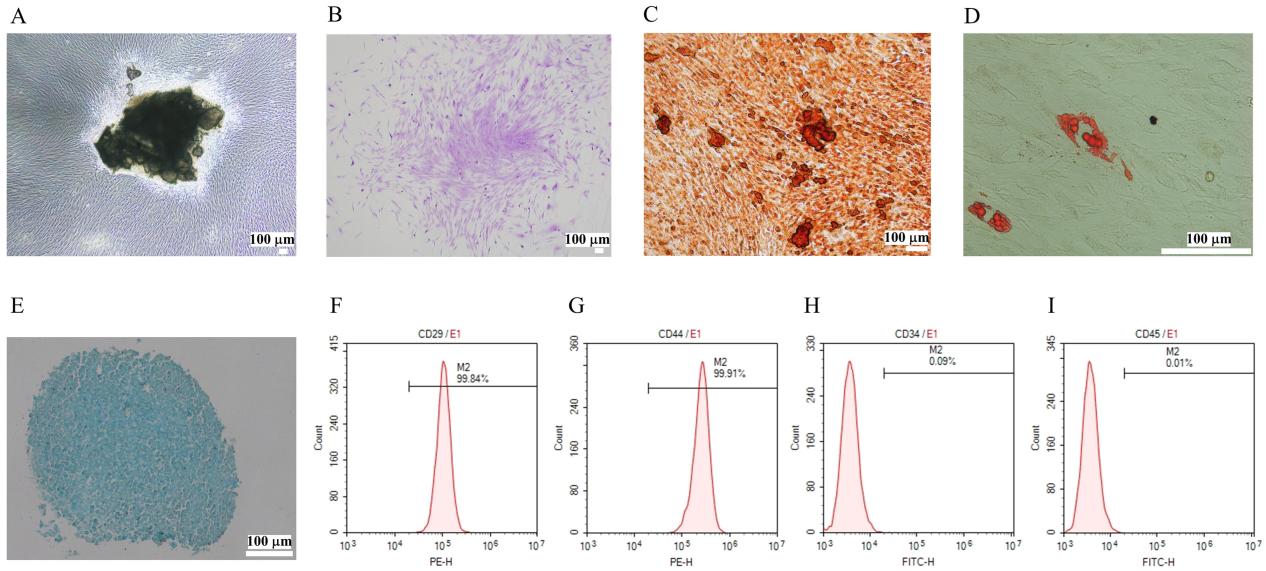

Fig. 1. Human periodontal ligament stem cells (hPDLSCs) characterization. (**A**) Primary hPDLSCs grew around the tissue mass and multiplied (scale bar, 100 μm). (**B**) Colonies formed by hPDLSCs (scale bar, 100 μm). (**C**) Osteogenic differentiation of hPDLSCs was demonstrated as red mineralized nodules (scale bar, 100 μm). (**D**) Adipogenic differentiation of hPDLSCs was demonstrated as red oil drops (scale bar, 100 μm). (**E**) Chondrogenic cell pellets (scale bar, 100 μm). (**F**–**I**) hPDLSCs were positive for CD 29 (99.84 %) and CD 44 (99.91 %) and negative for CD 34 (0.09 %) and CD 45 (0.01 %).

used to incubate the sections at 4 °C overnight. After washing with phosphate-buffered saline (PBS) five times (3 min each time), the tissue slices were incubated with secondary antibody (1:200) for 2 h and horseradish peroxidase for 30 min at room temperature. Proteins were visualized by the DAB solution kit (Vectorlabs, Newark, CA, USA).

The Impact of Brazilin on YAP in Vitro

Verteporfin (VP), a YAP pathway inhibitor, was selected to inhibit YAP. The hPDLSCs were cultured in a medium with or without Verteporfin. After seven days of induction, hPDLSCs were harvested and protein expression was analyzed by Western blot analysis. To demonstrate the role of YAP in the osteogenic differentiation of hPDLSCs, YAP was stably silenced by short hairpin RNA (shRNA) duplex oligo targeting YAP (pEF-1aF-GFP-puro) packaged to lentiviruses by Genechem Company (Shanghai, China). After 72 h, the transfection reagents-containing medium was changed to a normal culture medium.

Immunofluorescence Staining

After washing with PBS, the cells were fixed with paraformaldehyde for 10 min and then permeated with 1 % Triton X-100 for 10 min. Then, the samples were blocked with 5 % BSA for 30 min at room temperature. Subsequently, the samples were incubated with primary antibodies (YAP; 1:200; Cell Signaling Technology, Boston, MA, USA) at 4 °C overnight. Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:300; Abcam, Cambridge, UK) was incubated with the samples for 1 h at 37 °C in a dark room. The nuclei were stained with 4′,6-diamidino-phenylindole (DAPI; 1:500; Solarbio, Beijing, China). Images were taken with an inverted fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Statistical Analysis

All statistical analyses were calculated using GraphPad Prism 8.0.2 (GraphPad Software, Inc., San Diego, CA, USA). The data were analyzed using ANOVA and post hoc tests using the Dunnett test. In addition, the data are presented as the mean ± SD. The results were considered statistically significant with a p value; $p < 0.05$ was considered statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

Results

Characterization of hPDLSCs

hPDLSCs exhibited typical long spindle-shaped morphology and were arranged in a vortex, as observed by an inverted microscope (Fig. 1**A**). Colony formation assays confirmed that hPDLSCs were able to form cells clusters (Fig. 1**B**). The osteogenic/adipogenic/chondrogenic differentiation assay detected mineralized nodules, lipid droplets, and chondrogenic cells pellets after induction (Fig. 1**C**–**E**). Flow cytometry analyses showed that these cells were highly positive for the mesenchymal stem cell-positive markers CD 29 (95.92 %) and CD90 (98.48 %) but were negative for CD 34 (1.02 %) and CD 45 (0.98 %) (Fig. 1**F**–**I**). Taken together, these results confirmed that the isolated hPDLSCs were of mesenchymal origin and had powerful multipotency and self-renewal ability.

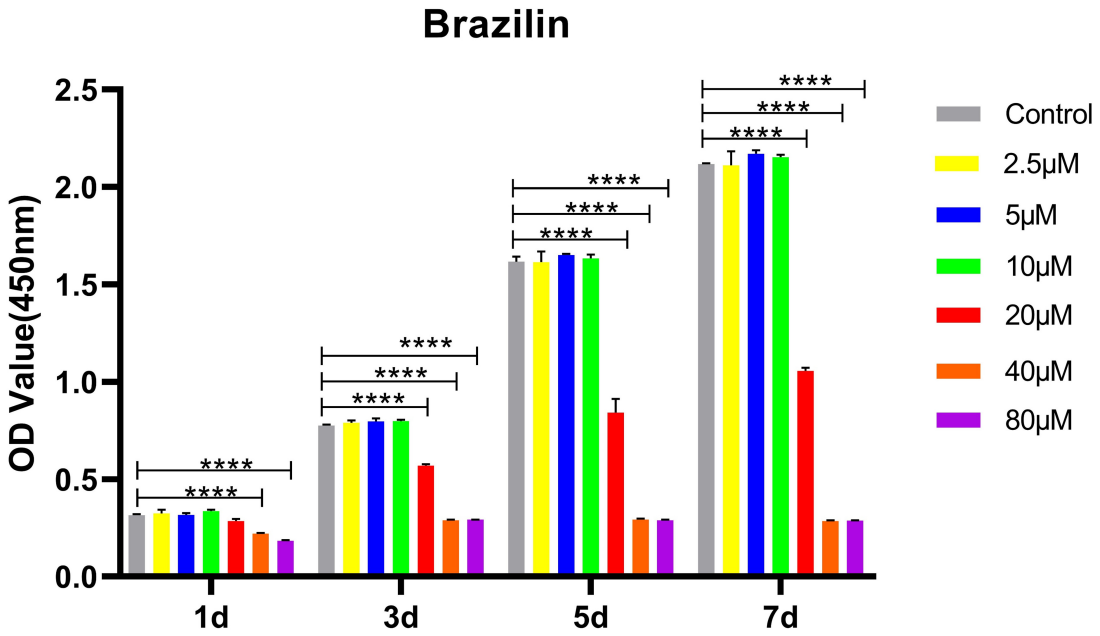

Fig. 2. Effects of *Brazilin* on cell viability. Cells were cultured with *Brazilin* at final concentrations of 2.5, 5, 10, 20, 40, and 80 μM for 1, 3, 5, and 7 d. OD, Optical Density; ****$p < 0.0001$.

Effects of Brazilin on Cells Viability

It was observed that hPDLSCs treated with 20 to 80 μM *Brazilin* exhibited smaller cells numbers from Day 3 compared with the control, *Brazilin* at 2.5 μM, 5 μM, and 10 μM exerted no significant cytotoxic effects on the viability of hPDLSCs from Days 1 to 7 (Fig. 2). Therefore, *Brazilin* at these concentrations was used in the subsequent study.

Effects of Brazilin on the Osteogenic Differentiation of hPDLSCs

As shown in Fig. 3**A**, the ALP staining results showed that the staining areas in the *Brazilin* group were significantly larger than those in the control group and osteogenic induction medium (OIM) group, treatment of hPDLSCs with *Brazilin* (2.5 μM, 5 μM, 10 μM) resulted in increased mineral nodules compared to the control group and OIM group according to the ARS. As presented in Fig. 3**B**, mRNA levels of osteogenic markers in hPDLSCs were enhanced compared to the expression in the control group after 7 and 14 d of osteogenic induction. When treated with 5 μM *Brazilin*, the mRNA expression of Runt-related transcription Factor 2 (*RUNX2*), Osterix (*OSX*), Osteocalcin (*OCN*), and Collagen type I (*COL1*) was enhanced compared to the expression in the OIM group on Day 14.

Effects of Brazilin on Inflammatory Mediator Production in Pg-LPS-Treated hPDLSCs

As Fig. 4 shows inflammatory mediators were significantly upregulated by 10 μg/mL Pg-LPS stimulation compared with the control group. Treatment with *Brazilin* at different concentrations caused a great decrease in IL-8 production compared with that of the LPS group. *Brazilin* inhibited IL-8 release in a dose-dependent manner with 10 μM Brazilian having the best inhibitory effect. *Brazilin* also had inhibitory effects on MMP1 and MMP3. The production of MMP1 was significantly inhibited by 5 μM *Brazilin*, and the expression of MMP3 was significantly inhibited by 2.5 μM, 5 μM, and 10 μM Brazilian with 5 μM *Brazilin* having the best inhibitory effect.

Effect of Brazilin in Promoting Osteogenic Differentiation in Inflammatory States

Following osteogenic induction for 14 d or 21 d, the hPDLSCs were positively stained by ALP or ARS (Fig. 5**A**). However, the ability of the cells to undergo osteogenic differentiation was largely impaired by the addition of LPS, whereas *Brazilin* was able to rescue the inflammation-induced reduction in osteogenesis. Similarly, the protein levels of OSX, and Osteopontin (OPN) in hPDLSCs and the mRNA levels of osteogenic genes (including *ALP*, *COLI*, *OSX*, and *OCN*) were significantly decreased compared with those under normal conditions at Days 7 (Fig. 5**B**,**C**). However, *Brazilin* cotreatment increased the expression of osteogenic genes that were damaged by LPS.

Micro-CT Analysis

As shown in Fig. 6**A**, the Micro-CT images showed that significant loss of alveolar bone occurred in the periodontitis group compared to the black group, the solvent group exhibited a similar degree of loss, and there was a reduction in the degree of alveolar bone loss in the *Brazilin* group. As illustrated in Fig. 6**B**, the CEJ-ABC

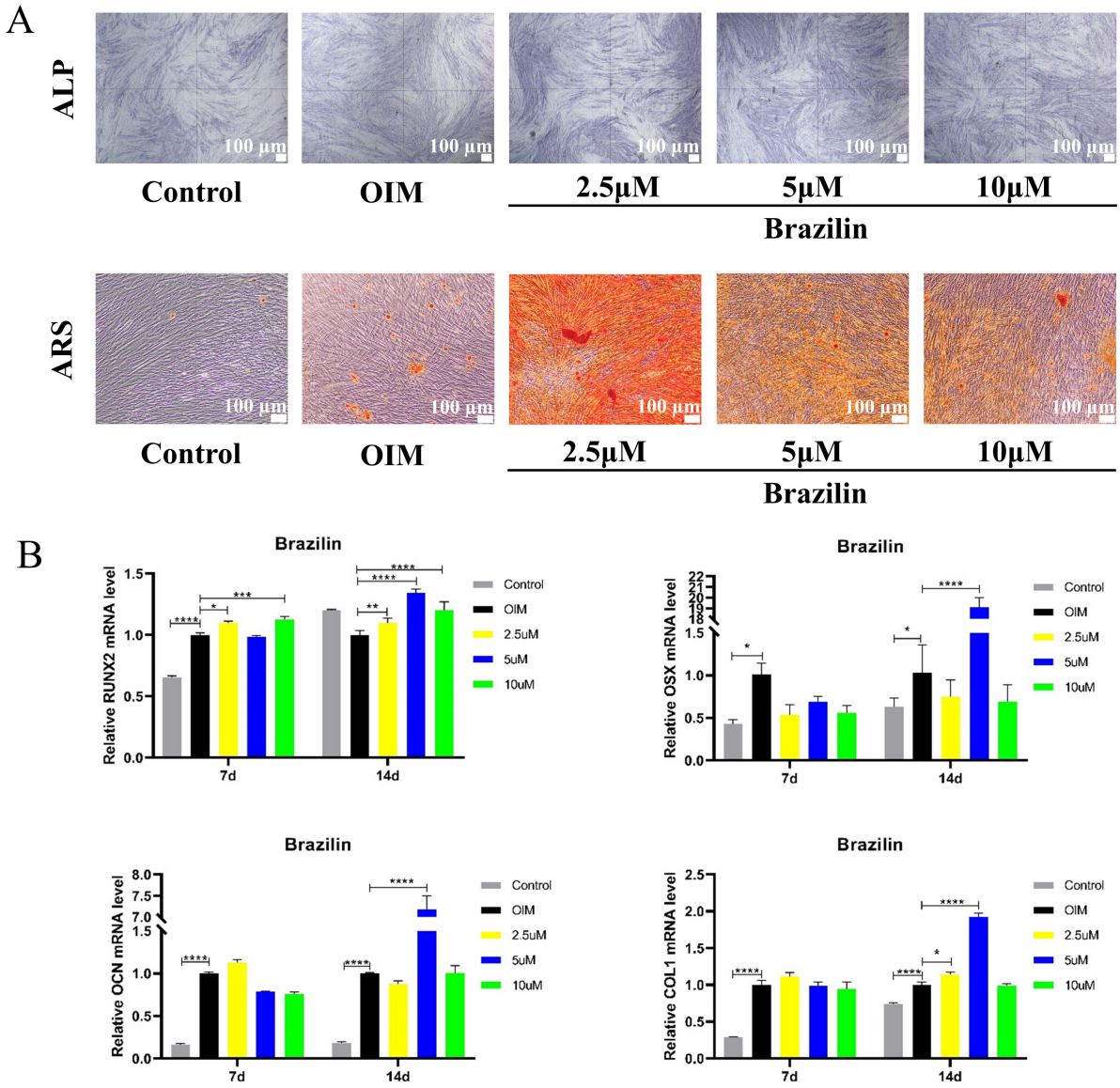

Fig. 3. Effects of *Brazilin* on the osteogenic differentiation of hPDLSCs. (**A**) Representative images of Alkaline Phosphatase (ALP) and Alizarin Red Staining (ARS) staining (14 d, scale bar, 100 μm). (**B**) The expression of Runt-related transcription Factor 2 (*RUNX2*), Osterix (*OSX*), Osteocalcin (*OCN*), and Collagen type I (*COL1*) was detected by Real-Time Polymerase Chain Reaction (RT-PCR) at 7 and 14 days after 5 μM *Brazilin* treatment. Data are expressed as the mean ± SEM from three independent experiments performed in triplicate. OIM, osteogenic induction medium; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

distance was almost two times longer in the periodontitis group than in normal control (NC) group, indicating the successful establishment of the periodontitis model. Notably, compared to the solvent group, the administration of the *Brazilin* group significantly reduced the CEJ-ABC distance in a dose-dependent manner, suggesting that *Brazilin* can inhibit the loss of alveolar bone *in vivo*.

Histological Analysis

Hematoxylin and eosin (HE) staining was performed to further histologically evaluate periodontium status in all groups (Fig. 6**A**). There was an apparent increase in the numbers of inflammatory cells in the periodontitis group and solvent group, indicating the status of persistent inflammation. In contrast, the treatments with a low *Brazilin* group and high *Brazilin* group decreased the number of infiltrated inflammatory cells, mitigated the inflammation, and improved the alveolar bone morphology to various degrees in the periodontitis. Newly formed bone was observed with Masson's trichrome staining in the low *Brazilin* group and high *Brazilin* group (Fig. 6**A**). Moreover, we confirmed that mineralized alveolar bone had formed in-

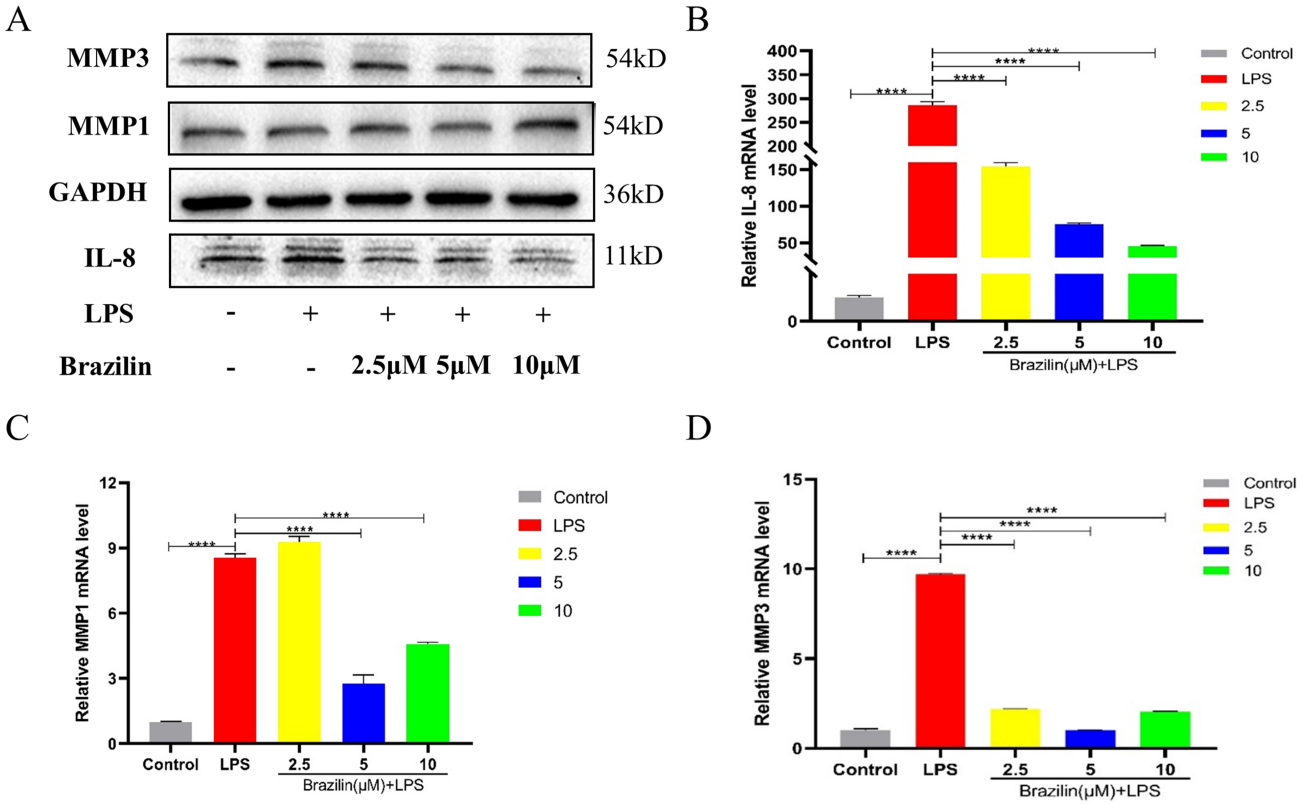

Fig. 4. Effects of *Brazilin* on inflammation mediators. (**A**) The effects of *Brazilin* (2.5 μM, 5 μM, 10 μM) on Interleukin-8 (IL-8), Matrix Metalloproteinases 1 (MMP1), and Matrix Metalloproteinases 3 (MMP3) expression were detected by Western Blot (WB) analysis. Expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (**B**–**D**) The effects of *Brazilin* (2.5 μM, 5 μM, 10 μM) on IL-8, MMP1, and MMP3 expression were detected by RT–PCR. Data are expressed as the mean ± SEM from three independent experiments performed in triplicate. LPS, lipopolysaccharide; ****$p < 0.0001$.

side that was treated with *Brazilin*. Immunohistochemical staining was conducted to further evaluate the inflammation condition and restoration of the alveolar bone condition. The staining sections showed that periodontitis induced the highest level of MMP1-positive cells in rats and the reduced amount of MMP1 was observed in the low *Brazilin* group and high *Brazilin* group, among which the low *Brazilin* group accomplished the most favorable outcome, the osteogenic marker OCN was increased in low and high *Brazilin* group (Fig. 6**C**).

Blocking YAP Attenuated the Brazilin-Induced Promotion of Osteogenesis of hPDLSCs

To elucidate the role of the YAP pathway in *Brazilin*-induced osteogenic promotion of hPDLSCs under inflammatory conditions, the specific inhibitor, VP, was used to block the YAP pathway. As the results were shown in Fig. 7**A**, we found that the LPS+VP+*Brazilin* groups repressed the *Brazilin*-enhanced ALP staining of hPDLSCs. ARS staining also revealed that the LPS+VP+*Brazilin* groups inhibited the enhanced *in vitro* mineralization by *Brazilin*. Similarly, verteporfin also partially blocks the osteogenic promotion of *Brazilin* in the inflammatory environment. As shown in Fig. 7**B**, *Brazilin* upregulated the protein levels of RUNX2, OPN, and OCN in hPDLSCs under inflammatory conditions. However, the positive effect of *Brazilin* on LPS-induced inflammation diminished in the YAP knockdown groups, indicating that *Brazilin* promotes osteogenic differentiation of hPDLSCs via YAP under inflammatory conditions. In the immunofluorescence staining results (Fig. 7**C**), compared with the LPS+*Brazilin* group, the fluorescent presence of YAP was still observed in the LPS+VP+*Brazilin* group after the addition of VP.

Deleting YAP Attenuated the Brazilin-Induced Promotion of Osteogenesis of hPDLSCs

To further elucidate the role of the YAP pathway in *Brazilin*-induced osteogenic promotion of hPDLSCs. YAP shRNA lentivirus was used to delete YAP expression in hPDLSCs; after 3 days of puromycin selection (1 μg/mL), the knock-down efficiency was verified by qRT-PCR and Western blot. As figure showed in Fig. 8**A**, both mRNA and protein expression of YAP decreased by YAP shRNA lentivirus compared to the NC group. As shown in Fig. 8**B**, the YAP knockdown groups repressed the *Brazilin*-enhanced ALP activity of hPDLSCs. Alizarin red staining also revealed that the YAP knockdown groups inhibited the enhanced *in vitro* mineralization by *Brazilin*. When

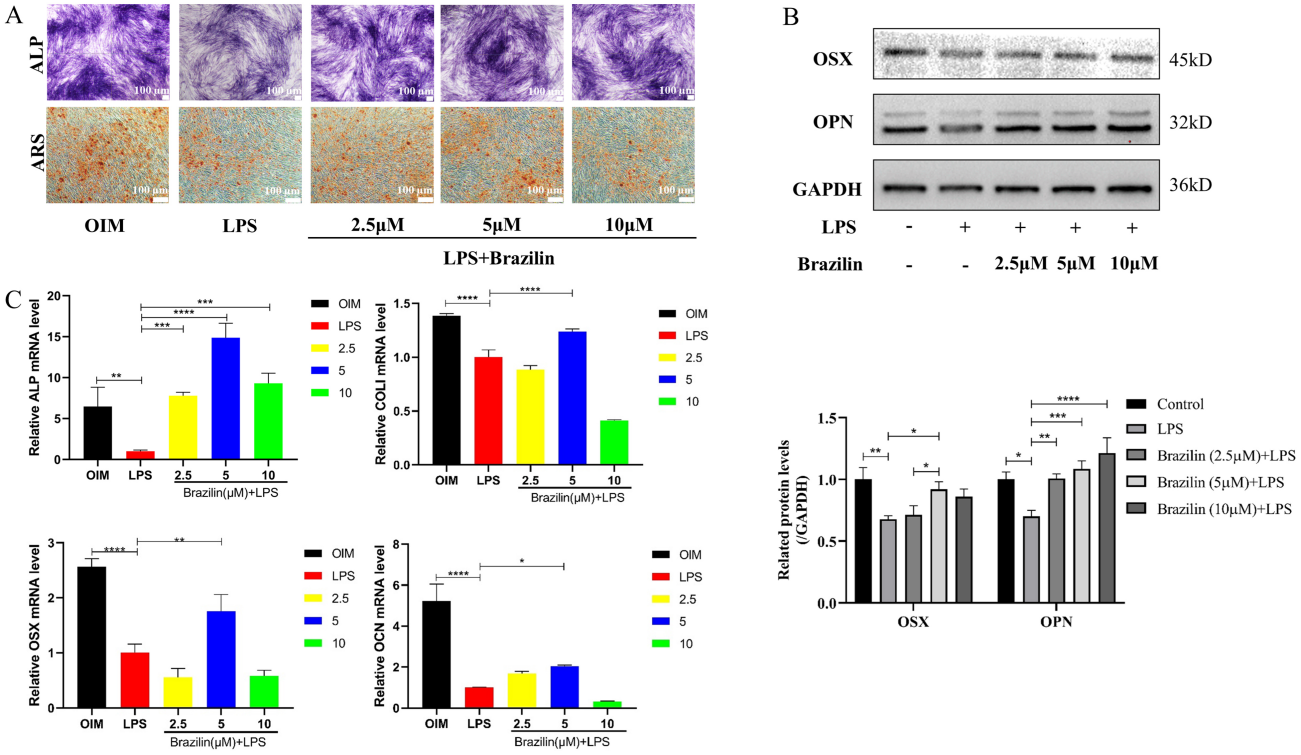

Fig. 5. Effects of *Brazilin* on osteogenic differentiation in Pg-LPS-treated hPDLSCs. (**A**) Representative images of ALP (14 d, scale bar, 100 μm) and ARS staining (21 d, scale bar, 100 μm). (**B**) The effects of LPS and *Brazilin* (2.5 μM, 5 μM, 10 μM) on OSX, and Osteopontin (OPN) expression were detected by WB analysis. Expression levels were normalized to GAPDH. (**C**) The expression of *ALP*, *COL1*, and *OSX*, and *OCN* was detected by RT–PCR at 7 and 14 days after LPS and 5 μM *Brazilin* cotreatment. Data are expressed as the mean ± SEM from three independent experiments performed in triplicate. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

YAP was knocked down, shYAP+*Brazilin* group showed weaker ALP staining and formed a less mineralized matrix (Fig. 8**B**) than the *Brazilin* group. The expression of RUNX2, OPN, and OCN in shYAP+*Brazilin* group decreased as compared with *Brazilin* group (Fig. 8**C**). After lentivirus transfection, observation with fluorescence microscope indicated that the transfection efficiency was over 90 %, but its nuclear translocation was partly blocked by YAP shRNA (Fig. 8**D**). Above results proved knocking down YAP inhibited the *Brazilin*-induced osteogenic differentiation of hPDLSCs.

Discussion

Inflammation-induced bone loss has recently become a research hotspot due to the profound effects of inflammatory responses on local and systematic bone metabolism. Periodontitis is considered to be one of the diseases with inflammatory responses and destruction of periodontal tissues, including evident alveolar bone loss, which can have varying effects on oral health and general health (Liljestrand *et al*., 2017; Yoshihara *et al*., 2004). hPDLSCs play a key role in periodontal healing and have become important seed cells for periodontal tissue regeneration (Zhu and Liang, 2015). However, recent results have shown that it is difficult to attain the desired tissue regeneration. The main problem is that the number of hPDLSCs in patients with periodontitis is decreased, and their functions are impaired, which impedes the regeneration of periodontal tissues. Inflammation control and periodontal tissue regeneration are the key issues in periodontitis treatment of hPDLSCs.

Nowadays, researchers are more interested in finding bioactive compounds from natural origin as drugs, owing to either the high cost of synthetic drugs or the side effects of the synthetic molecules. A mountain of studies has demonstrated that *Brazilin* plays an important role in eliminating inflammation and protecting against inflammatory bone loss (Nirmal *et al*., 2015). Mueller *et al*. (2016) evaluated *Brazilin* in RAW 264.7 macrophages and SW1353 chondrocytes and determined the secretion of the proinflammatory cytokines IL-6 and TNF-α and the anti-inflammatory IL-10. Interestingly, Jia *et al*. (2016) reported *Brazilin* can suppress the inflammatory response by inhibiting the activation of the Nuclear Factor-κB (NF-κB) signaling pathway. It is reported that *Brazilin* markedly attenuated mouse collagen-induced arthritis (CIA) and reduced the serum levels of inflammatory cytokines including TNF-α, IL-1β, and IL-6. And *Brazilin* prevented joint destruction, surface erosion, and enhanced bone formation as revealed by microstructural examinations (Jung *et al*., 2015). In this study, we explored the effect of *Brazilin* on anti-inflammatory and

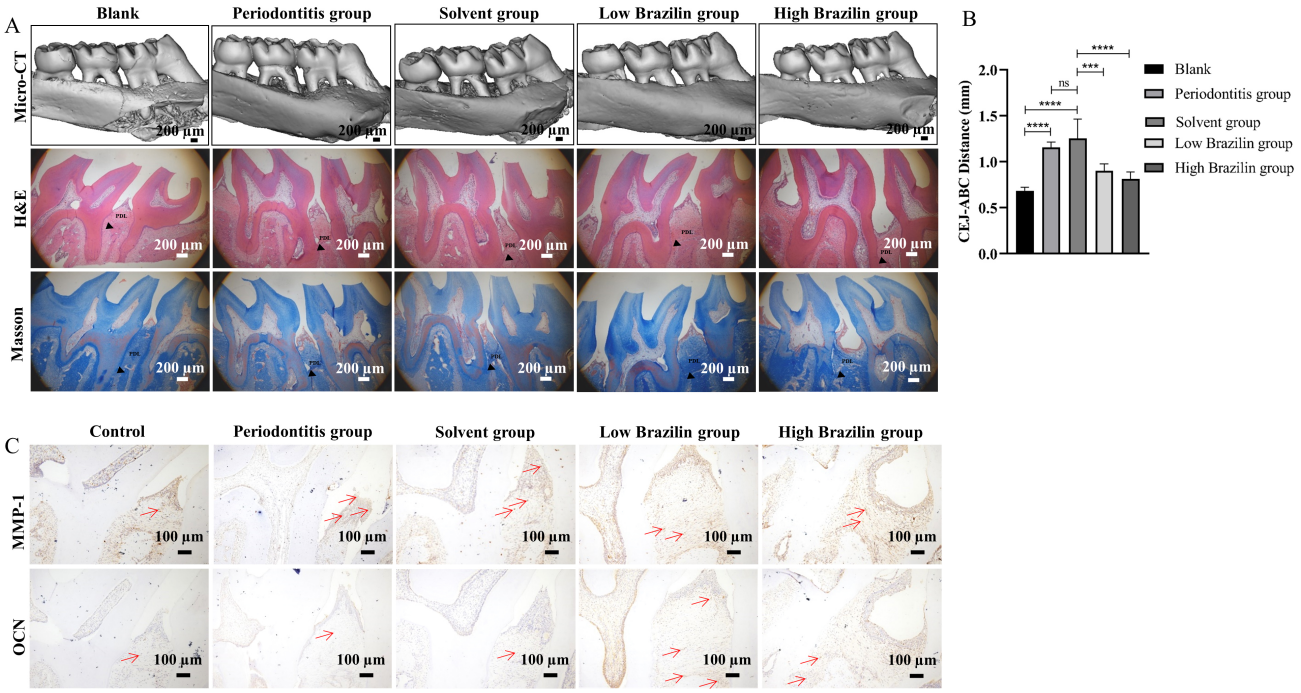

Fig. 6. The analysis of *Brazilin* ameliorated bone formation in rat periodontitis models. (**A**) Micro-CT image, HE staining images, and Masson's trichrome staining images of the periodontium after the treatment in decalcified sections. Black arrows point to the alveolar bone. PDL, periodontal ligament. (**B**) The distance of cemento-enamel-junction–alveolar bone crest (CEJ-ABC) was plotted. (**C**) The immunohistochemical analysis of MMP-1 and OCN positive cells (Red arrows indicate positive cells). ***$p < 0.001$, ****$p < 0.0001$, ns, non significance.

osteogenic differentiation in hPDLSCs under an inflammatory environment and the mechanism of *Brazilin* on the YAP pathway.

LPS from *P. gingivalis* is considered one of the main pathogenic factors for periodontal inflammation. In this study, hPDLSCs were treated with Pg-LPS (10 μg/mL) to stimulate the inflammatory microenvironment *in vitro*. The results showed that 10 μg/mL Pg-LPS initiates the production of inflammatory mediators such as interleukin-8 (IL-8), matrix metallopeptidase 1 (MMP1), and matrix metallopeptidase 3 (MMP3). As a major chemoattractant, IL-8 can induce the synthesis of matrix metallopeptidase, which can destroy normal periodontal tissue (Xiao *et al*., 2016). Therefore, suppressing these inflammatory mediators may help to restrict the progression of periodontal disease. In the present study, three concentrations of *Brazilin* significantly decreased the mRNA and protein levels of IL-8. 5 μM *Brazilin* showed the best inhibitory effect on MMP1 and MMP3. This result suggested the anti-inflammatory effects of *Brazilin*, which help to prevent periodontal destruction. Interestingly, we found that the drug cannot show more efficient inhibitory effects on MMP1 and MMP3 production when the concentration is too high or too low. The occurrence of this phenomenon may be related to the saturation of the receptors on the cells surface. Further increasing the concentration of the drug will no longer enhance its effect (Singh *et al*., 2016).

The development of clinical therapeutics for use in patients requires effective methods to rescue inflammation-mediated changes of hPDLSCs. Therefore, it is of great importance to investigate how to enhance the defective osteogenic differentiation capability of hPDLSCs in the inflammatory microenvironment. Runt-related transcription Factor 2 (RUNX2) was considered to be the central gene involved in osteoblast phenotype induction (Wei *et al*., 2015). Collagen type I (COL I) is an extracellular matrix protein that stimulates the adhesion and differentiation of osteoblasts (Biasutto and Zoratti, 2014). Osterix (OSX) is a novel osteoblast-specific transcription factor that is essential for osteoblast proliferation, differentiation, and bone formation (Hutchings *et al*., 2020). Osteocalcin (OCN) is a late marker of osteoblast maturation, playing a key role during the process of bone formation and mineralization (An *et al*., 2016). The results showed that 10 μg/mL Pg-LPS may hurt the osteogenesis capacity of hPDLSCs. However, *Brazilin* can significantly reverse the inhibition effect. In addition, through qPCR detection, the expression of the *RUNX2*, *COLI*, *OSX*, and *OCN* genes was significantly increased after 5 μM *Brazilin* treatment for 14 days in a noninflammatory environment. This result suggested that consecutive 5 μM *Brazilin* treatment with osteogenic induction could promote osteogenesis-related gene expression, including both early-stage genes and advanced-stage genes. Of note, compared with the noninflammatory envi-

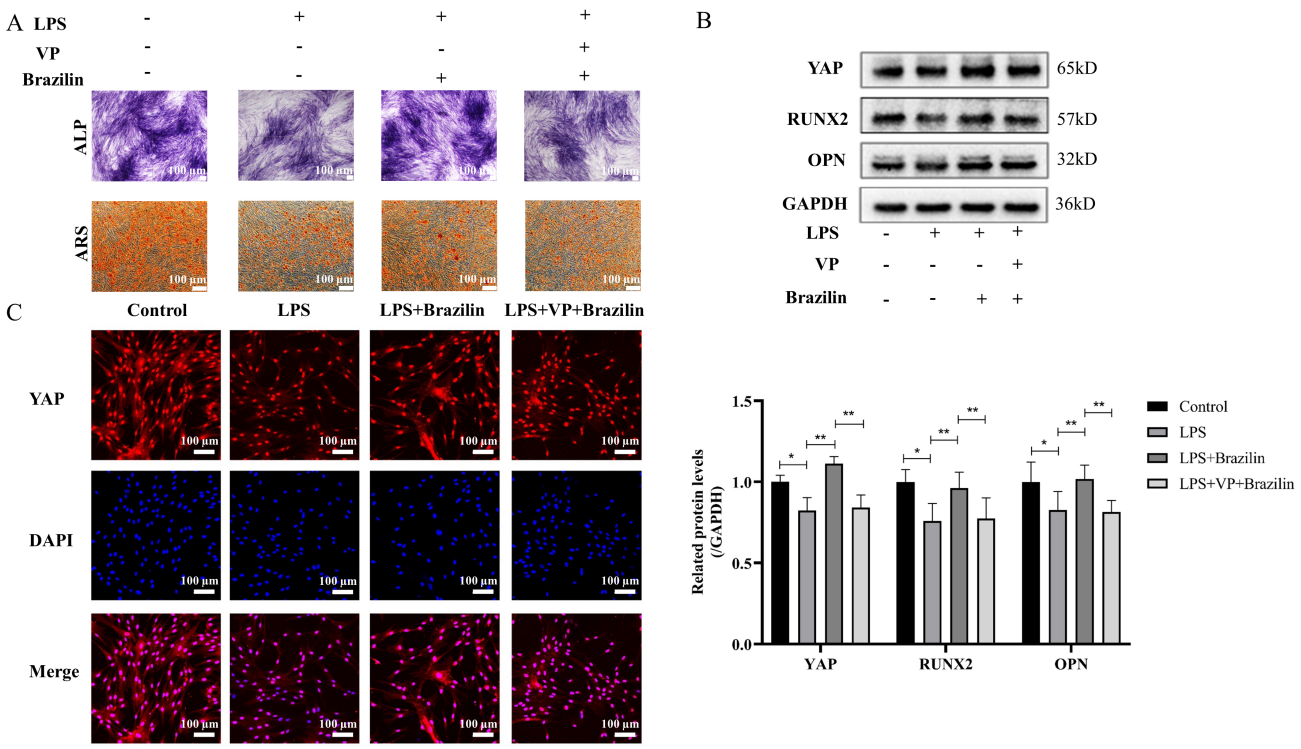

Fig. 7. Effects of Verteporfin on *Brazilin*-induced osteogenic promotion of PDLSCs. (**A**) Representative images of ALP (14 d, scale bar, 100 μm) and ARS staining. (**B**) Western blotting analysis of protein expression levels of the osteogenesis-associated genes after Yes-associated protein (YAP) expression was attenuated deleted by Verteporfin (VP). (**C**) Immunofluorescence staining of YAP. Data are expressed as the mean ± SEM from three independent experiments performed in triplicate. DAPI, 4′,6-diamidino-phenylindole; $*p < 0.05$, $**p < 0.01$.

ronment, the ability of *Brazilin* to promote osteogenic differentiation in an inflammatory environment was more obvious. To further investigate the actual effects of *Brazilin*, we established a rat model of periodontitis. *Brazilin* group outperformed the other group in HE, Masson staining, and Immunohistochemical staining, which demonstrated lower inflammation and more bone tissue production. Taken together, these findings demonstrate that *Brazilin* exhibited remarkable potential in the local application of periodontitis treatment.

The Hippo pathway plays an important role in many key processes, including oral and maxillofacial development and bone remodeling, whereas the YAP pathway plays an important role in the maintenance of bone homeostasis (Xiang *et al*., 2018). Several studies have shown that YAP is involved in stem cells and osteoblasts to promote osteogenic differentiation, especially in periodontal tissue remodeling, and regulates periodontal stem cells proliferation and differentiation (Sun *et al*., 2018; Wu *et al*., 2021; Zhang *et al*., 2016).

More importantly, in the inflammatory microenvironment, YAP not only reduces the inflammatory response but also inhibits inflammatory bone resorption (Lv *et al*., 2018; Yang *et al*., 2020). Following the use of verteporfin, a YAP inhibitor, RUNX2, OPN, and OCN expressions related to osteogenesis decreased as well as a reduced ALP staining and formation of mineralization nodules, however, in the group with the addition of *Brazilin*, there was a significant improvement in bone formation. Then we silent YAP caused by shRNA to get a similar result, which suggests that *Brazilin* may protect bone from inflammation via the YAP pathway and exert a promoting effect on osteogenesis. However, the mechanism of how *Brazilin* regulates the YAP pathway to promote osteogenesis in the inflammatory microenvironment needs to be investigated next. Taken together, we concluded that *Brazilin* can not only promote osteogenesis but can also reverse the adverse effects of inflammation on osteogenesis by exerting anti-inflammatory effects.

Conclusions

The results of *Brazilin* on hPDLSCs presented in this study will expand the knowledge of their role in anti-inflammatory and osteogenic differentiation. In particular, *Brazilin* has a positive effect on periodontal tissue regeneration and alleviated local inflammation. Notably, in a rat periodontitis model, *Brazilin* significantly inhibited alveolar bone loss by reducing inflammation and attenuating alveolar bone loss. We then preliminarily explored the effect of *Brazilin* on the YAP pathway *in vitro*, suggesting that *Brazilin* may be acting through the YAP pathway. This

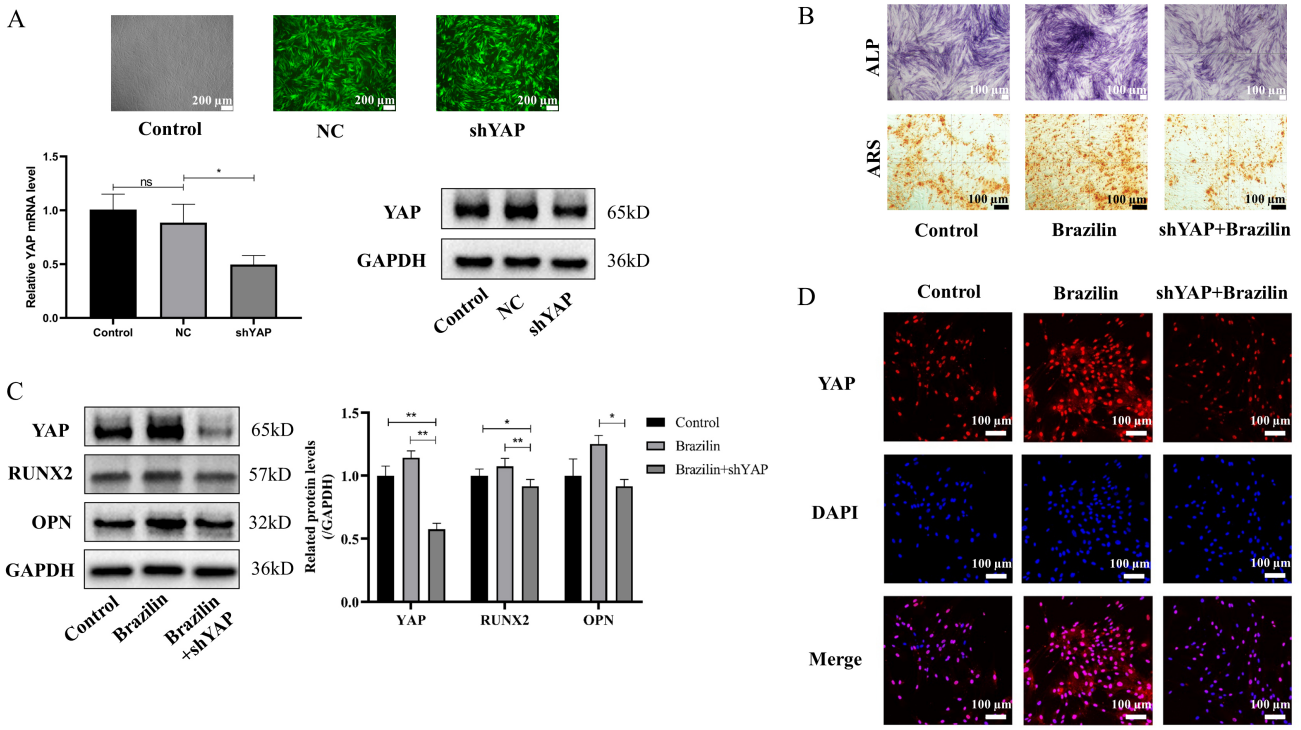

Fig. 8. Effects of shRNA on *Brazilin*-induced osteogenic promotion of hPDLSCs. (**A**) The expression levels of YAP mRNA and protein were deleted by shRNA. (**B**) Representative images of ALP (14 d, scale bar, 100 μm) and ARS staining. (**C**) Western blotting analysis of protein expression levels of the osteogenesis-associated genes after YAP expression was deleted by shRNA lentivirus. (**D**) Immunofluorescence staining of YAP. Data are expressed as the mean ± SEM from three independent experiments performed in triplicate. NC, normal control; *$p < 0.05$, **$p < 0.01$, ns, non significance.

study provides a novel idea for new drug research and development, as well as clinical applications for treating periodontitis.

List of Abbreviations

ALP, Alkaline Phosphatase; ARS, Alizarin Red Staining; BMSCs, bone marrow stem cells; *C. sappan*, *Caesalpinia sappan L*; CEJ-ABC, cementoenamel junction and the alveolar bone crest; CIA, collagen-induced arthritis; COL I, Collagen type I; DAPI, 4′,6-diamidinophenylindole; DMSO, dimethyl sulfoxide; HE, hematoxylin and eosin; IL-8, interleukin-8; hPDLSCs, Human periodontal ligament stem cells; LSP, lipopolysaccharide; MMP, matrix metallopeptidase; OIM, Osteogenic Induction Medium; OCN, Osteocalcin; OPN, Osteopontin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDL, periodontal ligament; PVDF, polyvinylidene difluoride; RUNX2, Runt-related transcription Factor 2; shRNA, short hairpin RNA; VP, Verteporfin; WB, Western Blot; YAP, Yes-associated protein.

Availability of Data and Materials

The data are available from the corresponding author on reasonable request.

Author Contributions

JQC & YLJ: performed the experiments, analyzed the data, and wrote the manuscript. XDZ, HXL & YYK: collected the data and reviewed the literature. JL, JZ & YHL: designed the experiments, reviewed and edited the manuscript, worked on funding acquisition, and supervised the whole work. All authors contributed to editorial changes in the manuscript, read and approved the final manuscript, and participated sufficiently in the work to take public responsibility for appropriate portions of the content.

Ethics Approval and Consent to Participate

The study protocol was approved by the Medical Ethics Committee of the Affiliated Stomatology Hospital of Kunming Medical University, China (KYKQ2021MEC025). All participating patients signed a written informed consent form.

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Conflict of Interest

The authors declare no conflict of interest.

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