

Berberine improves advanced glycation end products-induced osteogenic differentiation responses in human periodontal ligament stem cells through the canonical Wnt/ β -catenin pathway

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Abstract. The aim of the present study was to investigate the effects of advanced glycation end products (AGEs) and berberine hydrochloride (BBR) on the osteogenic differentiation ability of human periodontal ligament stem cells (hPDLSCs) *in vitro*, and their underlying mechanisms. hPDLSCs were subjected to osteogenic induction and were treated with AGEs or AGEs + BBR. Following varying numbers of days in culture, alkaline phosphatase (ALP) activity assays, ALP staining, alizarin red staining, ELISAs, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses were performed to determine the osteogenic differentiation ability of hPDLSCs; RT-qPCR, western blot analysis, and immunofluorescence staining were conducted to investigate the underlying mechanisms. The canonical Wnt/ β -catenin pathway inhibitor XAV-939 and agonist CHIR-99021 were used to determine the contribution of the canonical Wnt/ β -catenin pathway to differentiation. Treatment with AGEs resulted in reduced ALP

activity and Collagen I protein levels, decreased ALP staining, fewer mineralized nodules, and downregulated expression of osteogenic-specific genes [Runx-related transcription factor 2 (Runx2), Osterix, ALP, osteopontin (OPN), Collagen I and osteocalcin (OCN)] and proteins (Runx2, OPN, BSP and OCN); however, BBR partially rescued the AGE-induced decrease in the osteogenic potential of hPDLSCs. Furthermore, AGEs activated the canonical Wnt/ β -catenin signaling pathway and promoted the nuclear translocation of β -catenin; BBR partially attenuated this effect. In addition, XAV-939 partially rescued the AGE-induced reduction in the osteogenic potential of hPDLSCs, whereas CHIR-99021 suppressed the BBR-induced increase in the osteogenic potential of hPDLSCs. The present study indicated that AGEs attenuated the osteogenic differentiation ability of hPDLSCs, in part by activating the canonical Wnt/ β -catenin pathway; however, BBR attenuated these effects by inhibiting the canonical Wnt/ β -catenin pathway. These findings suggest a role for BBR in periodontal regeneration induced by hPDLSCs in patients with diabetes mellitus.

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Introduction

Periodontal disease is one of the most prevalent chronic diseases globally, and is associated with diabetes mellitus (DM) (1). A number of scientific reports have identified an association between periodontitis and systemic diseases, including arteriosclerosis, DM and heart diseases (1,2). Periodontitis and DM have been reported to be linked via a 'two-way relationship' (1). Diabetes-associated periodontitis poses a major threat to human health and results in a substantial economic burden on society.

Periodontal tissue damage induces periodontal ligament stem cells (PDLSCs) to regenerate the tooth-supporting apparatus (3). A previous study reported that the bone-regenerative

potential of PDLSCs in inflammatory microenvironments is reduced by alterations in their differentiation potential (3); however, the osteogenic differentiation potential of PDLSCs in patients with diabetes-associated periodontitis remains unclear. Various studies have demonstrated an increase in the synthesis of advanced glycation end-products (AGEs) in DM, and AGEs in periodontal tissues serve important roles in upregulating periodontal inflammation in patients with DM (1,4-6). Thus, it was hypothesized that AGEs serve a salient role in the osteogenic differentiation ability of human periodontal ligament stem cells (hPDLSCs). In the present study, the impact of AGEs on the osteogenic differentiation potential of hPDLSCs was investigated *in vitro*.

Extensive efforts to repair damaged periodontal tissues have been conducted in previous years (7-9). A selective estrogen receptor modulator, bisphosphate, and calcitonin have been reported to inhibit bone resorption; however, their clinical use is greatly restricted, as they only protect against bone damage without repairing damaged bone structures (10-12). Berberine hydrochloride (BBR), an isoquinoline alkaloid, is a traditional Chinese medicine that has historically been used to treat various infectious disorders in the past 3,000 years (13). BBR possesses numerous pharmacological properties, including antibacterial, anti-inflammatory, anticancer and cholesterol-reducing effects (13,14). It was revealed that BBR may improve glucose and lipid metabolism disorders (15); however, whether BBR may improve the osteogenic potential of hPDLSCs in an AGE-enriched microenvironment remains unclear. Therefore, the present study investigated the effects of BBR on the osteogenic differentiation ability of hPDLSCs in an AGE-enriched microenvironment.

Identification of the mechanisms underlying diabetes-associated periodontitis is required to determine its pathogenesis and provide effective treatment for patients. Previous studies have demonstrated that the Wnt signaling pathway serves an important role in the progression of bone regeneration (16-19). Wnt proteins transduce multiple signaling cascades, including the canonical Wnt/ β -catenin pathway, the Wnt/ Ca^{2+} pathway and the Wnt/polarity pathway (20). Among these pathways, the canonical Wnt/ β -catenin signaling pathway has been extensively studied. An *in vitro* study reported that the activation of canonical Wnt signaling promoted alkaline phosphatase (ALP) activity in osteogenic cultures of pluripotent mesenchymal cell lines (21). Conversely, a number of studies have indicated that the Wnt signaling pathway inhibits osteoblast differentiation (22-24). The precise mechanisms underlying the effects of Wnt signaling on bone regeneration remain unclear; however, it appears that its diverse effects are associated with cell type and differentiation status (24).

The present study aimed to investigate the effects of AGEs and BBR on the osteogenic differentiation ability of hPDLSCs, and the underlying molecular mechanisms responsible for these effects. It was hypothesized that AGEs may reduce the osteogenic differentiation ability of hPDLSCs by activating the canonical Wnt/ β -catenin signaling pathway, whereas the application of BBR may rescue the impaired osteogenic potential of hPDLSCs in an AGE-enriched microenvironment by inhibiting canonical Wnt/ β -catenin signaling.

Materials and methods

Antibodies and reagents. For cell culture, α -Minimum Essential Medium (α -MEM), M-199 and L-glutamine were purchased from Gibco (Gibco; Thermo Fisher Scientific, Inc.). Trypsin, Triton X-100, dimethyl sulfoxide and β -mercaptoethanol were obtained from Sigma-Aldrich (Sigma-Aldrich; Merck KGaA). BBR hydrochloride was acquired from Wako (Wako; Wako Pure Chemical Industries, Ltd). Natural AGE protein (cat. no. ab51995) was purchased from Abcam. XAV-939 (cat. no. S1180) and CHIR-99021 (cat. no. S1263) were obtained from Selleck Chemicals.

Primary and secondary antibodies were purchased from the following commercial sources: An antibody specific to glycogen synthase kinase 3β (GSK- 3β) was purchased from Cell Signaling Technology, Inc. (Cell Signaling Technology); antibodies specific to β -actin, and horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit immunoglobulin G (IgG) were purchased from Sigma-Aldrich (Sigma-Aldrich; Merck KGaA); antibodies specific to Runt-related transcription factor 2 (Runx2), osteopontin (OPN), bone sialoprotein (BSP), osteocalcin (OCN), wnt3a, β -catenin, CD73, CD90, CD105 and vimentin were purchased from Abcam; antibodies specific to CD34 were purchased from BD Biosciences; antibodies specific to cytokeratin-19 (CK-19) were purchased from Santa Cruz Biotechnology, Inc.; Alexa Fluor[®] 488-conjugated-goat anti-rabbit secondary antibodies were purchased from Invitrogen (Invitrogen; Thermo Fisher Scientific, Inc.). Other chemicals were of the highest grade available commercially.

Cell culture. hPDLSCs used in the present study were provided by the Oral Stem Cell Bank of Beijing (Beijing Tason Biotech Co., Ltd.). The cells were plated in 75 cm² polystyrene tissue culture flasks (Corning Inc.) at 37°C in a humidified atmosphere consisting of 95% air and 5% CO₂, and then subcultured at 3x10⁴ cells/cm² into 6-well plates (Corning Inc.) if required. The cells were cultured in α -MEM containing 10% fetal bovine serum (FBS; Biological Industries), 2 mmol/l L-glutamine and antibiotics with 100 U/ml penicillin (HyClone; GE Healthcare Life Sciences) and 100 μ g/ml streptomycin (HyClone; GE Healthcare Life Sciences). Following culturing with complete α -MEM for 24 h, the culture medium was replaced with osteogenic induction medium [α -MEM supplemented with 10% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mmol/l β -Glycerophosphate disodium salt hydrate (Biological Industries), 50 μ g/ml ascorbic acid (Biological Industries), 10 nmol/l dexamethasone (Biological Industries)]. The medium was changed every 48 h throughout the experiment. Cells were cultured for 7, 14 or 21 days, according to the particular experimental requirements. Passages 2-4 were used in experiments. Each experiment was repeated at least three times.

hPDLSCs were cultured in osteogenic induction medium containing 200 μ g/ml AGEs with or without 1 μ mol/l XAV-939, or 200 μ g/ml AGEs + 1 μ mol/l BBR with or without 1 μ mol/l CHIR-99021 according to the different experimental requirements. The concentrations of the drugs used in the present study were selected based on the Cell Counting Kit-8 (CCK-8) method described below, and were consistent with their use

in other studies (25-27). Throughout the study, in experiments involving XAV-939 or CHIR-99021, these were added to the culture medium 30 min prior to the addition of other drugs. All drug treatments were performed at 37°C unless otherwise stated.

Flow cytometric analysis of cell phenotype. hPDLSCs were identified through cell surface marker analysis by flow cytometry. The specific experimental procedures were as follows: Cells were cultured in 25 cm² polystyrene tissue culture flasks for 5 days in α -MEM, then they were washed twice carefully in heat-inactivated PBS, scraped off from the culture flasks, digested with 0.25% trypsin, and centrifuged at 300 x g for 5 min at 4°C. The cells were fixed with 4% paraformaldehyde (PFA) for 30 min at 4°C, permeabilized with 0.1% Triton X-100 for 10 min at room temperature, and blocked with a mixture of 0.5% bovine serum albumin (BSA) and 0.02% Tween-20 for 20 min at room temperature. Cells were then incubated at 37°C for 30 min with the following primary monoclonal and polyclonal antibodies: Mouse anti-CD73 (1:50; cat. no. ab91086), anti-CD105 (1:200; cat. no. ab114052) and anti-CD34 (1:50; cat. no. 555820); rabbit anti-CD90 (1:50; cat. no. ab133350) and anti-vimentin (1:200; cat. no. ab92547); and goat anti-CK-19 (1:100; cat. no. SC-33119). Cells were washed twice with PBS, then treated with Alexa Fluor 488-conjugated fluorescent secondary antibodies (1:400; cat. no. A-11029; mouse, 1:400; cat. no. A-11034; rabbit and 1:400; cat. no. A-11055; goat) for 30 min at 4°C in the dark. Cold PBS (200 μ l) was added to each tube following two washes in cold PBS. Finally, flow cytometric analysis was performed using a Beckman Coulter Epics XL (Beckman Coulter, Inc.). The results were analyzed using the EXPO 32 Analysis Software (Beckman Coulter, Inc.). Negative control experiments were performed according to the same protocol without the use of primary antibodies.

Osteogenic induction. All subsequent experiments were performed in the context of osteogenic induction; therefore, throughout the study, hPDLSCs were first seeded in 75 or 25 cm² polystyrene tissue culture flasks, or 6/24/96-well plates, in α -MEM at 37°C in a humidified atmosphere consisting of 95% air and 5% CO₂ overnight, and then the culture medium was replaced by osteogenic induction medium. The culture medium was replaced every 2 days. In this study, cells at passage 2-4 were used, and cultured at 37°C with 5% CO₂ for 5, 7, 14 or 21 days, depending on the subsequent experiment performed.

Cell viability assay. In the present study, various drugs, including AGEs, BBR, XAV-939 and CHIR-99021, were used to treat hPDLSCs. The selection of appropriate concentrations was determined via a cell viability assay using Cell Counting Kit-8 (CCK-8). The specific protocol was: hPDLSCs were cultured in 96-well plates at a density of 5x10³ cells/well, with ~150 μ l cell suspensions evenly distributed in each well. The culture medium was replaced with osteogenic induction medium after 24 h. Then, cells in different 96-well plates were treated with various drugs or drug combinations, including AGEs (0, 100, 200 and 400 μ g/ml), BBR (0, 0.1, 1, 3 and 10 μ mol/l), XAV-939 (0, 0.1, 1 and 10 μ mol/l) and CHIR-99021 (0, 0.1, 1 and 10 μ mol/l) for 0, 1, 3, 5 and 7 days. The culture

medium was replaced three times per week, as required. Subsequently, 15 μ l CCK-8 reagent (Dojindo Molecular Technologies, Inc.) was added to each well. Following incubation for 3 h at 37°C, the optical density (OD) was detected at an excitation wavelength of 450 nm on a microplate reader (Thermo Fisher Scientific, Inc.). The concentrations that did not significantly affect cell viability in these assays (data not shown), were selected for subsequent experiments: 200 μ g/ml AGEs, 1 μ mol/l XAV-939, 1 μ mol/l BBR, and 1 μ mol/l CHIR-99021.

ALP staining. Depending on the experimental requirements, hPDLSCs were divided into the following groups: osteogenic induction medium (OSTEO); OSTEO + AGEs; OSTEO + AGEs + BBR; OSTEO + AGEs + XAV-939; OSTEO + AGEs + BBR + CHIR-99021. Cells were seeded in 6-well plates (3x10⁴ cells/cm²); following 14 days of osteogenic induction, ALP staining was performed using a Cell Alkaline Phosphatase Activity Stain kit (Genmed Scientifics, Inc.) according to the manufacturer's protocols. ALP staining was observed under an inverted microscope (Olympus Corporation) and a total of 5 views per field were acquired.

ALP activity assay. According to the manufacturer's protocols, the ALP activity of cells was measured using a Cell Alkaline Phosphatase Activity Colorimetric Assay kit (Genmed Scientifics, Inc.). Cells were divided into groups as aforementioned. The specific operation procedures were as follows: hPDLSCs were seeded in 24-well plates (3x10⁴ cells/cm²) following culturing for 14 days with the various drug treatments. Cells were collected with cell scrapers after they were washed in Genmed reagent A and then mixed with Genmed reagent A again prior to centrifugation at 300 x g for 5 min at 4°C. The cell culture supernatant was discarded, and Genmed reagent B was added to lyse cells (15 sec), with subsequent incubation on ice for 30 min. Then, cells were subjected to additional centrifugation (300 x g, 5 min, 4°C). Finally, cell culture supernatants were collected, with one portion used for protein concentration detection (bicinchoninic acid assay) and the remainder frozen at -70°C for further ALP analysis. The ALP activity of each cell was determined from the OD value detected by a microplate reader at 405 nm, using p-nitrophenol as a standard, according to the manufacturer's protocols. The ALP activity of each sample was normalized to its protein concentration and presented as OD405/min/mg protein. The experiment was repeated three times.

Alizarin red staining for mineralization. Cells were divided into treatment groups as aforementioned. Cells were seeded in 6-well plates (3x10⁴ cells/cm²); following 21 days of osteogenic induction, alizarin red staining was performed using a Cell Alizarin Red Stain kit (Genmed Scientifics, Inc.), according to the manufacturer's protocols. Bright red stained areas indicated calcified nodules. Alizarin red staining was observed under an inverted microscope (Olympus Corporation) and a total of 5 views per field were acquired.

ELISA for human collagen type I. Cells were divided into treatment groups as aforementioned. Cells were seeded in 96-well plates (3x10⁴ cells/cm²); following 14 days of

Table I. Oligonucleotide primer sequences used in reverse transcription-quantitative PCR analysis.

Gene	Primer sequence (5'-3')	GenBank number	Product size (bp)
Runx2	F: CCGCCTCAGTGATTTAGGGC R: GGGTCTGTAATCTGACTCTGTCC	NM_001015051.3	132
Osterix	F: CTCACGACCTGTCCTGTCTT R: CAGCTCCACTCCTGTTCCAC	NM_001300837.1	151
ALP	F: AGATGTTTCGACGCCCCCTAA R: TGTGTTTCCCAGGAGAGAATGGAG	NM_031313.2	194
OPN	F: CATAACAAGGCCATCCCCGTT R: TGGGTTTCAGCACTCTGGTC	NM_000582.2	112
Collagen I	F: GCCAAGACGAAGACATCCCA R: GGCAGTTCTTGGTCTCGTCA	NM_000088.3	156
OCN	F: ATTGTGGCTCACCTCCATCA R: AGGGCTATTTGGGGGTCATC	NM_199173.5	119
β -catenin	F: GCGCCATTTTAAGCCTCTCG R: GAGTAGCCATTGTCCACGCT	NM_001098209.1	234
LRP5	F: CTGCTGGGGGACTTCATCTAC R: GATGCTGCTCTCTCCAGTGTA	NM_001291902.1	176
DKK-1	F: CAGGCGTGCAAATCTGTCTC R: GGAATACCCATCCAAGGTGCT	NM_012242.3	191
GSK-3 β	F: GACTAAGGTCTTCCGACCCC R: GATGGTAGCCAGAGGTGGATT	NM_001146156.1	226
β -actin	F: AGGCTCTTTTCCAGCCTTCC R: AATGCCAGGGTACATGGTGG	NM_001101.4	152

Runx2, runt-related transcription factor 2; ALP, alkaline phosphatase; OPN, osteopontin; Collagen I, collagen type I; OCN, osteocalcin; LRP5, low-density lipoprotein receptor-related protein 5; DKK-1, dickkopf-1; GSK-3 β , glycogen synthase kinase 3 β ; F, forward; R, reverse.

osteogenic induction, the cell culture supernatant in each well was collected to quantify the levels of osteoblast-associated protein human collagen type I (Collagen I). Each sample was analyzed in duplicate. An ELISA was performed to detect the expression levels of Collagen I in each group using a Human Collagen type I ELISA kit (cat. no. CSB-E08082h; Cusabio Technology LLC), according to the manufacturer protocols. This experiment was repeated three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The mRNA expression levels of Runx2, Osterix, ALP, OPN, Collagen I, OCN, β -catenin, low-density lipoprotein receptor-related protein 5 (LRP5), dickkopf-1 (DKK-1) and GSK-3 β were determined by RT-qPCR analysis. Cells were divided into treatment groups as aforementioned. hPDLSCs in each group were harvested from 25 cm² polystyrene tissue culture flasks following 7 days of osteogenic induction. Total RNA of hPDLSCs was extracted and DNase-treated using a RNeasy Mini kit (Qiagen GmbH) according to the manufacturer's protocols, and then an ultraviolet spectrophotometer was used to assess the purity and quantity of the RNA preparation. RNA (2 μ g) was reverse transcribed into complementary DNA (cDNA) using Promega Reverse Transcription System (cat. no. Promega A3500; Promega Corporation) with the

following parameters: 60 min at 42°C, 5 min at 95°C, and 3 min at 4°C. 40 μ l cDNA was used for DNA amplification. Gene expression levels were quantified via qPCR analysis with SYBR Green Realtime PCR Master Mix (Toyobo Life Science), according to the manufacturer's protocols, in an ABI PRISM 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was conducted as follows: 60 sec at 95°C, then 40 cycles of 15 sec at 94°C, 15 sec at 60°C and 30 sec at 72°C, and a final melt curve stage of 15 sec at 95°C, 60 sec at 60°C, 30 sec at 95°C and 15 sec at 60°C. β -actin was used as an internal reference; the mRNA levels of target genes were normalized to β -actin. The relative mRNA levels were quantified using the $2^{-\Delta\Delta C_q}$ method (28). The primer sequences used in this study for β -actin, Runx2, Osterix, ALP, OPN, Collagen I, OCN, β -catenin, LRP5, DKK-1 and GSK-3 β are listed in Table I. All experiments were performed three times.

Western blot analysis. Cells were divided into treatment groups as aforementioned. hPDLSCs in each group were harvested from 75 cm² polystyrene tissue culture flasks following 7 days of osteogenic induction prior to protein extraction. Protein extraction was conducted on ice or at 4°C. In brief, cells were washed twice with cold PBS prior to disruption in SDS lysis buffer (cat. no. KGP706; KeyGen

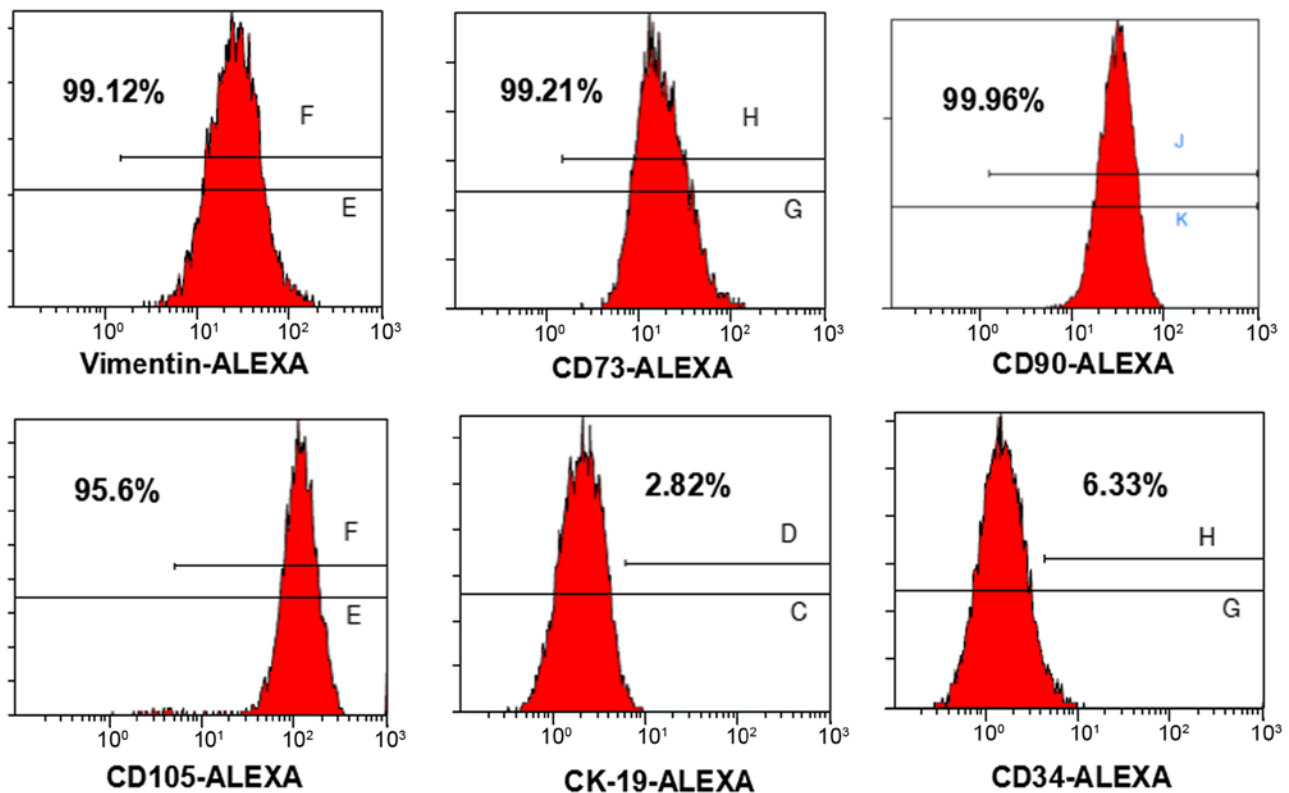


Figure 1. Analysis of cell surface markers in hPDLSCs. The expression of various cell markers was investigated in hPDLSCs using flow cytometry. hPDLSCs, human periodontal ligament stem cells; CD, cluster of differentiation; CK-19, cytokeratin-19.

Biotech Co., Ltd.). Then, cell lysates were collected, placed at 4°C for 30-60 min and then centrifuged at 4°C for 5 min (13,800 x g). Cell supernatants were collected for protein concentration determination using a bicinchoninic acid assay, according to the manufacturer's protocols. Then, protein denaturation was conducted by boiling in 6X SDS sample buffer for 5 min prior to protein (40 µg) separation via 10% SDS-PAGE. The separated proteins were then transferred onto PVDF membranes (EMD Millipore). Membranes were blocked with 5% skim milk for 1 h at room temperature, and subsequently incubated with primary antibodies at 4°C overnight. The primary antibodies were: Anti-β-actin (1:2,500; cat. no. A5316); anti-Runx2 (1:460; cat. no. ab23981); anti-BSP (1:200; cat. no. ab52128); anti-OPN (1:2,000; cat. no. ab91655); anti-OCN (1:500; cat. no. ab13420); anti-β-catenin (1:1,000; cat. no. ab32572); anti-GSK3β (1:1,000; cat. no. 12456) and anti-wnt3a (1:500; cat. no. ab28472). Membranes were then incubated with HRP-conjugated rabbit anti-mouse IgG (1:10,000; cat. no. A9044) and HRP-conjugated goat anti-rabbit IgG (1:20,000; cat. no. A0545) secondary antibodies at room temperature for 1 h. The labeled protein bands were visualized using Immobilon® enhanced chemiluminescence western HRP substrate (EMD Millipore). All experiments were repeated three times.

Immunofluorescence (IF) assay. To detect nuclear translocation of β-catenin, IF staining was performed following 7 days of osteogenic induction of hPDLSCs. Cells were divided into treatment groups as aforementioned. Cells were

cultured in 96-well plates at a density of 5×10^3 cells/well, and then rinsed twice in warm PBS. Then, cells were fixed with 4% PFA for 20 min at room temperature, permeabilized with 0.1% Triton X-100 for 8-10 min at 4°C and blocked with a mixture of 0.2% BSA and 0.02% Tween-20 for 20 min at room temperature. Cells were then incubated with rabbit anti-β-catenin monoclonal antibody (1:250; cat. no. ab32572) overnight at 4°C, followed by Alexa Fluor 488-conjugated-goat anti-rabbit secondary antibody (1:400; cat. no. A-11034) for 35 min at 37°C in the dark. Following incubation with DAPI (1:100) for 2 min at room temperature in the dark for cell nucleus identification, cells were washed twice for 5 min in PBS. Cells were observed, and images were captured and analyzed under an inverted immunofluorescence microscopy (Olympus Corporation). A total of 5 views per field were acquired.

Statistical analysis. Statistical analysis in this study was performed using GraphPad Prism 7 (GraphPad Software, Inc.). Comparisons between groups were performed using one-way analysis of variance followed by Tukey's multiple comparison test, and all values in this study were presented as the mean ± standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Flow cytometry analysis of hPDLSCs. All of the hPDLSCs cultured in the present study positively expressed mesenchymal stem cell (MSC)-associated surface markers,

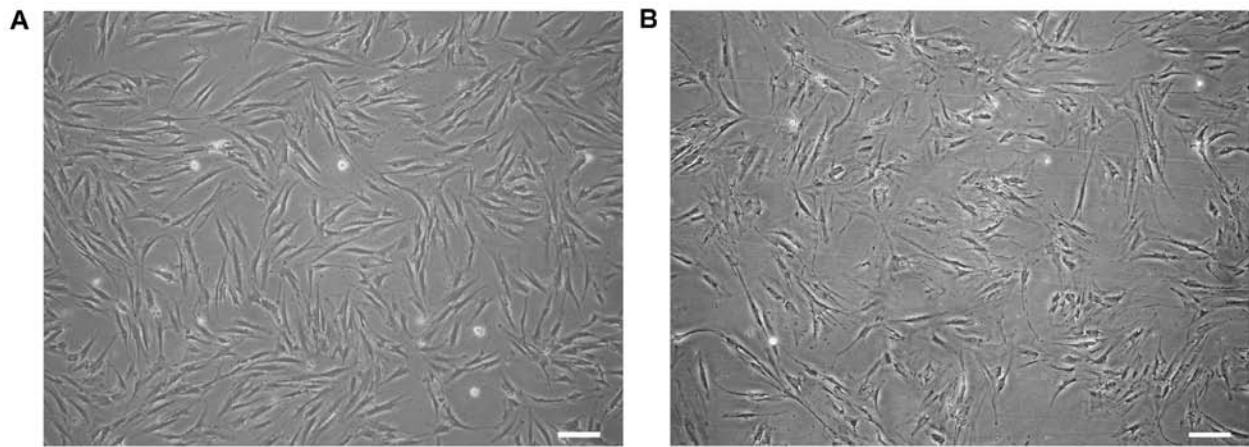


Figure 2. Morphology of hPDLSCs prior to and following osteogenic induction. (A) Morphology of hPDLSCs in standard culture conditions. (B) Morphology of hPDLSCs following culturing in osteogenic induction medium for 3 days. Scale bar, 200 μ m; magnification, x40. hPDLSCs, human periodontal ligament stem cells.

including CD73, CD90 and CD105, but were negative for the hematopoietic marker CD34 (Fig. 1). Additionally, hPDLSCs expressed vimentin but not CK-19 (Fig. 1). The results indicated that the hPDLSCs used in the study were pure mesenchymal cells that were derived from the mesoderm, and not the ectoderm.

Morphological alterations in hPDLSCs following osteogenic induction. Cells underwent notable morphological alterations following osteogenic induction. Prior to osteogenic induction, hPDLSCs grew by static adherence and remained in the form of a monolayer. The cells established a typical long spindle shape with oval nuclei comprising 2-3 nucleoli, as observed under an inverted phase contrast microscope (Fig. 2A). Following osteogenic induction, the cells exhibited adherent growth characteristics and diverse morphology. They also exhibited noticeably increased local aggregation. Similar to osteoblasts, they appeared as thickened fusiform, polygonal or irregular triangular cells with pseudopodia (Fig. 2B).

Osteogenic differentiation of hPDLSCs is impaired in an AGE-enriched microenvironment. To determine the effects of AGEs on the osteogenic differentiation ability of hPDLSCs, ALP activity assays, ALP staining, alizarin red staining, ELISA, and RT-qPCR and western blot analyses were performed following treatment with osteogenic induction medium for various numbers of days. The OSTEOP group served as the control. It was demonstrated that the osteogenic potential of hPDLSCs significantly decreased in the OSTEOP + AGEs group compared with the control group (Fig. 3). The ALP activity of hPDLSCs in the OSTEOP + AGEs group was significantly decreased compared with the control group following 14 days of osteogenic induction ($P < 0.01$; Fig. 3A). The degree of ALP staining was markedly decreased in the AGEs-enriched microenvironment following 14 days of osteogenic induction compared with the control (Fig. 3B). Following 21 days of culture in the osteogenic induction medium, the hPDLSCs in the two groups formed mineralized nodules that exhibited positive alizarin red staining; however, the cells in the OSTEOP+AGEs

group formed fewer mineralized nodules compared with the control group (Fig. 3C). Following 14 days of osteogenic induction, ELISA revealed that the levels of Collagen I in the OSTEOP + AGEs group were significantly decreased compared with the control ($P < 0.01$; Fig. 3D). Additionally, RT-qPCR and western blot analyses revealed that the expression levels of osteogenesis-associated genes Runx2, Osterix, ALP, OPN, Collagen I and OCN were significantly downregulated in the OSTEOP + AGEs group compared with the control (Fig. 3E). Finally, the expression levels of osteogenesis-associated proteins Runx2, OPN, BSP and OCN were markedly downregulated in the OSTEOP + AGEs group (Fig. 3F). These findings suggested that the administration of AGEs reduced the osteogenic differentiation ability of hPDLSCs.

BBR reverses the AGE-induced inhibition of the osteogenic differentiation of hPDLSCs. The effect of BBR was then examined on the AGE-induced reduction in the osteogenic potential of hPDLSCs. The results revealed that BBR could partially rescue the AGE-induced inhibition of the osteogenic differentiation ability of hPDLSCs (Fig. 3). As presented in Fig. 3A, the ALP activity of hPDLSCs in the OSTEOP + AGEs + BBR group was significantly increased compared with the OSTEOP + AGEs group ($P < 0.01$). Additionally, as presented in Fig. 3B and C, BBR notably promoted the expression of ALP and formation of mineralized nodules in hPDLSCs. Collagen I levels were significantly upregulated in the OSTEOP + AGEs + BBR group compared with the OSTEOP + AGEs group ($P < 0.01$; Fig. 3D). In addition, RT-qPCR and western blot analyses demonstrated that the expression levels of osteogenesis-associated genes and proteins in the OSTEOP + AGEs + BBR group were significantly upregulated following osteogenic induction for 7 days compared with the OSTEOP + AGEs group (Fig. 3E and F). Collectively, the results suggested that administration of BBR in an AGE-enriched microenvironment attenuated the AGE-induced decrease in the osteogenic differentiation ability of hPDLSCs.

AGEs decrease the osteogenic differentiation ability of hPDLSCs via the activation of the canonical Wnt/ β -catenin pathway. To investigate the mechanism by which AGEs reduce

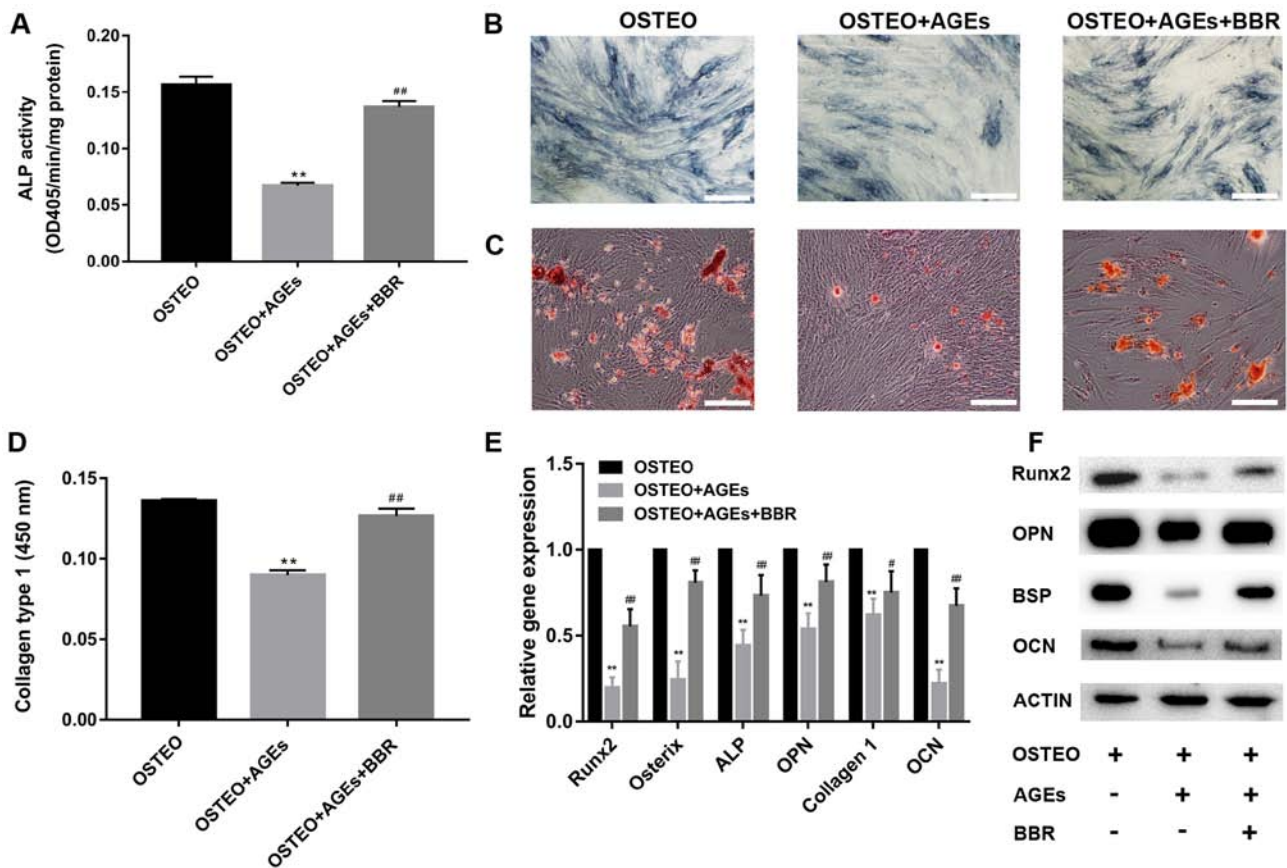


Figure 3. Effects of AGEs and BBR on the osteogenic differentiation of hPDLSCs. (A) ALP activity of hPDLSCs (n=3). (B) ALP staining of hPDLSCs (scale bar, 100 μ m; magnification, x100). (C) Alizarin red staining of hPDLSCs (scale bar, 100 μ m; magnification, x100). (D) Levels of Collagen I measured by ELISA (n=3). (E) Reverse transcription-quantitative polymerase chain reaction analysis of mRNA expression levels for osteoblast marker genes Runx2, Osterix, ALP, OPN, Collagen I and OCN (n=3). (F) Western blot analysis of osteoblast marker proteins Runx2, OPN, BSP, and OCN. Data are presented as the mean \pm standard deviation. **P<0.01 vs. OSTEO; #P<0.05, ##P<0.01 vs. OSTEO+AGEs. AGEs, advanced glycation end products; BBR, berberine hydrochloride; hPDLSCs, human periodontal ligament stem cells; ALP, alkaline phosphatase; Collagen I, collagen type I; Runx2, runt-related transcription factor 2; OPN, osteopontin; OCN, osteocalcin; BSP, bone sialoprotein; OSTEO, osteogenic induction medium; ACTIN, β -actin.

the osteogenic differentiation ability of hPDLSCs, RT-qPCR and western blot analyses were conducted following 7 days of osteogenic induction to determine the expression levels of canonical Wnt/ β -catenin pathway-related genes and proteins, including β -catenin, LRP5, GSK-3 β , DKK-1 and wnt3a. It was revealed that AGE administration significantly upregulated the mRNA expression levels of β -catenin and LRP5, and down-regulated those of GSK-3 β and DKK-1 (Fig. 4A). Additionally, the protein expression levels of wnt3a and β -catenin were notably increased, while those of GSK-3 β were decreased, in the OSTEO + AGEs group compared with the OSTEO group (Fig. 4B). IF staining was then conducted to evaluate the nuclear translocation of β -catenin, as a marker of Wnt/ β -catenin pathway activation. The results indicated that AGEs promoted the nuclear translocation of β -catenin compared with control (Fig. 4C).

To determine whether activation of the canonical Wnt/ β -catenin pathway was required in the AGE-induced suppression of the osteogenic potential of hPDLSCs, the small molecule inhibitor XAV-939 was used. As presented in Fig. 5A-D, addition of XAV-939 resulted in attenuation of the effects of AGEs on ALP levels, nodule formation and Collagen I levels. Furthermore, XAV-939 reversed the decreased expression of osteoblast markers in hPDLSCs compared with the

OSTEO + AGEs group, with most genes and proteins tested being upregulated following XAV-939 treatment except for OPN (Fig. 5E and F). The results revealed that AGEs decreased the osteogenic differentiation ability of hPDLSCs partially by activating the canonical Wnt/ β -catenin pathway.

BBR increases the osteogenic differentiation ability of hPDLSCs in an AGE-enriched microenvironment by inhibiting the canonical Wnt/ β -catenin pathway. The mechanism by which BBR rescued the inhibited osteogenic potential of AGEs-treated hPDLSCs was investigated. As presented in Fig. 6A and B, a significant decrease in the gene expression levels of β -catenin and LRP5 (P<0.01), and notable decrease in the protein expression levels of wnt3a and β -catenin was observed in the OSTEO + AGEs + BBR group compared with the OSTEO + AGEs group. Additionally, the gene expression levels of GSK-3 β and DKK-1, and the protein expression levels of GSK-3 β were markedly increased in the OSTEO + AGEs + BBR group compared with the OSTEO + AGEs group (Fig. 6A and B). IF staining revealed that BBR decreased the nuclear translocation of β -catenin compared with the OSTEO + AGEs group (Fig. 6C).

To further examine whether the canonical Wnt/ β -catenin pathway was involved in the BBR function in hPDLSCs, a

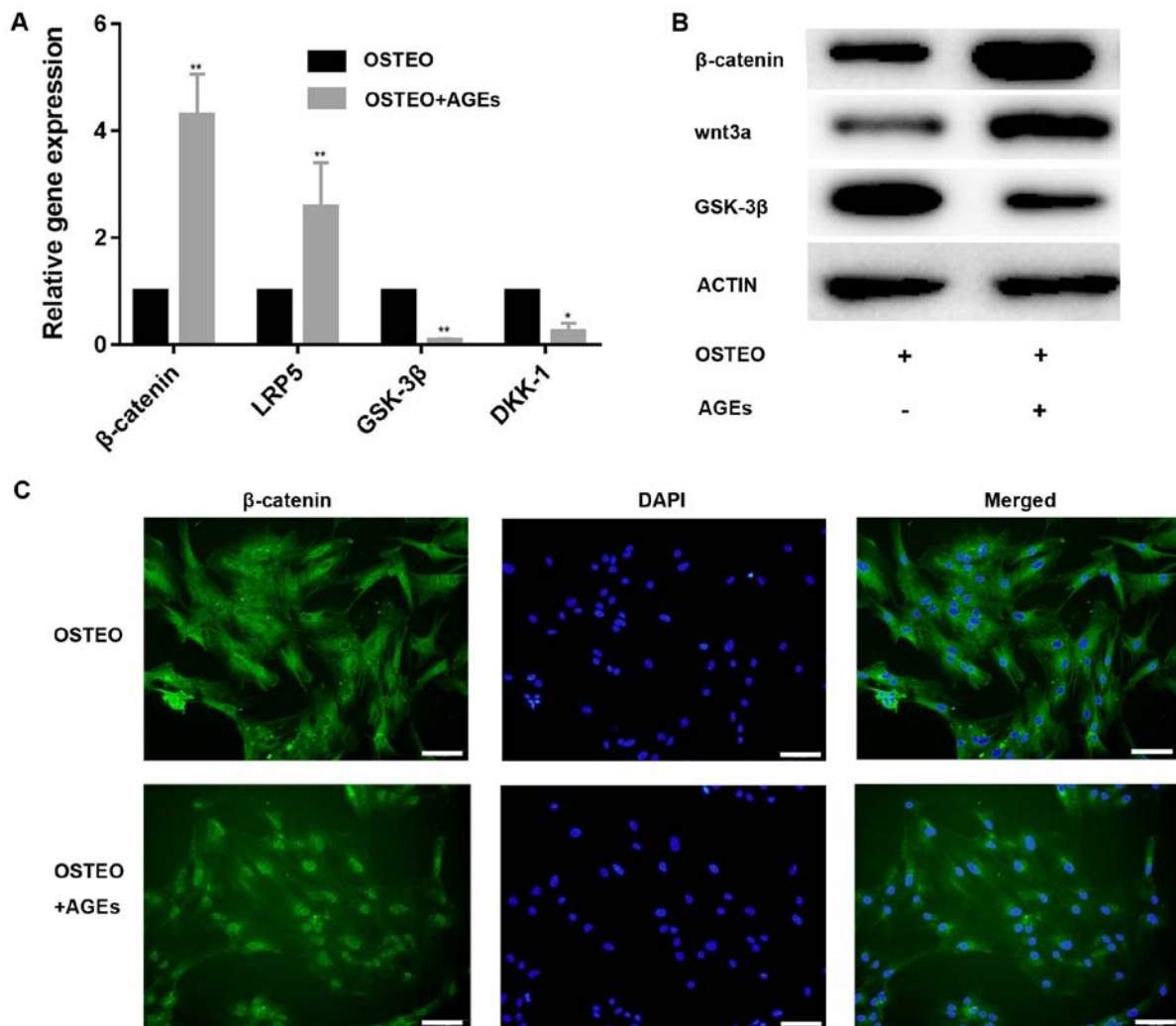


Figure 4. Effects of AGEs on the expression levels of canonical Wnt/ β -catenin pathway-associated genes and proteins in hPDLSCs. (A) Reverse transcription-quantitative polymerase chain reaction analysis of Wnt/ β -catenin pathway-associated genes β -catenin, LRP5, GSK-3 β and DKK-1 ($n=3$). (B) Western blot analysis of Wnt/ β -catenin-pathway related proteins wnt3a, β -catenin and GSK-3 β . (C) Immunofluorescence staining demonstrating the nuclear translocation of β -catenin (scale bar, 100 μ m; magnification, $\times 100$). Data are presented as the mean \pm standard deviation. * $P<0.05$, ** $P<0.01$ vs. OSTEO. AGEs, advanced glycation end products; hPDLSCs, human periodontal ligament stem cells; LRP5, low-density lipoprotein receptor-related protein 5; GSK-3 β , glycogen synthase kinase 3 β ; DKK-1, dickkopf-1; OSTEO, osteogenic induction medium; ACTIN, β -actin.

small molecule activator CHIR-99021 was used. As presented in Fig. 7A-F, the addition of CHIR-99021 reversed the effects of BBR on ALP activity, nodule formation and Collagen I levels in AGE-treated hPDLSCs, and resulted in a significant decrease in the gene expression of all osteoblast markers of hPDLSCs compared to with OSTEO + AGEs + BBR group. Of note, the activity levels of ALP, the levels of Collagen I in the supernatant, and the mRNA expression levels of Runx2 and Osterix remained significantly increased compared with in the OSTEO + AGEs group ($P<0.01$; Fig. 7A, D and E). The present results indicated that BBR promoted the osteogenic differentiation ability of hPDLSCs in an AGE-enriched microenvironment at least in part by inhibiting the canonical Wnt/ β -catenin signaling pathway.

Discussion

Periodontal disease is a widespread, infectious and inflammatory oral disease that, not only seriously affects

human oral health, but also aggravates the pathogenesis of systemic diseases, such as DM and atherosclerotic cardiovascular diseases (29,30). Conventional treatments for periodontal disease, including scaling and root planning, are effective, but fail to achieve ideal periodontal tissue regeneration or control inflammation (31). The regeneration of impaired periodontal tissues remains a major challenge in clinical practice due to the complex structure of the periodontium (32). Therefore, to develop novel and more effective periodontal therapeutic regimens, improved understanding of the molecular and cellular mechanisms involved in the development of periodontal disease are required. Stem cell-based regenerative approaches combined with the use of emerging biomaterials are a promising therapeutic approach for periodontal disease (32). As a novel population of MSCs isolated from the periodontal ligament (PDL), hPDLSCs possess self-renewal capacity and superior multidirectional differentiation ability to regenerate periodontal tissues and maintain periodontal ligament integrity (33). Additionally, some studies have identified

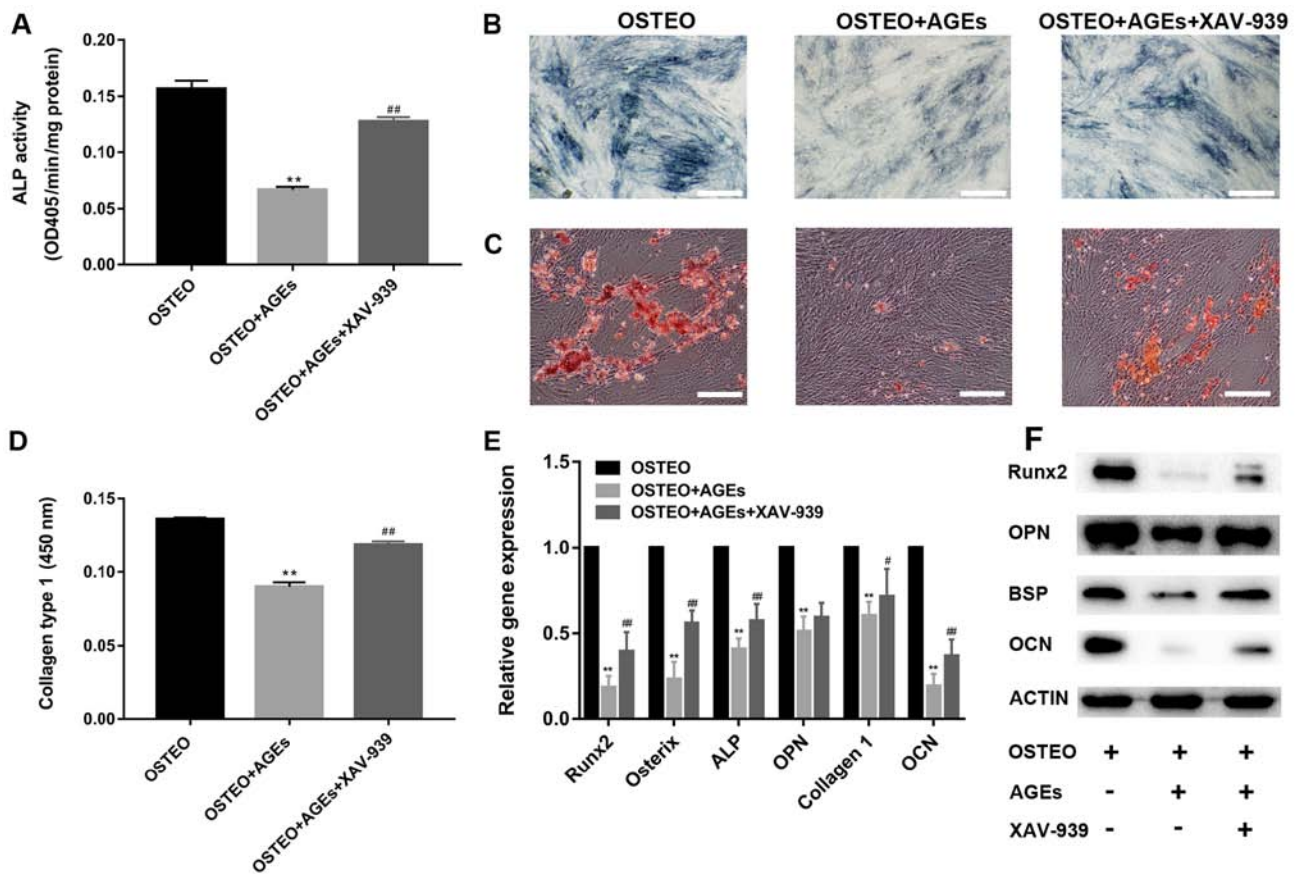


Figure 5. Effects of XAV-939 on the osteogenic differentiation of hPDLSCs in AGEs microenvironment. (A) ALP activity of hPDLSCs (n=3). (B) ALP staining of hPDLSCs (scale bar, 100 μ m; magnification, x100). (C) Alizarin red staining of hPDLSCs (scale bar, 100 μ m; magnification, x100). (D) Levels of Collagen I measured by ELISA (n=3). (E) Reverse transcription-quantitative polymerase chain reaction analysis of osteoblast marker genes Runx2, Osterix, ALP, OPN, Collagen I and OCN (n=3). (F) Western blot analysis of osteoblast marker proteins Runx2, OPN, BSP, and OCN. Data are presented as the mean \pm standard deviation. **P<0.01 vs. OSTEO; *P<0.05, ##P<0.01 vs. OSTEO + AGEs. hPDLSCs, human periodontal ligament stem cells; AGEs, advanced glycation end products; ALP, alkaline phosphatase; Collagen I, collagen type I; Runx2, runt-related transcription factor 2; OPN, osteopontin; OCN, osteocalcin; BSP, bone sialoprotein; OSTEO, osteogenic induction medium; ACTIN, β -actin.

various advantages of hPDLSCs compared with human bone marrow mesenchymal stem cells (34,35). Thus, hPDLSCs have been regarded as reliable seed cells for periodontal tissue regeneration (36,37).

An increasing number of studies have demonstrated the close association between DM and periodontal disease (2,38,39). Among the aggravating factors of DM, AGEs are considered to be salient factors (40). It was previously revealed that a decrease in the number, and suboptimal function of hPDLSCs in patients with periodontitis indicated poor repair capacity with respect to tissue regeneration (41); however, whether AGEs induce adverse effects on the function of hPDLSCs in individuals with diabetes-associated periodontitis remains unclear. Wang *et al* (25) introduced 200 μ g/ml AGEs into cell culture medium to mimic DM. In the present study, the impact of AGEs on the osteogenic differentiation potential of hPDLSCs *in vitro* was investigated via a variety of techniques. hPDLSCs were first induced by osteogenic induction medium, and alterations in cell morphology indicated that the osteogenesis was induced, supporting the use of this medium for subsequent experiments (Fig. 2). The findings of the present study indicated that the administration of AGEs reduced the osteogenic differentiation ability of hPDLSCs, consistent with previous studies (42,43).

Based on the aforementioned reasons, the target in periodontal regeneration with DM is to rescue the impaired osteogenic differentiation ability of hPDLSCs. BBR, an isoquinoline alkaloid, has been used as an antidiarrheal, anti-inflammatory, antihypertensive and antiarrhythmic agent (44,45). A previous study also indicated that BBR was effective against glucose and lipid metabolism disorders (15). In addition, BBR can alleviate the risk of osteoporosis, increase bone density, inhibit osteoclast activity, and serve a role in bone protection (46); however, the effects of BBR on the osteogenic differentiation ability of hPDLSCs remain unclear. Therefore, the present study investigated the effect of 1 μ mol/l BBR on AGEs-induced impairment of the osteogenic differentiation ability of hPDLSCs. It was demonstrated that the expression levels of osteoblast-associated genes Runx2, Osterix, ALP, OPN, Collagen I and OCN were upregulated following BBR application, but were not restored entirely to control levels, suggesting that BBR only partially reversed the decreased osteogenic potential of hPDLSCs in an AGEs-enriched microenvironment. These genes are expressed during different stages of osteogenic differentiation, and regulate the secretion and mineralization of bone matrix. The rescued expression of the genes suggested that BBR promoted bone regeneration at various stages.

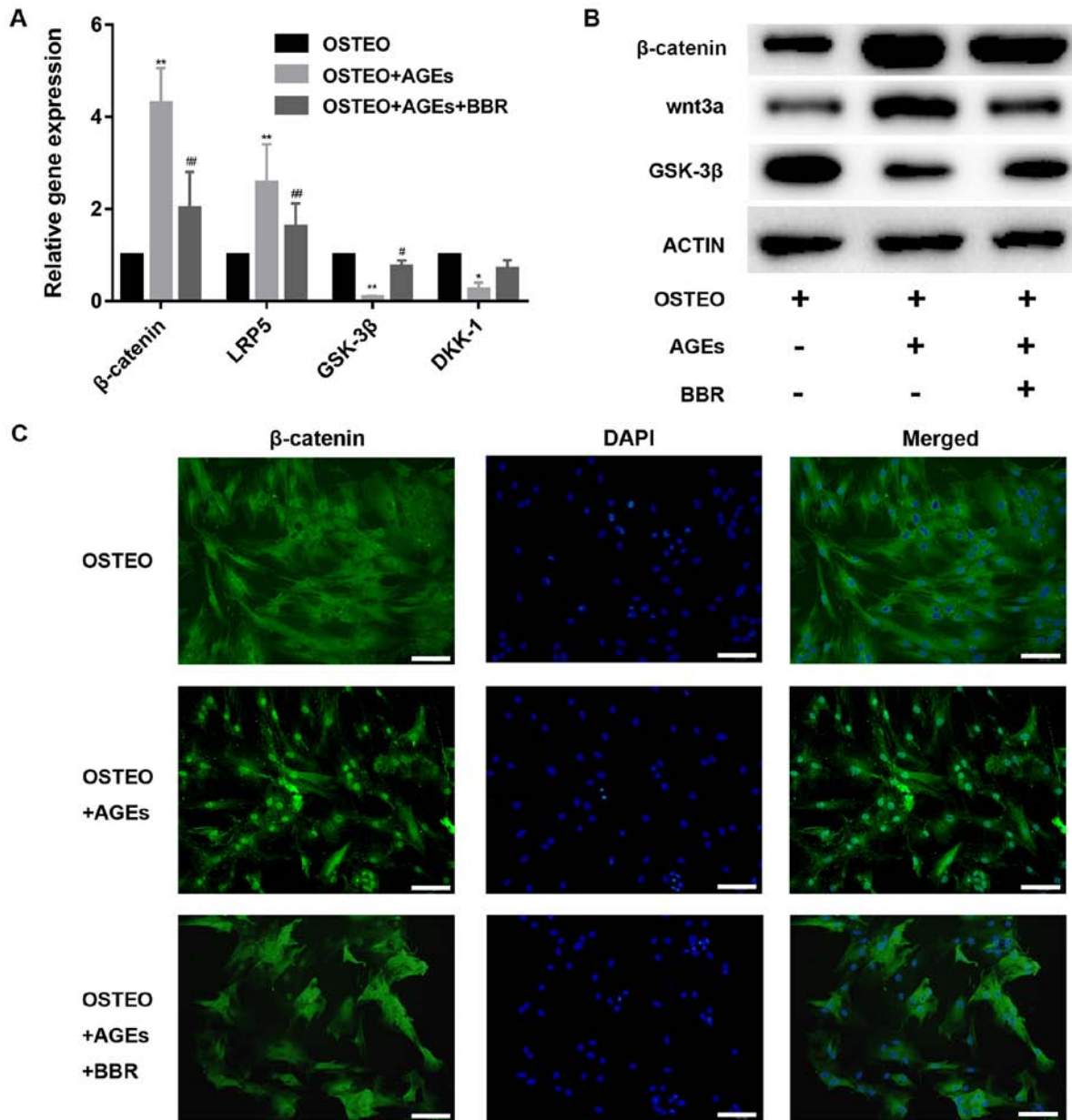


Figure 6. Effects of BBR on the expression levels of canonical Wnt/β-catenin pathway-associated genes and proteins in hPDLSCs. (A) Reverse transcription-quantitative polymerase chain reaction analysis of Wnt/β-catenin pathway-associated genes β-catenin, LRP5, GSK-3β and DKK-1 (n=3). (B) Western blot analysis of Wnt/β-catenin pathway-related proteins wnt3a, β-catenin and GSK-3β. (C) Immunofluorescence staining demonstrating the nuclear translocation of β-catenin (scale bar, 100 μm; magnification, x100). Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. OSTEO; #P<0.05, ##P<0.01 vs. OSTEO + AGEs. BBR, berberine hydrochloride; hPDLSCs, human periodontal ligament stem cells; LRP5, low-density lipoprotein receptor-related protein 5; GSK-3β, glycogen synthase kinase 3β; DKK-1, dickkopf-1; OSTEO, osteogenic induction medium; AGEs, advanced glycation end products; ACTIN, β-actin.

The precise molecular mechanisms underlying the osteogenic differentiation ability of hPDLSCs are yet to be fully determined. The multi-directional differentiation potential of stem cells can be modulated by different signaling pathways, including the Wnt/β-catenin signaling pathway (47). Wnts are a family of 19 secreted glycoproteins that mediate the developmental and post-developmental physiology by regulating cellular processes, including proliferation, differentiation and apoptosis (16,48). When the canonical Wnt/β-catenin signaling pathway is activated, GSK-3β is inhibited. Then, β-catenin accumulates, and is translocated to the nucleus and binds to T-cell factor/lymphoid enhancer-binding factor transcription factors, resulting in the transcription of Wnt downstream target

genes (49). Conversely, when the canonical Wnt/β-catenin signaling pathway is inhibited, β-catenin becomes a part of the cytosolic protein complex that consists of axin, adenomatous polyposis Collagen I and GSK-3β. In this protein complex, GSK-3β phosphorylates β-catenin, resulting in its ubiquitination and subsequent degradation by the proteasome (50). A genetic study has reported that the Wnt signaling pathway serves an important role in bone homeostasis (3). de Boer *et al* (22) reported that Wnt3a strongly inhibited the dexamethasone-induced expression of the osteogenic marker ALP. Liu *et al* (42) investigated the multiple differentiation potential of PDLSCs in donors with chronic periodontitis and DM, and observed that DKK-1-mediated inhibition of WNT signaling rescued the

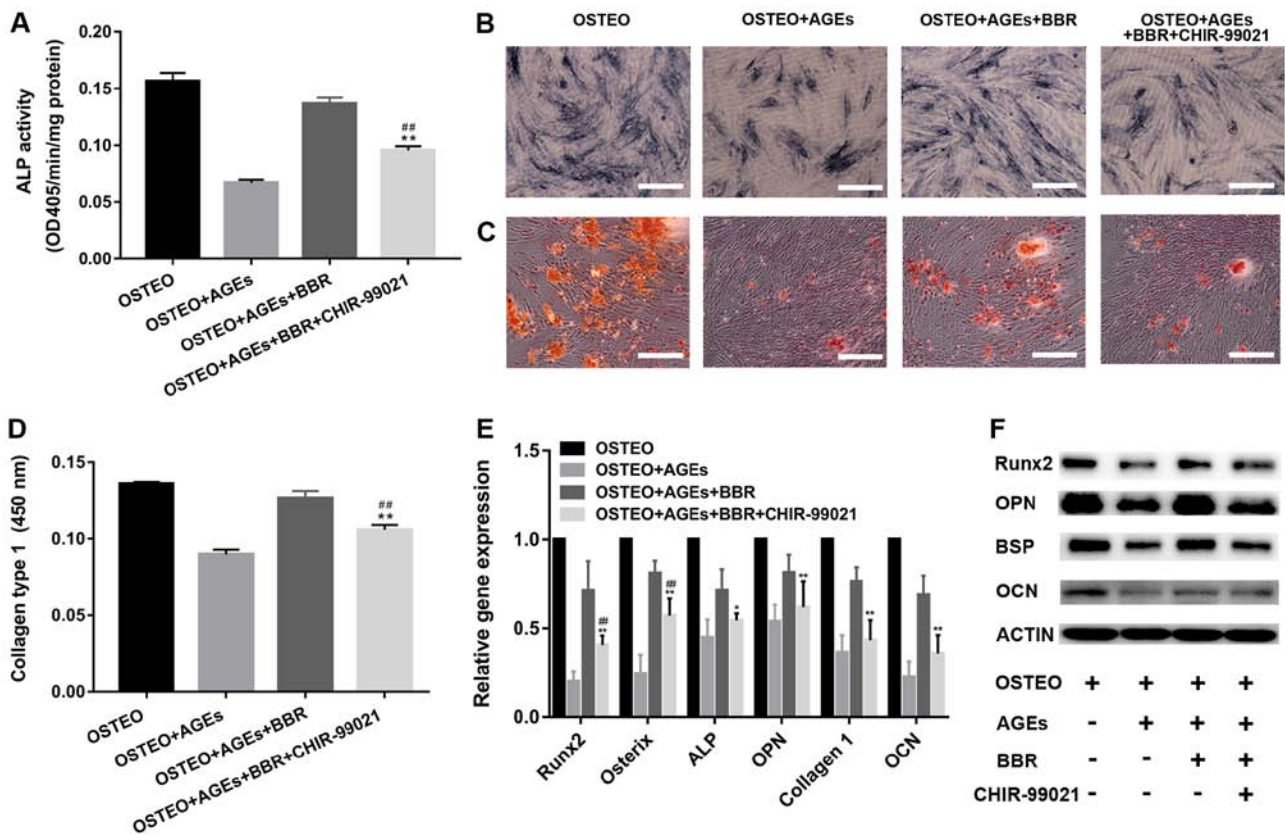


Figure 7. Effects of CHIR-99021 on the osteogenic differentiation of hPDLSCs. (A) ALP activity of hPDLSCs (n=3). (B) ALP staining of hPDLSCs (scale bar, 100 μ m; magnification, x100). (C) Alizarin red staining of hPDLSCs (scale bar, 100 μ m; magnification, x100). (D) Levels of Collagen I measured by ELISA (n=3). (E) Reverse transcription-quantitative polymerase chain reaction analysis of osteoblast marker genes Runx2, Osterix, ALP, OPN, Collagen I and OCN (n=3). (F) Western blot analysis of osteoblast marker proteins Runx2, OPN, BSP, and OCN. Data are presented as the mean \pm standard deviation. * P <0.05, ** P <0.01 vs. OSTEO + AGEs + BBR; ## P <0.01 vs. OSTEO + AGEs. hPDLSCs, human periodontal ligament stem cells; ALP, alkaline phosphatase; Collagen I, collagen type I; Runx2, runt-related transcription factor 2; OPN, osteopontin; OCN, osteocalcin; BSP, bone sialoprotein; OSTEO, osteogenic induction medium; AGEs, advanced glycation end products; BBR, berberine hydrochloride; ACTIN, β -actin.

potential of PDLSCs in patients with co-morbid periodontitis and DM. Considering the important role of the Wnt signaling pathway in bone remodeling, the present study investigated the effects of AGEs and BBR on canonical Wnt/ β -catenin signaling to determine the molecular mechanisms underlying these effects. The results revealed that AGEs significantly activated the canonical Wnt/ β -catenin pathway by upregulating the gene expression levels of β -catenin and LRP5, and the protein expression levels of wnt3a and β -catenin, and downregulating the gene expression levels of GSK-3 β and DKK-1, and the protein expression levels of GSK-3 β compared with the control group. Furthermore, AGEs promoted the nuclear translocation of β -catenin compared with the control. Conversely, the application of BBR induced opposing effects on Wnt/ β -catenin pathway-associated genes and proteins in AGEs-treated cells.

To further verify whether the canonical Wnt/ β -catenin pathway was indeed involved in regulating the osteogenic potential of hPDLSCs, an inhibitor and an activator of Wnt/ β -catenin signaling were utilized in the present study. XAV-939 selectively suppresses the transcription of Wnt/ β -catenin by inhibiting tankyrase 1/2, and is often used to inhibit the Wnt/ β -catenin signaling pathway in osteogenesis or stem cells (51). CHIR-99021 is a highly specific inhibitor of GSK3, thereby activating the Wnt/ β -catenin signaling pathway; CHIR-99021 has been applied as an agonist of Wnt/ β -catenin signaling (27,52,53). Therefore,

XAV-939 and CHIR-99021 were selected to further investigate the role of canonical Wnt/ β -catenin pathway in osteogenic differentiation responses. The data in Fig. 5A-F revealed that XAV-939 reversed the decreased expression of the majority of osteoblast markers of hPDLSCs in the OSTEO + AGEs group, with the exception of OPN. This may be because bone matrix proteins, including OPN, BSP and OCN, are produced by mature osteoblasts during bone formation and tissue mineralization, and the expression of OPN is generally regarded as an intermediate stage marker of osteogenic differentiation (54-56). The addition of XAV-939 may not affect this intermediate stage osteoblast marker; however, the underlying mechanism requires further investigation. Overall, it was demonstrated that AGEs decreased the osteogenic differentiation ability of hPDLSCs partially by activating canonical Wnt/ β -catenin pathway. Additionally, as presented in Fig. 3A-F, BBR increased the expression of all osteoblast markers of hPDLSCs compared with the OSTEO + AGEs group; however, the data in Fig. 7A-F revealed that the addition of CHIR-99021 resulted in a significant decrease in the expression of all osteoblast markers of hPDLSCs compared to the OSTEO + AGEs + BBR group. Of note, the activity levels of ALP, the supernatant levels of Collagen I, and the mRNA expression of Runx2 and Osterix remained significantly increased compared with the OSTEO + AGEs group. These results indicated that CHIR-99021 only partially

reversed the effects of BBR on the osteogenic differentiation of AGEs-treated hPDLSCs, suggesting that BBR increased the osteogenic differentiation ability of hPDLSCs in an AGE-enriched microenvironment partially by inhibiting the canonical Wnt/ β -catenin pathway. There may be additional signaling pathways involved in this process, which require further investigation in subsequent studies. The results suggested that canonical Wnt/ β -catenin signaling may serve an important role in the osteogenic differentiation ability of hPDLSCs. It was hypothesized that regulation of canonical Wnt/ β -catenin pathway may affect the noncanonical Wnt/ Ca^{2+} pathway, which in turn may lead to an increase in the osteogenic potential of hPDLSCs; however, the specific mechanism requires further investigation.

In conclusion, the present *in vitro* study demonstrated that AGEs attenuated the osteogenic differentiation ability of hPDLSCs, partially by activating the canonical Wnt/ β -catenin pathway; however, the application of BBR reversed the inhibition of the osteogenic potential of hPDLSCs in an AGEs-enriched microenvironment, partially by inhibiting the canonical Wnt/ β -catenin pathway. These results suggested that BBR may induce potential therapeutic effects by promoting the osteogenic differentiation ability of hPDLSCs in patients with diabetes-associated periodontitis. Further *in vivo* studies should be conducted to validate the clinical efficacy of BBR as an adjuvant to attenuate damage to periodontal tissues in treating diabetes-associated 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

JZ and BHX were involved in the experimental design and revised the important technical and theoretical content of the manuscript; LNZ drafted the manuscript; LNZ and ZW performed the experiments; LNZ, XXW and KYL collected and analyzed the data and assisted in drafting the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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