Pterostilbene attenuates the proliferation and differentiation of TNF-α-treated human periodontal ligament stem cells

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Abstract. Periodontitis is a common chronic inflammatory oral disease. The objective of periodontal treatment is to control infection whilst regenerating damaged periodontal tissue. The present study aimed to determine the potential effects of pterostilbene (PTE), a representative stilbene compound, on the proliferation and differentiation of human periodontal ligament stem cells (hPDLSCs). Different concentrations (1, 5, 10 and 20 µM) of PTE were applied to hPDLSCs, after which Cell Counting Kit-8 and western blotting assays were performed to examine the protein levels of Ki67, PCNA, p-ικB and IkB. Bcl-2, Bax, cleaved caspase3. The effect of PTE on the release of inflammatory factors, including IL-1β, IL-6 and IL-10 was assessed by RT-qPCR. The apoptosis of TNF-α-induced hPDLSCs was evaluated by TUNEL assay and western blotting. Additionally, the role of PTE in hPDLSC mineralization was evaluated using alizarin red staining. The expression levels of mineralization indices, including RUNX2 and ALP were subsequently determined using western blotting. Subsequently, the target of PTE was predicted using TargetNet database and AutoDock v4.2 software and verified using western blotting. The results of the present study revealed that PTE promoted the formation of mineral nodules in TNF-α-induced hPDLSCs. PTE was also demonstrated to promote the formation of mineral nodules in TNF-α-induced hPDLSCs. The Targetnet database, along with molecular docking, indicated that histone deacetylases (HDACs) were the probable targets of PTE upstream of regulating periodontitis. The results of western blotting implied that TNF-α significantly increased expression levels of HDAC2, 4, 6 and 8, whilst PTE treatment markedly decreased HDAC4, 6 and 8 expression in a concentration-dependent manner compared with the TNF-α group, which further confirmed these conclusions. In summary, results of the present study revealed that PTE promoted TNF-α-induced hPDLSC proliferation and differentiation, whilst alleviating inflammation and apoptosis. PTE also inhibited the expression of HDACs, which may be involved in the mechanism of periodontitis.

Introduction

Periodontitis is a common chronic inflammatory oral disease (1). With the gradual onset of inflammation, periodontitis causes destruction of the periodontal ligament, cementum and alveolar bone (2). If treatment is not applied in a timely manner, periodontitis will cause further periodontal detachment and bone loss, eventually leading to early tooth loss (3). Due to the importance of periodontal tissues, the objective of periodontal treatment is to first control the infection and inhibit disease progression, then to regenerate the damaged periodontal tissue and reconstruct the missing tooth support structures (4). At present, conventional treatments for periodontal disease are relatively basic in nature, including cleansing, curettage and root leveling (5). Although these can control inflammation and prevent the progression of periodontal disease, they cannot completely restore the damaged tooth support structure (5). In addition, although the emergence of guided tissue regeneration methods has improved the treatment of periodontal disease, its ultimate effect remains limited and unstable (6,7). Furthermore, indications for guided tissue regeneration must be strictly screened and clinical predictability of its application is poor, limiting its application further (6,7). Over the previous decade, with the development of regenerative medicine, stem cell-based tissue engineering technology has provided an important means of repairing damaged tissues whilst preserving the highly ordered surrounding internal environment (8-11). This observation has been reported by a number of previous preclinical studies (8-11). In particular, human periodontal ligament stem cells (hPDLSCs) have been demonstrated to favorably regenerate supporting tissues...
surrounding the tooth in vivo (12). Therefore, hPDLSCs are increasingly being applied for the regeneration treatment of bone loss caused by periodontal disease (12).

Stilbene compounds are general names given to a series of compounds with the stilbene group as the parent backbone, which serve important roles in pharmacology. For example, several stilbene-based drugs such as raloxifene, toremifene and tamoxifen, have been approved clinically for treating cancer (16,17). Previous studies have revealed that PTE can prevent hypoxia-reoxygenation injury by activating sirtuin 1 in cardiomyocytes (18), in addition to improving cardiac function and reducing oxidative stress in animal models of ischemia-reperfusion injury (19). Another previous study suggested that PTE reduced high-fat-induced atherosclerosis in mice by inhibiting various proinflammatory cytokines, including TGF-β, TNF-α and interleukins (20). In particular, it was also demonstrated that PTE can reduce the release of inflammatory factors, including IL-1β, IL-6 and TNF-α by macrophages induced by oral symbiotic bacteria (21). Therefore, the present study hypothesized that PTE can prevent the onset of periodontitis.

TNF-α is one of the pivotal endogenous mediators of periodontitis (22). Therefore, TNF-α was used to induce hPDLSCs in the present study to assess the effects of PTE on cell proliferation and differentiation. Results of the present study may expand the current understanding of the efficacy of PTE and provide a novel direction for the design of treatment strategies for periodontitis.

Materials and methods

Cell culture and grouping. hPDLSCs (cat. no. BJ-ATCC0562; primary cells; Shanghai Bangjing Industry Co., Ltd.) were cultured in α-MEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in 5% CO2 at 37°C. PTE (purity ≥97%; Sigma-Aldrich; Merck KGaA) was dissolved in DMSO (purity >99.8%; Shanghai Aladdin Biotechnology) according to the manufacturer's instructions. PTE was dissolved in DMSO containing TNF-α (50 ng/ml). PTE was centrifuged at 10,000 x g for 15 min at 4°C to collect the supernatant. ALP activity was measured according to the manufacturer's protocol of the ALP activity detection kit (cat. no. P0321; Beyotime Institute of Biotechnology). The OD value was recorded at 405 nm using a microplate reader (Dojindo Molecular Technologies, Inc.).

Bioinformatics analysis. TargetNet (www.targetnet.scbdd.com) is a platform that can be used for drug target prediction (25). The structure of PTE was input with default parameters (area under curve ≥0.7; fingerprint type: extended connectivity fingerprint 4) to obtain a report of probable target proteins.

Molecular docking technology is a theoretical simulation method used for studying the interactions among molecules, such as ligands and receptors, in addition to predicting the nature of their binding mode and affinity (26). The structure of PTE was first imported into the OpenBabel v2.2.0 software (Free software foundation, Inc.) for hydrogenation and conversion into its 3D structure. The 3D structure of various histone deacetylase (HDAC) proteins, including HDAC2 (PDB ID: 6WBW), HDAC4 (PDB ID: 6FYZ), HDAC6 (PDB ID: 5EDU) and HDAC8 (PDB ID: 5D1B), were downloaded from the Protein Data Bank website (https://www.rcsb.org/). To reduce the influence of binding force prediction, water molecules and existing ligands in the protein structure were deleted using PyMOL v2.2.0 software (DeLano Scientific, LLC). The position of the original ligand was set as a docking site before the docking between PTE and HDACs was performed using AutoDock v4.2 software (Scripps Institute) via automatic calculation of binding free energy PTE and HDACs in the running process of the system. All docking calculations were performed using a Lamarckian genetic algorithm. The default FlexX parameters were used.

Cell Counting Kit-8 (CCK-8) assay. Cell viability was measured using CCK-8 assay kit (Dojindo Molecular Technologies, Inc.). hPDLSCs were seeded into 96-well plates at a density of 3x10⁴ cells/well. After allowing for cell adherence, the original culture medium was replaced with medium containing PTE (0, 1, 5, 10 and 20 µM) and/or TNF-α (100 ng/ml). After 48 h of incubation at 37°C, 10 µl CCK-8 was added to each well, after which samples were incubated at 37°C for an additional 2 h. Optical density (OD) values were recorded at 450 nm using a microplate reader (Dojindo Molecular Technologies, Inc.).

Alkaline phosphatase (ALP) activity assay. hPDLSCs were seeded into six-well plates at a density of 2x10⁵ cells/well. After cells reached 60-70% confluence, the culture medium was replaced with osteogenesis induction medium [α-MEM supplemented with 10% FBS, 10 mM β-glycerophosphate (China Pharmaceutical Shanghai Chemical Reagent Co., Ltd.), 50 mg/l ascorbate and 2 mg/l dexamethasone (both Sigma-Aldrich; Merck KGA) containing TNF-α (100 ng/ml) and PTE (0, 5, 10 and 20 µM). Briefly, the cells were lysed with 0.05% Triton X-100 (Sigma-Aldrich) at 4°C for 2 h. On day 7 following osteogenic induction at 37°C, the cell lysate was centrifuged at 10,000 x g for 15 min at 4°C to collect the supernatant. ALP activity was measured according to the manufacturer's protocol of the ALP activity detection kit (cat. no. P0321; Beyotime Institute of Biotechnology). The OD value was recorded at 405 nm using a microplate reader (Dojindo Molecular Technologies, Inc.).

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay. The apoptosis of hPDLSCs cells was assessed using a TUNEL assay (Beyotime Institute of Biotechnology) according to the manufacturer's instructions.
After 24 h cell culture, transfected cells were fixed with 1% paraformaldehyde at room temperature for 15 min and 50 µl TUNEL was used to incubate cells for 1 h at 37˚C, followed by staining of nuclear DNA with 10 µg/ml DAPI at 37˚C for 2-3 min and mounted with glycerol gelatin (Sigma-Aldrich; Merck KGaA). The cells were analyzed from three fields of view using a fluorescence microscope (magnification, x200; Olympus Corporation).

Reverse transcription-quantitative PCR (RT-qPCR). hPDLSCs were incubated in six-well plates at a density of 2x10^5 cells/well. TNF-α (100 ng/ml) and PTE (0, 5, 10 and 20 µM) were added to the plates at 37˚C until cells reached 60-70% confluence. Cells were subsequently incubated at 37˚C for 48 h. For the determination of ALP and runt-related transcription factor 2 (RUNX2) expression levels, cells were collected on day 7 of osteogenic induction. Total RNA was extracted from the cultured cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and reversed transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc.) in accordance with the manufacturer's protocol. qPCR reactions were performed using the SYBR Green Master Mix (Biosharp Life Sciences) on a 7500 Real-time system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, followed by 45 cycles of 95˚C for 30 sec, 55˚C for 30 sec, 72˚C for 30 sec and 72˚C for 7 min. Relative mRNA expression was quantified using the 2^-∆∆Cq method (27) and normalized to GADPH. The primer sequences used for qPCR are listed in Table I.

Western blotting. The treatment of hPDLSCs was the same as that described in RT-qPCR. Protein lysates from hPDLSCs were prepared using RIPA lysis buffer (Beyotime Institute of Biotechnology) and protein concentration was measured using a BCA kit (Sigma-Aldrich; Merck KGaA). The membranes were then incubated with HRP-conjugated goat anti-rabbit (1:10,000; Abcam) secondary antibodies for 2 h at room temperature and developed using an ECL kit (Biosharp Life Sciences). ImageJ v1.8.0 software (National Institutes of Health) was applied for image analysis and relative protein expression was normalized to GAPDH.
Alizarin red staining. hPDLSCs were seeded into 12-well plates at a density of 1x10^5 cells/well. After the cells reached 60-70% confluency, the culture medium was replaced with osteogenesis induction medium containing TNF-α (100 ng/ml) and PTE (0, 5, 10 and 20 µM). At 21 days after osteogenic induction at 37˚C, cells were washed once with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. 0.04 M Alizarin Red S staining solution (cat. no. C0148S; Beyotime Institute of Biotechnology) was subsequently added for 30 min at room temperature. Images were observed under a light microscope (magnification, x200; Olympus, Corporation).

Statistical analysis. All experimental data are presented as the mean ± SD from three independent experiments. GraphPad Prism 8.0 statistical software (GraphPad Software, Inc.) was used to analyze the data. Additionally, the data were in accordance with the normal distribution by Shapiro-Wilk test. One-way ANOVA and Tukey's post hoc test was conducted to compare differences between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

PTE promotes TNF-α-induced hPDLSC viability and alleviates inflammation. The effect of different concentrations of PTE (0, 1, 5, 10 and 20 µM) on the viability of hPDLSCs was determined using a CCK-8 assay. The results revealed that PTE at 20 µM concentrations were used in subsequent experiments and labeled as L, M and H groups, respectively. Following TNF-α induction, the effects of these three concentrations of PTE on cell viability were assessed using a CCK-8 assay. The results indicated that cell viability was significantly decreased in the TNF-α group compared with the control group, but subsequent PTE treatment reversed this reduction in cell viability in a concentration-dependent manner (Fig. 1C). The expression levels of Ki67 and PCNA were then measured using western blotting. The data revealed that the expression levels of each protein were significantly decreased following TNF-α treatment. However, PTE significantly reversed these reductions in Ki67 and PCNA expression (Fig. 1D). These results suggested that PTE prevented hPDLSCs from TNF-α-induced damage and upregulated cell viability.

The expression levels of certain inflammatory factors, including IL-1β, IL-6 and IL-10, were next assessed in hPDLSCs by performing RT-qPCR. The results revealed that the levels of IL-1β and IL-6 were significantly increased, whilst that of IL-10 was significantly decreased, in the TNF-α group compared with the control group. Furthermore, PTE treatment significantly downregulated IL-1β and IL-6 levels whilst upregulating IL-10 expression compared with the TNF-α group (Fig. 2A). In addition, western blotting revealed that PTE inhibited the TNF-α-induced phosphorylation of p65, with M and H groups reaching significance (Fig. 2B).

PTE reduces apoptosis and promotes TNF-α-induced hPDLSC differentiation. Cell apoptosis was assessed using TUNEL and western blotting assays. The number of apoptotic cells

Table I. Sequences of the primers.

| Gene  | Sequence (5'-3') | NCBI Reference sequence | Product size |
|-------|-----------------|-------------------------|--------------|
| IL-1β | CTACCTGTCCCTGCTTGTTGA | NM_000576.3 | 153 |
|       | GGAACGTGGGCGACCTCAA  |                        |              |
| IL-6  | CTTTGGTCCTGTTGCTTCT  | NM_000600.5 | 233 |
|       | CAGTGCCTCTTTGCTTTC   |                        |              |
| IL-10 | AGACAGAATGGCAAAAGAAGGC | NM_000572.3 | 148 |
|       | TCGAAGCATGGTACGAGGTCAGTT  |                    |              |
| ALP   | CTTGTGCTGGACGGACCC   | NM_000478.6 | 231 |
|       | GGCACAGACTTGGGCTTCTTT |                        |              |
| RUNX2 | CGATGGGACCGACCTATTAA  | NM_001015051.4 | 129 |
|       | GTCGCCAACAGATTTCATCCA |                        |              |
| GAPDH | GCACCGTCAAGGCTGAGAAC  | NM_001256799.3 | 138 |
|       | TGGTGAAGACGCGCAGTGGGA  |                        |              |

ALP, alkaline phosphatase; RUNX2, runt-related transcription factor 2.
emitting green fluorescence in the TNF-α group was increased significantly compared with the control group. Additionally, as the PTE concentration increased, the number of apoptotic cells decreased significantly (Fig. 2C). Furthermore, the expression levels of Bax and cleaved caspase 3 were significantly increased in the TNF-α group compared with the control. However, they were significantly decreased in the PTE-treated groups compared with the TNF-α group (Fig. 2D). Bcl2 expression levels demonstrated the opposite trend following TNF-α and PTE treatment (Fig. 2D). The results of these assays suggested that PTE alleviated TNF-α-induced hPDLSC apoptosis.

The role of PTE in the mineralization capacity of hPDLSCs was subsequently evaluated using alizarin red staining. While clear red mineralized nodules were observed in the control group, no staining could be observed in the TNF-α group. However, PTE treatment promoted the formation of mineral nodules in TNF-α-induced hPDLSCs (Fig. 3A). The expression levels of mineralization indices were determined using western blotting and RT-qPCR. The protein and mRNA expression levels of ALP and RUNX2 were both significantly downregulated in the TNF-α group, which was significantly reversed in the three PTE groups (Fig. 3B and C). The activity of ALP was similarly decreased significantly by TNF-α, which was also significantly reversed by all three doses of PTE (Fig. 3D).

HDACs are probable targets of PTE. The targets of PTE were predicted using the Targetnet database. The results determined HDAC2, 4, 6 and 8 to be probable targets of PTE. Therefore, the possible association between PTE and these proteins were calculated using the molecular docking module. The whole protein was presented in green, whilst the main skeleton of PTE was presented in brown. The blue solid line represented hydrogen bonds, the green dashed line represented π-stacking and the gray dashed line represented hydrophobic interactions. The docking values between PTE and HDAC2, 4, 6 and
were -6.0, -7.3, -6.4 and -6.4 kcal/mol, respectively, which indicated that the complex formation is thermodynamically spontaneous and implied the high stability of PTE-HDAC2, 4, 6 and 8 complexes (Fig. 4). Following computer prediction, the expression levels of these four proteins were determined using western blotting. TNF-α significantly increased the expression levels of all four of the HDAC isoforms tested, whilst PTE treatment markedly decreased HDAC4, 6 and 8 expression in a concentration-dependent manner compared with the TNF-α group. However, no significant difference could be observed for HDAC2 between the TNF-α and PTE groups (Fig. 5).

**Discussion**

hPDLSCs derived from human periodontal tissues are a type of mesenchymal stem cell that is readily obtainable in clinical practice (28). They can be obtained following wisdom teeth extraction or premolars that are extracted by orthodontists using minimally invasive methods (29). This availability render hPDLSCs attractive sources of autologous stem cells. A previous study reported that the addition of hPDLSCs to biological materials is beneficial to promote bone healing in rat skull defect models (30). In addition, a number of in vitro and in vitro studies have demonstrated that hPDLSCs exhibit potent self-renewal and multi-lineage differentiation capacities (31,32). Their ability to differentiate into osteoblasts makes them considerably superior options to stem cells derived from other oral tissues, including the dental pulp, gums and dental follicles (33). These characteristics also mean that hPDLSCs have been extensively applied for the regeneration treatment of periodontal disease-induced periodontal cord and bone loss (12). Therefore, the present study selected hPDLSCs to assess the possible role of PTE in periodontitis, which
revealed that PTE increased the viability of hPDLSCs in a concentration-dependent manner.

During the progression of periodontitis, the increased expression of TNF-α further promotes the secretion of other inflammatory mediators, adhesion molecules and cytokines that are closely associated with bone resorption (22). This aggravates the clinical symptoms of periodontitis (22). TNF-α was used in the present study to treat hPDLSCs, after which the effects of PTE on the cell viability, inflammatory factor release, apoptosis and differentiation of hPDLSCs were assessed.

Figure 4. Molecular docking results of PTE with HDACs. The full field of view and enlarged partial fields of view of PTE with (A) HDAC2, (B) HDAC4, (C) HDAC6 and (D) HDAC8. The whole protein was presented in green, whilst the main skeleton of PTE was presented in brown. The blue solid line represented hydrogen bonds, the green dashed line represented π-stacking and the gray dashed line represent hydrophobic interactions. PTE, pterostilbene; HDAC, histone deacetylase.

Figure 5. Expression levels of HDAC2, 4, 6 and 8 were determined using western blotting. ***P<0.001; *P<0.05, **P<0.01 and ***P<0.001. HDAC, histone deacetylase; L, low dose; M, medium dose; H, high dose.
was revealed to upregulate the expression levels of proliferation markers Ki67 and PCNA, and reduce IL-1β and IL-6 levels, whilst increasing IL-10 levels. It has been previously demonstrated that IL-10 serves an anti-inflammatory role by strongly inhibiting the synthesis of IL-1, IL-6, IL-8 and TNF-α on a transcriptional level, regardless of whether it is endogenous or exogenous (34-36). The results of the present study revealed that PTE increased the viability of TNF-α-induced hPDLCs and reduced the release of inflammatory cytokines. In addition, results from TUNEL staining and western blotting demonstrated that PTE significantly inhibited the apoptosis of TNF-α-induced hPDLCs. A previous study also revealed that PTE inhibited inflammation and apoptosis in mouse models of pulmonary fibrosis (37). ALP has been considered to be an early osteogenic marker, whilst RUNX2 regulates the differentiation of pluripotent stem cells into osteoblasts (38). In the present study, PTE significantly upregulated the expression of ALP and RUNX2 on day 7 of osteogenic induction in TNF-α-induced hPDLCs, suggesting that PTE promoted hPDLC differentiation.

Using the Targetnet database, PTE was predicted to target histone family protein HDACs. Previous studies have revealed that HDAC inhibitors can promote the proliferation of human dental pulp stem cells and the differentiation of odontoblasts (39,40). Furthermore, HDAC2 inhibition has been demonstrated to reduce cytokine production and osteoclast bone resorption (41). A previous study also reported that microRNA-22 targets HDAC6 to promote hPDLC differentiation (42). In the present study, according to computer simulation software, it was determined that PTE could bind to HDACs through intermolecular forces. Additionally, the docking value between PTE and HDAC4 was the most optimal among all HDACs. Western blotting revealed that PTE treatment significantly downregulated the protein expression levels of HDAC4, 6 and 8, with HDAC4 being the most optimal among all HDACs. Western blotting revealed that PTE treatment significantly downregulated the protein expression levels of HDAC4, 6 and 8, with HDAC4 and 6 exhibiting relatively higher concentration-dependence. These results suggest that PTE may participate in the regulation of periodontitis by downregulating HDAC expression. The downregulation of HDACs reduces the removal of acetylated groups on the lysine residues of histones, destabilizing the binding between histones and DNA (43). This detachment promotes the combination of the DNA promoter regions with transcription factors (44), which increases the transcription of genes related to proliferation and differentiation (45,46). This may be the mechanism through which PTE can regulate proliferation and differentiation via HDACs. However, results of the present study are limited to the preliminary exploration of this mechanism. Additional experiments are required to verify the association between PTE and HDACs in periodontitis. Furthermore, in addition to osteogenic differentiation, cartilage and adipogenic multidirectional differentiation are also important indices of the differentiation potential of stem cells. Future studies on the effects of PTE on these two aspects also warrant further investigation.

In conclusion, the present study revealed that PTE promoted TNF-α-induced hPDLC viability and differentiation, whilst alleviating inflammation and apoptosis. PTE also inhibited the expression of HDACs, which may be its mechanism of action in periodontitis. It is hoped that findings of the present study may promote the development of novel treatment strategies for periodontitis.

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

Authors’ contributions
MY, GW and YL conceived this study. MY, GW and JN performed the experiments. MP analyzed the data and YL performed bioinformatics analysis. YL revised the manuscript for important intellectual content. All authors have read and approved the final manuscript. MY and GW confirm the authenticity of the raw data.

Ethics approval and consent to participate
The Ethics Committee of Shanghai Huangpu District 2nd Dental Disease Prevention and Treatment Institute (Shanghai, China) waived the requirement for ethics approval for using the purchased human periodontal ligament stem cells.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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