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Baicalein enhances the osteogenic differentiation of human periodontal ligament cells by activating the Wnt/ β -catenin signaling pathway

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Highlights

- Baicalein enhanced the osteogenic differentiation of hPDLs.
- The optimal dosage of baicalein was 10 μ M.
- DKK-1 significantly reversed the effects of baicalein on hPDLs.
- The effects are exerted by activating the Wnt/ β -catenin signaling pathway.

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ABSTRACT

Objective: Periodontium regeneration is one of the most important processes for periodontitis therapy. Human periodontal ligament cells (hPDLs) play a vital role in the repair and regeneration of periodontal tissues. Our study aimed to investigate the mechanisms underlying the promotion of hPDLs osteogenic differentiation by baicalein.

Design: hPDLs were obtained from periodontal ligament (PDL) tissues by primary culture. The MTT assay was used to determine the growth curves of hPDLs treated with different concentrations of baicalein (1.25, 2.5, 5, or 10 μ M). Alkaline phosphatase (ALP) staining and Alizarin red S staining were performed to assess osteogenic differentiation of hPDLs administered baicalein. Osteogenic differentiation-related gene and protein expression levels and Wnt/ β -catenin pathway signal changes were assessed by qRT-PCR and Western blotting analysis.

Results: The results showed that baicalein decreased the growth of hPDLs slightly and increased ALP activity and calcium deposition in a dose-dependent manner. The expression of runt-related transcription factor 2 (RUNX2), bone morphogenetic protein 2 (BMP2), Osterix (OSX) and osteocalcin (OCN) were elevated after baicalein administration. Moreover, baicalein strongly activated the Wnt/ β -catenin pathway and up-regulated the expression of β -catenin, lymphoid enhancer factor 1 (LEF1) and Cyclin D1. Dickkopf-related protein 1 (DKK-1) significantly reversed the effects of baicalein on hPDLs.

Conclusions: Our findings indicated that baicalein enhanced the osteogenic differentiation of hPDLs via the activation of the Wnt/ β -catenin signaling pathway, which may represent a potential candidate for periodontitis therapy.

Keywords:

Baicalein

Osteogenic differentiation

Wnt/ β -catenin signaling

Periodontal ligament cells

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1. Introduction

Periodontitis is regarded as the second most common dental disease worldwide (Schulze-Spate et al., 2015). Periodontitis is a set of inflammatory diseases that affect the periodontium, including periodontal ligament (PDL), cementum, and alveolar bone. Periodontitis involves the progressive loss of the alveolar bone around the teeth, and if left untreated can lead to the loosening and subsequent loss of teeth (Nanci & Bosshardt, 2006). The ultimate goal of periodontal therapy is to control periodontal tissue inflammation and to produce predictable regeneration of the part of the periodontium that has been destroyed as a result of the periodontal disease. However, non-surgical approach, surgical approach or adjunct antibiotic therapy may not be effective for certain patients, and additional bone regeneration therapy is inevitable to enable the reconstruction of the functional periodontium (Greenstein, 2000; Shimono et al., 2003; Tsalikis, Sakellari, Dagalis, Boura, & Konstantinidis, 2014). Unfortunately, there is no effective method for periodontitis treatment to date. Human periodontal ligament cells (hPDLs) are the main functional cells in the PDL. hPDLs differentiate into cementoblasts or osteoblasts that can generate mineralized tissues (Lekic, Rojas, Birek, Tenenbaum, & McCulloch, 2001). Therefore, hPDLs play a vital role in the repair and regeneration of periodontal tissues (Isaka et al., 2001). A strategy that can enhance the osteogenic differentiation of hPDLs has been proposed as the treatment for periodontitis.

Baicalein (5,6,7-trihydroxyflavone) is an active ingredient extracted from the traditional Chinese herb *Scutellaria baicalensis* Georgi, which has been reported to possess anti-inflammatory, anti-oxidant, anti-cancer and anti-type 2 diabetes properties and is widely used to treat various diseases (Lin, Shen, Lin, Yang, & Chen, 2007; Li-Weber, 2009; Fan et al., 2013; Fu et al., 2014). Baicalein has been reported to stimulate osteoblastic MC3T3-E1 cell differentiation, inhibit osteoclast

differentiation and induce mature osteoclast apoptosis to promote bone metabolism (Kim, Lee, Kim, Min, & Kim, 2008; Kim et al., 2008). A recent study indicated that the active ingredients (baicalein and wogonin) from *Scutellaria baicalensis* Georgi influenced the promotion of proliferation and matrix calcification of hPDLs (Liu et al., 2014). However, the effects of baicalein on the underlying signal transduction pathways have not been elucidated.

The Wnt/ β -catenin signaling pathway is perceived as a key for the optimization of the therapeutic approaches used to treat various skeletal diseases (Tang et al., 2014; Yorgan & Schinke, 2014). The Wnt/ β -catenin signaling pathway plays an important role in the promotion of osteogenic differentiation and bone formation (Day, Guo, Garrett-Beal, & Yang, 2005; Rossini, Gatti, & Adami, 2013). Osteoanabolic agents that improve the function of the Wnt/ β -catenin pathway may have a promising future for osteoporosis treatment (Bauman & Cardozo, 2015). Activating β -catenin-dependent transcription is the vital step in the activation of the canonical Wnt signaling pathway (Clevers & Nusse, 2012). Therefore, we hypothesize that the Wnt/ β -catenin signaling pathway participates in this process and that baicalein affects the osteogenic differentiation of hPDLs.

In this study, we used baicalein to enhance and regulate the osteogenic differentiation potential of hPDLs. Additionally, we investigated the role of baicalein in the regulation of the signaling pathways involved in the osteogenic differentiation of hPDLs.

2. Materials and Methods

The study was approved by the Medical Ethics Committee of Wenzhou Medical University and the Hospital of Stomatology. The donors' informed written consent was obtained before extraction.

2.1 Primary culture and identification of hPDLs

hPDLs were obtained from extracted healthy third molars from 6 donors aged 18 to 30 years. The teeth were rinsed 3 times with sterile phosphate-buffered saline (PBS), and then the PDL was detached from the middle third of the root surface and

sliced into small pieces (1mm³). PDL tissues were digested with type I collagenase (Sigma, USA) for 30 minutes at 37°C and then cultured in α -minimum essential medium (α -MEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Millipore, USA), 2 mM L-glutamine, 100 U/mL of penicillin, and 100 mg/mL of streptomycin (Gibco, USA) at 37°C in a humidified atmosphere with 5% CO₂. When the cells reached approximately 80% confluence, they were washed with PBS and detached with 0.25% trypsin plus 0.05% EDTA (Sigma, USA) for passaging. Cells at passages 2-5 were used for the experiments. The medium was renewed every 3 days throughout the experiments. hPDLCs were identified through immunohistochemical staining for vimentin and cytokeratin(1:200, mouse, vimentin, BM0135, cytokeratin, BM0030, BosterBio, Wuhan, China) (Li, Han, Li, Wang, & Xu, 2013).

2.2 Treatment of hPDLCs with baicalein

Baicalein ($\geq 90\%$ purity, Sigma, USA) was dissolved in dimethylsulfoxide (DMSO, Sigma, USA) and filtered through a 0.22 μ m strainer (Millipore, USA). Baicalein was serially diluted in α -MEM to final concentrations of 0, 1.25, 2.5, 5, and 10 μ M. The concentration of DMSO did not exceed 0.1%, and medium containing 0.1% DMSO was used as a control (0 μ M baicalein) in all experiments. The hPDLCs were seeded in 6-well plates at a density of 2×10^5 cells/well and cultured until they reached 60-70% confluence. Subsequently, the cells were separately incubated with baicalein-containing medium at the above-mentioned dilutions for 3, 7 or 11 days.

2.3 Cell proliferation analysis

The effects of baicalein on cell proliferation were assessed using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. The hPDLCs (5×10^3 /well) were plated in a 96-well plate. After 12 h of incubation, the cells were treated with different concentrations of baicalein (0, 1.25, 2.5, 5, or 10 μ M) for 1, 3, 5 or 7 days. After treatment, 20 μ L of 1 mg/ml MTT (Sigma, USA) solution was added to each well, and the plates were incubated in the dark for 4 h at 37 °C. The MTT was transformed by living cells (but not dead cells) into formazan crystals, which were

dissolved in 150 μ L of DMSO by gentle shaking for 10 minutes. Finally, the optical density was measured at 490 nm, and the cell viability was normalized as the relative ratio of the absorbance to the absorbance of the control.

2.4 Alkaline phosphatase (ALP) assay

According to the results of pre-experiment, hPDLCS incubated with baicalein for 3 days presented higher ALP expression compared to 7 and 11 days. So PDLCS were incubated with baicalein for 3 days and then induced to differentiate in osteogenic medium (10^{-5} mM dexamethasone, 50 μ g/mL ascorbic acid, and 5 mM β -glycerophosphate) (Sigma, USA). After 7 days of osteogenic induction, the cells were washed 3 times with pre-cooled PBS and fixed with 4% paraformaldehyde (Beyotime, China) for 10 min at 4°C. Then, ALP staining was performed using the BCIP/NBT Alkaline Phosphatase Color Development kit according to the manufacturer's instructions (Beyotime, China). ALP activity was measured after the cells were incubated with baicalein for 3 days. The cells were washed 3 times with PBS and lysed with 1% Triton X-100 for 30 min. Then, the cells were harvested and measured with an ALP kit (Jiancheng Bioengineering, Nanjing, China) according to the manufacturer's instructions.

2.5 Alizarin red S staining

According to the results of pre-experiment, hPDLCS incubated with baicalein for 11 days formed more mineralized nodules. So the cells were treated with baicalein for 11 days and then differentiated in osteogenic medium for 21 days. Alizarin red S staining was performed to evaluate calcium deposition in the mineralized nodules formed by the hPDLCS. After 21 days of osteogenic induction, the cells were fixed with 4% paraformaldehyde and stained with 1 mg/mL Alizarin red S solution (pH 4.3) (Sigma, USA) for 30 min. Then, the cells were rinsed 3 times with deionized water. The images of stained cells were captured under a phase contrast microscope with a digital camera (Nikon, Japan). Calcium deposition was quantitatively determined by the cetylpyridinium chloride (CPC) method. The cells were destained with

cetylpyridinium chloride (Sigma, USA), and then the absorbance was measured at 520 nm using a multifunctional microplate reader (TECAN, Switzerland). The absorbance was used to compare the concentration of Alizarin red S staining between groups.

2.6 Quantitative Real-time PCR (qRT-PCR) assay

Primers were designed and synthesized by Invitrogen, and the sequences of certain genes (including RUNX2, OCN, BMP2, OSX and Cyclin D1) were listed in Table 1. After the cells were treated with different concentrations of baicalein for 3, 7 and/or 11 days, total RNA was isolated from each group with the TRIzol reagent (Invitrogen, USA). The purity and concentration of the total RNA were calculated through the absorbance at 260 and 280 nm. Then, 1 μ g of RNA was reverse-transcribed into cDNA using the PrimeScript™ RT Reagent Kit (Takara, Japan) according to the manufacturer's protocol. qRT-PCR was performed with the LightCycler 480 SYBR Green I Master (Roche, USA) on a LightCycler 480 system (Roche, USA); the program consisted of one cycle of 30 s at 95°C for activation, followed by 40 cycles of 5 s at 95°C for denaturing, 20 s at 60°C for annealing, and 20 s at 75°C for extension. To avoid primer dimer formation and false priming, melting curves were generated for all reactions. Each reaction was performed in triplicate with the same sample of cDNA, and the expression levels of the target genes were normalized to the expression of the internal control gene β -actin. The data were analyzed as fold changes according to the formula $2^{-\Delta\Delta Ct}$.

2.7 Western blotting analysis

After 11 days of baicalein treatment for each group, the cells were lysed in ice-cold RIPA Lysis Buffer (Fude, China) containing 1mM PMSF and centrifuged at 10,000 x g for 20 min at 4°C. The protein concentration was quantified using the BCA assay kit (Fude, China). After the boiled proteins were separated by 8% SDS-PAGE, the proteins were transferred to a PVDF membrane. The membrane was blocked in 3% nonfat milk for 2 h and then incubated with primary antibodies (1:1000, rabbit,

β -catenin, #8480, LEF1, #2230, Cyclin D1, #2978, β -actin, #4970, Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. After washing 3 times with TBST for 15 min, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:10000, goat anti-rabbit, β -catenin, LEF1, Cyclin D1, β -actin, Cell Signaling Technology, Inc., Danvers, MA, USA) at room temperature for 2 h. The membrane was washed with TBST 3 times for 15 min and then photographed with the Bio-Rad ChemiDoc™ XRS+ using the ECL enhanced chemiluminescence kit (Millipore, USA). The band intensities were determined using Bio-Rad ChemiDoc XRS+ Imaging System and Quantity One software.

2.8 Statistical analysis

Every experiment was repeated three times independently, and the cells may or may not come from the same donor. But we undoubtedly use the same donor's cells among different groups in the same experiment. The experimental results were expressed as the mean \pm standard deviation (SD), and the significance of differences within groups was analyzed by one-way ANOVA with the LSD test. All statistical analyses were executed with the SPSS 19.0 software, with $P < 0.05$ considered statistically significant.

3. Results

3.1 Morphology and identification of PDL cells

The cells in the primary culture grew radially around the PDL tissue after one week (Fig.1A), and the passaged cells were spindle-shaped (Fig.1B). The immunohistochemical staining revealed that the cells were positive for vimentin(Fig.1C) but negative for cytokeratin (Fig.1D). Based on the cell morphology and scratched position of the PDL, these cells could be identified as hPDLs.

3.2 Effect of baicalein on the proliferation of hPDLs

The effect of various concentrations (1.25-10 μ M) of baicalein on the proliferation

of hPDLCs was evaluated on days 1, 3, 5 and 7 (Fig. 2A). On day 1, baicalein in the range of 1.25-10 μM slightly inhibited the cell growth rate. On days 3, 5 and 7, the number of hPDLCs was decreased in the presence of baicalein in a dose-dependent manner; however, the decrease was not significant.

3.3 Effect of baicalein on the ALP activity of hPDLCs

The effect of baicalein on the early osteogenic differentiation of hPDLCs was assessed by staining for ALP (Fig. 2B) and testing the ALP activity (Fig. 2C). hPDLCs administered baicalein presented higher ALP expression and activity levels in a dose-dependent manner. The ALP activity of hPDLCs treated with 10 μM baicalein (B10) was significantly increased ($p < 0.05$) and was higher than that in B1.25 group ($p < 0.05$). However, there was no significant difference in the ALP activity of hPDLCs following the addition of 1.25 μM baicalein compared to the control ($P > 0.05$)

3.4 Effect of baicalein on calcium deposition

The effect of baicalein on the late osteogenic differentiation of hPDLCs was evaluated by the formation of mineralized nodules (Fig. 2D) and calcium deposition (Fig. 2E). After 21 days of osteogenic differentiation, red mineralized nodules were visualized by Alizarin red S staining. The stain color grew deeper and thicker with the increasing concentration of baicalein, indicating that the amount of accumulated mineral matrix deposition was increased. We also quantified the calcium content using a destaining method. Compared to the control group, Alizarin red S deposition was remarkably increased in the presence of baicalein in a dose-dependent manner ($p < 0.05$). Notably, cells treated with 10 μM baicalein (B10) produced more calcium deposition than the cells in the 1.25, 2.5, and 5 μM baicalein groups (B1.25, B2.5, and B5) ($p < 0.05$).

3.5 Effect of baicalein on the expression of osteogenic markers

qRT-PCR was used to detect the expression of several osteogenic marker genes,

including runt-related transcription factor 2 (RUNX2) (Fig.3A), bone morphogenetic protein 2 (BMP2) (Fig.3B), Osterix (OSX) (Fig.3C) and osteocalcin (OCN) (Fig.3D), following administration of baicalein to the hPDLCs for 3, 7 and 11 days. RUNX2 mRNA expression was increased in hPDLCs cultured with 10 μ M baicalein on days 3 and 7 and was significantly upregulated by baicalein treatment on day 11. The RUNX2 mRNA expression in the B5 and B10 groups was 2-fold higher as that of the control on day 11 ($p < 0.05$). The BMP2 mRNA level in the B10 group was significantly increased compared to the control ($p < 0.05$); the mRNA expression level was approximately 8-fold that of the control on day 11 and was higher than the levels detected in the B1.25, B 2.5 and B5 groups ($p < 0.05$). After baicalein administration, OSX expression was reduced compared to the control in all groups with the exception of the B10 group on day 3; however, the OSX expression was dramatically elevated on days 7 and 11 ($p < 0.05$). Indeed, the expression level detected in the B10 group was almost 21-fold that of the control group ($p < 0.05$) and was much higher than the other baicalein-treated groups ($p < 0.05$). On day 3, only 10 μ M baicalein slightly increased the expression of OCN ($p < 0.05$); however, the expression was obviously upregulated on days 7 and 11 ($p < 0.05$). Similarly, the expression of OCN was significantly increased in the B10 group compared to the B1.25, B2.5 and B5 groups ($p < 0.05$). Thus, baicalein elevated the expression of osteogenic markers, especially at the 10 μ M dosage.

3.6 Activation of Wnt/ β -catenin signaling during baicalein-stimulated osteogenic differentiation

The effect of baicalein on the Wnt signaling pathway in hPDLCs was studied on the 11th day. According to the Western blotting results, three Wnt pathway-related regulators (β -catenin, LEF1 and Cyclin D1) were up-regulated by the 5 μ M and 10 μ M baicalein treatments, particularly 10 μ M (Fig.4A). The mRNA level of the Wnt pathway target gene Cyclin D1 was markedly regulated in the presence of 10 μ M baicalein ($p < 0.05$) (Fig.4B). To confirm whether the canonical Wnt/ β -catenin pathway was involved in the osteogenesis of hPDLCs, the Wnt inhibitor DKK-1 (100

ng/ml) was added to the 10 μ M baicalein-treated hPDLs and cultured for 11 days. We found that treatment of the cells with DKK-1 decreased the baicalein-stimulated protein expression of the Wnt pathway-related regulators β -catenin, LEF1 and Cyclin D1 (Fig.4C). Additionally, the mRNA expression of Cyclin D1 was reduced by 40% compared to the 10 μ M baicalein-treated hPDLs ($p < 0.05$) (Fig.4D).

3.7 Suppression of Wnt/ β -catenin signaling by DKK-1 suppressed osteogenic differentiation of hPDLs

We found that treatment of the hPDLs with DKK-1 down-regulated the 10 μ M baicalein-stimulated ALP activity (Fig.5A, B) and calcium deposition (Fig.5C, D) ($p < 0.05$). Moreover, the positive role of baicalein in the mRNA expression of osteogenic markers (Fig.5E) including RUNX2, BMP2, OSX and OCN was significantly blocked ($p < 0.05$) in the presence of DKK-1.

4. Discussion

hPDLs have been represented as a promising resource to support periodontal regeneration and repair (Wolf et al., 2013). However, the capacity for tissue regeneration of hPDLs in a chronic inflammatory microenvironment can be interfered (Xia et al., 2016). Nokhbehshaim et al. (Nokhbehshaim et al., 2011) observed that EMD-stimulated matrix mineralization and COL1 synthesis by PDL cells were diminished under IL-1 β -induced inflammatory conditions, resulting in effects on periodontal tissue regeneration. Given the vital role of hPDLs in bone regeneration, it is possible that agents that can enhance the osteogenesis of hPDLs may be of value in bone regeneration in periodontitis. Recently, some studies have reported that some varieties of flavonoid compounds (i.e., quercetin, naringin and 6-hydroxyflavone) have been demonstrated to be potential therapeutic agents for the treatment of osteoporosis and other bone disorders by augmenting bone formation (Lai et al., 2014; Liu, Li, & Yang, 2014; Zhou & Lin, 2014). *Scutellaria baicalensis* Georgi (Chinese name Huang-qin) contains abundant flavonoids, such as baicalin, baicalein, and wogonin. These flavonoids were demonstrated to benefit the synthesis

of both collagen and total protein in gingival fibroblasts (Chung, Park, & Bae, 1995). Moreover, baicalin was reported to up-regulate the collagen I mRNA and total protein levels and inhibit the expression of MMP-1/TIMP-1 in PDLCs, which was suggested to might be effective in periodontitis (Cao, Li, & Zhu, 2010). Baicalin could protect against periodontal tissue damage in ligature-induced periodontitis in rats (Cai, Li, Du G, & Cao, 2008). However, no study has clearly investigated the effect of baicalein on the osteogenic differentiation of hPDLCs and its underlying signal mechanism. Accordingly, we assessed the effects of baicalein on the osteogenic differentiation of hPDLCs in primary cultures in vitro. In the present study, we showed that baicalein promoted the osteogenic differentiation of hPDLCs and the canonical Wnt/ β -catenin signaling pathway facilitated the process.

The MTT assay indicated that baicalein slightly inhibited the viability of hPDLCs, which was similar to the finding that baicalein slightly attenuated the growth rate of MC3T3-E1 cells (Kim, Lee, Kim, Min, & Kim, 2008).

ALP is a vital enzyme that appears during the early phase of osteoblast differentiation and can accelerate the mineralization of the bone matrix (Bellows, Aubin, & Heersche, 1991; Serigano et al., 2010). In the present study, ALP was treated as a marker of the early osteogenic differentiation of hPDLCs. The late stage of osteogenesis of hPDLCs subjected to baicalein treatment was represented by the expression of extracellular matrix mineralization and calcium deposition. In our study, we discovered that baicalein administration augmented the ALP activity and calcium deposition of hPDLCs in a dose-dependent manner. Previous study demonstrated that ALP activity was increased in osteoblastic MC3T3-E1 cells treated with 10 μ M baicalein (Kim, Lee, Kim, Min, & Kim, 2008). It also found that baicalein induced the calcium deposition of MC3T3-E1 cells in a dose-dependent manner, at concentrations ranging from 1.25 to 10 μ M.

Several osteogenic marker genes were also evaluated to determine whether baicalein was effective during the process of osteogenic differentiation of hPDLCs. BMP2, a member of the TGF- β family, promotes osteoblast differentiation and maturation, participates in the development and reconstruction of bone and cartilage,

and accelerates the repair of bone defects (Tsuji et al., 2006; Lowery et al., 2011). RUNX2 is a specific transcription factor that can induce osteoprogenitors or preosteoblasts to differentiate into osteoblasts and then proceed to regulate the maturation of osteoblasts (Zhang et al., 2011; Watanabe et al., 2013). OSX plays an important role in the initial regulation of bone tissue formation and reconstruction (Sun, Wang, & Hao, 2008). OCN is the most abundant non-collagenous protein in bone and is a biological indicator used to assess bone formation during the late differentiation stage. There is a possibility that OCN can help regulate bone matrix mineralization (Thomas, Baker, Eisman, & Gardiner, 2001; Komori, 2006). In this study, the PCR analysis indicated that baicalein promoted the transcription of BMP2, RUNX2, OCN and OSX. It should be noted that higher concentrations of baicalein (5 or 10 μM) significantly enhanced the osteogenic differentiation of hPDLs, especially in 10 μM , in a time-dependent manner. Interestingly, on day 3, the expressions of RUNX2, OCN and OSX were down-regulated after low concentrations of baicalein treatment. Zhou et al. (Zhou & Lin, 2014) found that low concentrations of quercetin also inhibited the transcription of these osteogenic markers on adipose-derived stem cells (ASCs) differentiation, which is similar to baicalein. Further studies are required to elucidate the possible mechanism of the inhibitory effects.

The effects of the Wnt signaling pathway on osteogenic differentiation and bone formation have been universally reported (Day, Guo, Garrett-Beal, & Yang, 2005; Rossini, Gatti, & Adami, 2013; Saidak et al., 2015). A previous study indicated that the activation of Wnt/ β -catenin signaling was involved in the osteogenesis of human periodontal ligament fibroblasts (Heo, Lee, & Lee, 2010). Activation of canonical Wnt/ β -catenin signaling reversed H_2O_2 -induced decreases in the proliferation and differentiation of human periodontal ligament fibroblasts (Kook et al., 2015). Some studies have suggested that the Wnt/ β -catenin signaling pathway is linked to osteogenesis via the activation of β -catenin and that an accumulation of activated β -catenin may be sufficient to activate LEF1 and ultimately up-regulate the expression of its target gene Cyclin D1 (Shtutman et al., 1999; Rossini, Gatti, & Adami, 2013). In the present study, the levels of β -catenin, LEF1 and Cyclin D1 were

enhanced after baicalein administration, indicating that Wnt/ β -catenin signaling was involved in the baicalein-enhanced osteogenesis of hPDLs.

The Wnt/ β -catenin signaling is activated by extracellular Wnt proteins but also occurs in response to other mechanical strain and chemical (Heo & Lee, 2011; Ying et al., 2014). In order to further investigate whether baicalein promotes osteogenic differentiation via Wnt/ β -catenin signaling pathway, the specific inhibitor of Wnt/ β -catenin signaling, DKK-1, was applied. DKK1 inhibits the accumulation of stabilized β -catenin by competitively binding to the LRP5 receptor and thereby blocking the interaction between Wnt2, Frizzled and LRP5/6 and resulting in the block of the Wnt/ β -catenin signaling pathway (Ai, Holmen, Van Hul W, Williams, & Warman, 2005). The inhibition of Wnt/ β -catenin signaling by DKK-1 abolished the baicalin-induced osteogenic effects (Guo et al., 2011). In our study, DKK-1 reduced the levels of β -catenin and LEF1, which subsequently depressed the transactivation of the target gene Cyclin D1. We also found that hPDLs treated with DKK-1 reversed the baicalein-stimulated ALP activity and formation of mineralization. These results are consistent with previous reports suggesting that the addition of DKK-1 decreased β -catenin accumulation, ALP activity and matrix mineralization in osteoblasts (Qiang, Barlogie, Rudikoff, & Shaughnessy Jr, 2008; Guo et al., 2011). RUNX2 can be promoted by the up-regulation of β -catenin due to Wnt signaling activation and contribute to osteoblast differentiation (Gaur et al., 2005; Hamidouche et al., 2008). OSX and OCN act as downstream genes of RUNX2 (Ducy, Zhang, Geoffroy, Ridall, & Karsenty, 1997; Nakashima et al., 2002). Moreover, OSX is not expressed in Runx2/Cbfa1-null mice (Nakashima et al., 2002). In our study, the positive role of baicalein on these osteogenic markers was partially blocked by the inhibition of the β -catenin/LEF-1 pathway. These findings revealed that baicalein up-regulated the expression of osteogenic markers through the activation of the β -catenin/LEF-1 pathway. BMP2 is a family member of another critical pathway (the bone morphogenetic proteins, BMPs) that enhances osteogenic differentiation. A study showed that DKK-1 abrogated BMP2-mediated osteoblast differentiation (Qiang, Barlogie, Rudikoff, & Shaughnessy Jr, 2008). Interestingly, the ascendant trend in

BMP2 expression due to baicalein administration was reversed by the addition of DKK-1. The cross interaction between the Wnt/ β -catenin and BMP2 signaling pathways for osteoblast differentiation is worth further study.

Taken together, blocking β -catenin in hPDLs mostly prevented the positive effect of baicalein on osteogenic differentiation. We have not evaluated how DKK1 blocks the effect of baicalein on the osteogenic differentiation of hPDLs, but we believe that baicalein administration increases the expression of canonical Wnt proteins, which subsequently elevates the expression of osteogenic-related factors and results in the osteogenesis of hPDLs.

There are some limitations to this study. PDLs are a group of mixed population, which have limited differentiation potential as it contains high proportion of terminally differentiated cells. However, Periodontal ligament stem cells (PDLSCs), a newly subpopulation of MSCs, had been isolated from PDL tissues and PDLs and had exhibited stronger osteogenic differentiation potentials than PDLs. PDLSCs may be the better candidate for the study of periodontal regeneration (Alvarez, Lee, Wang, & Hong, 2015).

5. Conclusions

In summary, the present study demonstrated that baicalein (one of main effective components of *Scutellaria baicalensis* Georgi) could promote the osteogenesis of hPDLs via the activation of the Wnt/ β -catenin signal transduction pathway. Based on the results of this study, baicalein-enhanced osteogenesis of hPDLs might be an attractive and promising treatment strategy to enhance alveolar bone formation and reconstruction in periodontitis.

Conflicts of Interest

The authors have declared that no competing interests exist.

6. Acknowledgments

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Table 1 Primer Sequences

Gene	Forward (5'-3')	Reverse(5'-3')
<i>RUNX2</i>	CCCGTGGCCTTCAAGGT	CGTTACCCGCCATGACAGTA
<i>BMP2</i>	TATTTGGATAAGAACCAGACATTG	GAAAGAAGAACAACAAACCATCA
<i>OSX</i>	ACCTACCCATCTGACTTTGCTC	CCACTATTTCCCACTGCCTTG
<i>OCN</i>	AGCAAAGGTGCAGCCTTTGT	GCGCCTGGGTCTCTTCACT
<i>Cyclin D1</i>	TGATGCTGGGCACTTCATCTG	TCCAATCATCCCGAATGAGAGTC
<i>β-actin</i>	TGGCACCCAGCACAAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA

Figure Legends

Fig.1 (A) Primary culture of hPDLCs on the 7th day ($\times 100$); (B) morphology of hPDLCs at Passage 1 ($\times 100$); immunohistochemical staining of hPDLCs: (C) positive staining for vimentin ($\times 100$); (D) negative staining for cytokeratin ($\times 100$).

Fig.2 Effects of baicalein on the proliferation and osteogenic differentiation of hPDLCs. (A) the proliferation of hPDLCs was analyzed by MTT assay; (B) ALP staining and (C) ALP activity of hPDLCs after baicalein treatment; (D) Alizarin red S staining and (E) calcium deposition of hPDLCs after baicalein treatment. B0, control; B1.25, 1.25 μM baicalein; B2.5, 2.5 μM baicalein; B5, 5 μM baicalein; B10, 10 μM baicalein; *: compared to control, $p < 0.05$; #: compared to 10 μM baicalein group (B10), $p < 0.05$.

Fig.3 qRT-PCR analysis of baicalein-enhanced osteogenic differentiation of hPDLCs: the mRNA expressions of osteogenic-related genes (A) RUNX2, (B) BMP2, (C) OSX and (D) OCN. B0: control; B1.25, 1.25 μM baicalein; B2.5, 2.5 μM baicalein; B5, 5 μM baicalein; B10, 10 μM baicalein; *: compared to control, $p < 0.05$; #: compared to 10 μM baicalein group (B10), $p < 0.05$.

Fig.4 qRT-PCR and Western blotting analysis of baicalein-enhanced Wnt/ β -catenin signaling of hPDLCs: (A) the protein expressions of β -catenin, LEF1 and Cyclin D1; (B) the mRNA expression of Wnt pathway target gene Cyclin D1; B0: control; B5, 5 μM baicalein; B10, 10 μM baicalein. (C) DKK-1 blocks the protein expressions of β -catenin, LEF1 and Cyclin D1 and (D) the mRNA expression of the target gene Cyclin D1; Control; DKK-1, 100 ng/mL human recombinant DKK-1; B10, 10 μM baicalein; B10+DKK-1, 10 μM baicalein plus 100 ng/mL human recombinant DKK-1. *: compared to control, $p < 0.05$; #: compared to 10 μM baicalein group (B10), $p < 0.05$.

Fig.5 DKK-1 partially blocked the positive effect of baicalein on the osteogenic differentiation of hPDLCs: (A) ALP staining and (B) ALP activity; (C) alizarin red S

staining and (D) calcium deposition assay; (E) qRT-PCR assay: the mRNA expressions of osteogenic-related genes RUNX2, BMP2, OSX and OCN. Control; DKK-1, 100 ng/mL human recombinant DKK-1; B10, 10 μ M baicalein; B10+DKK-1, 10 μ M baicalein plus 100 ng/mL human recombinant DKK-1; *: compared to control, $p < 0.05$; #: compared to 10 μ M baicalein group (B10), $p < 0.05$.

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Figure 1

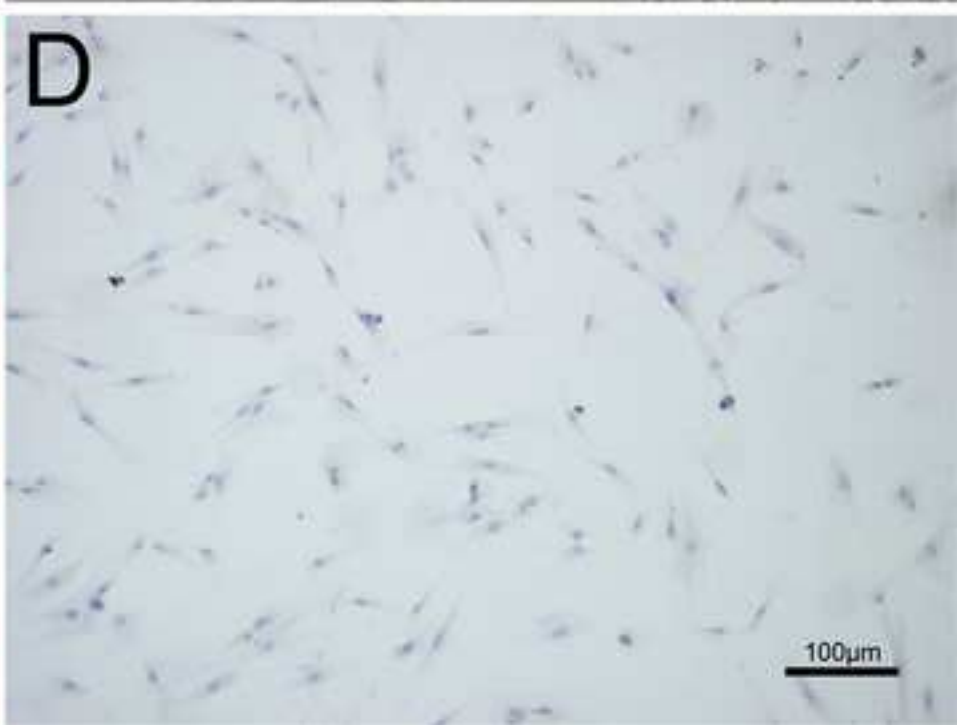
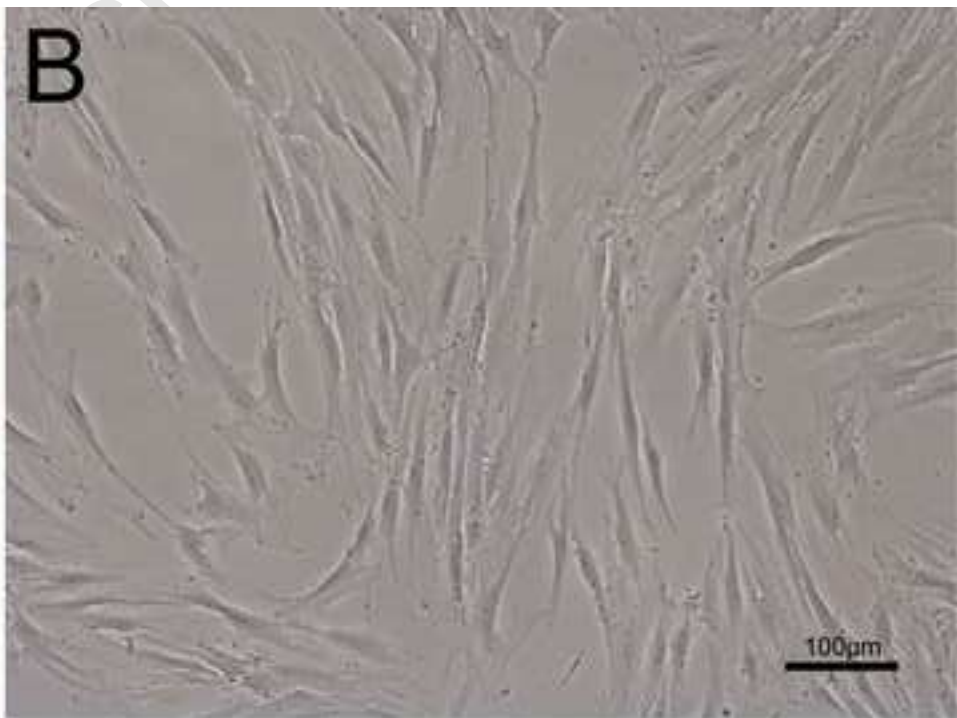
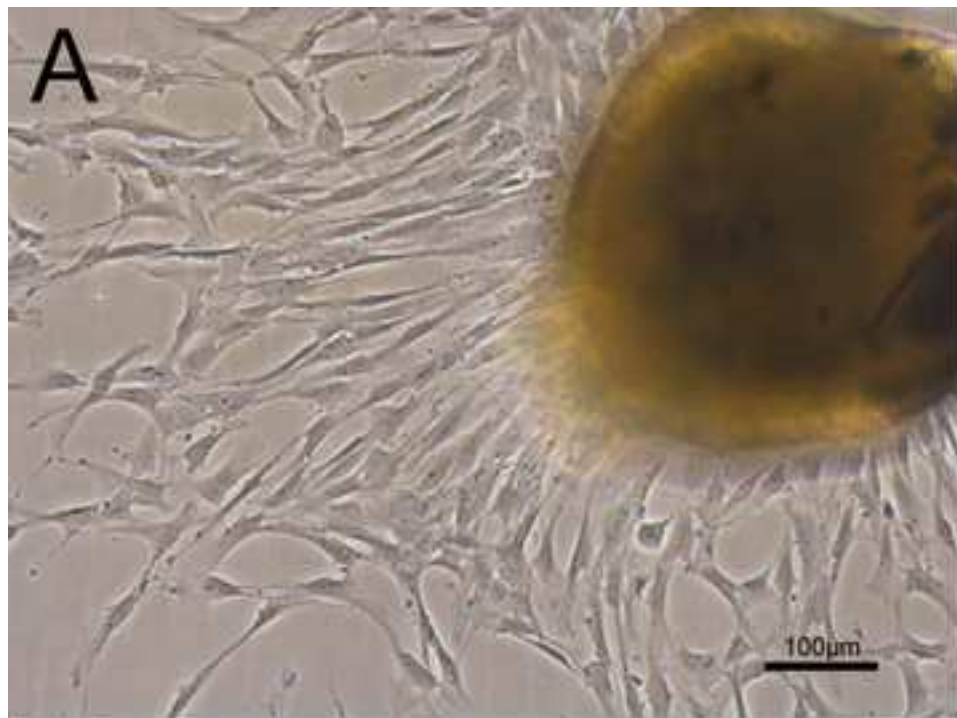


Figure2

