Salvianolic Acid C Attenuates LPS-Induced Inflammation and Apoptosis in Human Periodontal Ligament Stem Cells via Toll-Like Receptors 4 (TLR4)/Nuclear Factor kappa B (NF-κB) Pathway

Background: Periodontitis is a chronic inflammatory disease that causes gingival detachment and disintegration of alveolar bone. Salvianolic acid C (SAC) is a polyphenol compound with anti-inflammatory and antioxidant activities that is isolated from Danshen, a traditional Chinese medicine made from the roots of Salvia miltiorrhiza Bunge. The aim of this study was to investigate the mechanisms of underlying its protective effects and its inhibition effect on inflammation and apoptosis in human periodontal ligament stem cells (hPDLSCs).

Material/Methods: LPS-induced hPDLSCs, as a model mimicking an inflammatory process of periodontitis in vivo, were established to investigate the therapeutic effect of SAC in periodontitis. The inflammatory cytokines secretion and oxidative stress status were measured by use of specific commercial test kits. The hPDLSCs viability was analyzed by Cell Counting Kit-8 assay. The cell apoptosis and cell cycle were assayed with flow cytometry. Expressions levels of proteins involved in apoptosis, osteogenic differentiation, and TLR4/NF-κB pathway were evaluated by Western blotting. Alkaline phosphatase (ALP) activity was detected by ALP assay kit and ALP staining. The mineralized nodules formation of hPDLSCs was checked by Alizarin Red S staining.

Results: Our results showed that LPS induced increased levels of inflammatory cytokines and oxidative stress and mediated the phosphorylation and nuclear translocation of NF-κB p65 in hPDLSCs. SAC reversed the abnormal secretion of inflammatory cytokines and inhibited the TLR4/NF-κB activation induced by LPS. SAC also upregulated cell viability, ALP activity, and the ability of osteogenic differentiation. The anti-inflammation and TLR4/NF-κB inhibition effects of SAC were reversed by TLR4 overexpression.

Conclusions: Taken together, our results revealed that SAC effectively attenuates LPS-induced inflammation and apoptosis via the TLR4/NF-κB pathway and that SAC is effective in treating periodontitis.

MeSH Keywords: Apoptosis • Inflammation • Periodontal Ligament • Toll-Like Receptor 4

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/918940
**Background**

Periodontitis, characterized by tooth mobility and bleeding from gingiva, is a chronic inflammatory disease that causes gingival detachment and disintegration of alveolar bone [1]. It was recently reported that periodontitis broadly affects up to 50% of adults [2]. Previous studies have demonstrated periodontitis is related to systemic conditions such as cardiovascular disease, diabetes mellitus, adverse pregnancy outcomes, and osteoporosis [3]. Periodontitis is related to immune reactions and is affected by exposure to endotoxins from Gram-negative bacterial species in the dental biofilm [4]. Currently, there are still no clinically effective therapeutic strategies for periodontitis.

Human periodontal ligament stem cells (hPDLSCs), which are a type of mesenchymal stem cell, play important roles in maintaining periodontal homeostasis and inducing tissue regeneration because of their superior ability for differentiation to cementum, bone, periodontal ligament, and cementoblasts [5–7]. This suggests that hPDLSCs could be useful in therapy of periodontal diseases by regeneration, reconstruction, and fixation of periodontal tissue [8]. Recent studies have demonstrated that endotoxins produced by periodontal pathogens can disrupt microenvironment homeostasis and destroy periodontal tissue through suppressing the viability of hPDLSCs [4,9]. Periodontal inflammation plays important roles in progression of periodontitis, but the underlying mechanism of pathogenesis remains to be investigated. Lipopolysaccharide (LPS), which is a potent stimulator of inflammation, can produce proinflammatory cytokines such as TNF-α, IL-6, and IL-1β, resulting in disturbance of periodontal ligament cell differentiation, and further leads to deep periodontal tissue destruction and periodontitis [10–12]. In addition, Toll-like receptors (TLRs) were demonstrated to be related to the recognition of bacterial species wall components [13]. Several reports have reported that Toll-like receptors 4 (TLR4) is a gene product that regulates LPS signaling [14]. A previous study showed that TLR4, which is a signaling receptor of bacterial endotoxins, can be activated by LPS in hPDLSCs [15]. TLR4 is also responsible for the apoptosis of various cell types [16]. TLR4 expression in human periodontal ligament mesenchymal stem cells (PDL-MSCs) plays important roles in inflammation [13], but further investigations are needed to elucidate the roles of TLR4 in hPDLSCs with endotoxin stimulation.

The roots of *Salvia miltiorrhiza* Bunge, also known as Danshen, is a traditional Chinese medicine for treatment of inflammatory diseases. It contains 2 pharmacologically active compounds: salvianolic acids and tanshinones. Salvianolic acid C (SAC) is a polyphenol compound isolated from Danshen and has anti-inflammatory and antioxidant bioactivities [17]. SAC significantly inhibits NF-κB activity and protein and mRNA expression of proinflammatory mediators, including IL-6, IL-8, and IL-1β, in endothelial cells [18]. SAC is also reported to attenuate mitochondrial inflammation, oxidative stress, and caspase-mediated apoptosis through inactivation of Keap1/Nrf2/HO-1 signaling in acute liver injury [19]. Thus, we hypothesized that SAC would have a therapeutic effect on periodontitis.

In the present study, we evaluated the inhibitory effect of SAC on LPS-induced periodontal inflammation and investigated the molecular mechanism underlying its anti-inflammatory and antiapoptotic effects on human periodontal ligament stem cells.

**Material and Methods**

**Cell culture and treatment**

Human periodontal ligament stem cells (hPDLSCs) were purchased from American Type Culture Collection and were cultured in complete α-minimum essential media (Gibco, Gaithersburg, MD, USA), supplemented with 10% FBS, L-glutamine (5 mM), streptomycin (100 µg/mL), and penicillin (100 units/mL) at 37°C in a 5% CO₂ humidified atmosphere. The cells were maintained with or without LPS (1 µg/mL) for 24 h and pretreated with or without SAC (0, 0.1, 1, and 5 mM) for 1 h. The experiment was divided into 6 groups: Control group (culture medium only), SAC negative control group (5 mM SAC), LPS stimulation model group (1 µg/mL LPS), SAC low dose group (1 µg/mL LPS+0.1 mM SAC), SAC middle dose group (1 µg/mL LPS+1 mM SAC), and SAC high dose group (1 µg/mL LPS+5 mM SAC). SAC (shown in Figure 1A) was obtained from Chem Faces Pharmaceutical Company.

**Measurement of inflammatory cytokines and oxidative stress**

Cells in logarithmic growth phase were treated with or without LPS and SAC in 12-well plates. Inflammatory cytokines TNF-α (ab181421), IL-6 (ab46027), IL-1β (ab46052) in the media were quantified through ELISA kits (Abcam, USA) following the manufacturer’s instructions. The optical density was measured using an automatic biochemistry analyzer.

The amount of ROS in cell supernatants was detected by 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Beyotime Biotechnology). The cells were stained with DCFH-DA (20 μM) in the dark for 30 min at 37°C. Subsequently, absorbance was measured by a microplate reader (Thermo Fisher Scientific, USA). The NO produced by hPDLSCs was detected using an NO kit (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's protocols. Briefly, 0.1 mL of sample and 0.4 mL of mixed reagent were added and mixed evenly in a 6-well plate. Then, the fluorescence intensity was quantified by a microplate reader. For inducible NO synthase (iNOS) activity assay,
hPDLSCs were disrupted and centrifuged to obtain cell supernatant. The supernatant was mixed and incubated with the reaction buffer at 37°C for 30 min. Then, absorbance was measured on an absorbance microplate reader.

**Cell viability analysis**

Cell Counting Kit-8 assay was performed to measure the hPDLSCs viability according to standard protocol. hPDLSCs were incubated with fresh medium in 96-well plates (5×10³ cells/well), then treated with or without LPS (1 μg/ml) and SAC (5 mM). Cells were maintained for 24 h and then CCK-8 solution (10 μl) was separately added to each well. Following incubation at 37°C for 1 h, the absorbance was measured using a microplate reader at 450 nm.

To further confirm the proliferation rate, cell cycle at G1, S, and G2 was detected with a CytoFLEX flow cytometer. Briefly, hPDLSCs (1×10⁵ cell/mL) was fixed in cold 70% ethanol at 4°C for 1 h and then incubated with RNase A (100 μl) at 37°C for 0.5 h. Finally, hPDLSCs were stained with propidium iodide (500 μl) and captured at 488 nm on a flow cytometer.
Flow cytometry

The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Sigma-Aldrich, Tokyo, Japan) was employed to assay the cell apoptosis following the manufacturer’s instructions. HPDLSCs were incubated with or without 1 μg/mL LPS and 5 mM SAC for 24 h. After H2O2 treatment, HPDLSCs were collected and centrifuged at 1500 rpm for 10 min. Afterwards, hPDLCs (1×10^4 cells/mL) were washed with PBS and re-suspended with binding buffer (1×). Then, hPDLCs were mixed gently with Annexin V-FITC (5 μL) and cultured for 40 min at room temperature. PI staining solution (10 μL) was added and maintained for 35 min in the dark. The Annexin V-FITC-positive and PI-negative cells were identified as apoptotic cells, and the percentage of apoptotic cells in each group was analyzed with a flow cytometer.

Western blotting

After treatment, hPDLCs were washed with cold PBS and lysed in RIPA lysis buffer with the supplied with protease/phosphatase inhibitor cocktail. Protein concentrations were measured by BCA protein assay kits. Total protein (25 μg) was fractionated with SDS-PAGE gels and transferred onto PVDF membranes. The PVDF membranes were blocked with 5% skim milk and incubated with primary antibodies at 4°C overnight. Rabbit anti-Bcl2 antibodies (1: 1000), rabbit anti-Bax antibodies (1: 1000), rabbit anti-Phospho-cleaved caspase3 antibodies (1: 500), rabbit anti-caspase3 antibodies (1: 1000), rabbit anti-bmp2 antibodies (1: 1000), rabbit anti-Oct4 antibodies (1: 1000), rabbit anti-Sox2 antibodies (1: 1000), rabbit anti-Runx2 antibodies (1: 1000), rabbit anti-MyD88 antibodies (1: 1000), rabbit anti-NF-kB p65 antibodies (1: 500), rabbit anti-Phospho-NF-kB p65 antibodies (1: 1000), rabbit anti-TLR4 antibodies (1: 1000), and rabbit anti-GAPDH antibodies (1: 1000) were obtained from Cell Signaling Technology. After washing 3 times with PBS, appropriate secondary antibodies were applied for 2 h, and signals were analyzed using the Tanon-5200 Chemiluminescence Imager with enhanced chemiluminescence Western blotting substrate (Millipore, Billerica, MA).

Quantitative real-time PCR (RT-qPCR)

Total RNA of hPDLCs was extracted by the Trizol Reagent (Ambion, USA) following the manufacturer’s instructions. Then, RNAs (1 μg) were reverse transcribed with Reverse Transcription regents (TaKaRa, Otsu, Shiga, Japan) to synthesize cDNAs. RT-qPCR was performed using a System 7500 instrument with the following PCR amplification conditions: 95°C for 2 min and then 40 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s, followed by 78°C for 5 min. The mRNA level was normalized by the GAPDH level and was calculated using the 2^(-ΔΔCt) method. The primer sequences of TLR4 for qPCR amplification were as follows: TLR4, forward, 5’-GAATGAGGACTGGTGAGAAC-3’, reverse, 5’-CTCAGCAAGACCTTCCACCT-3’, GAPDH, forward, 5’-CTCACGGATGCACCAATGT-3’, reverse 5’-CGCCGGCTCACAATGTTCAT-3’.

Detection of ALP activity

Cells were cultured with 1 μg/mL LPS, or a combination of LPS and SAC (5 mM) for 24 h. ALP staining of hPDLCs was performed according to protocols described previously [20,21]. In brief, ALP activity of cells was analyzed by ALP assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions and histochemical staining was performed using the azo dye coupling method. Fixed hPDLCs were flooded with fresh stock substrate solution consisting of Tris buffer (pH=9.1) containing N, N-dimethylformamide, and naphthol AS phosphates. Then, hPDLCs were cultured in solution (stock substrate with 0.1% fast blue BB salt) at room temperature for 30 min in the dark. The prepared cells were observed and photographed under an inverted microscope at 400× magnification.

Alizarin Red S staining

Alizarin Red S staining (Sigma-Aldrich; Merck KGaA) was performed to detect the mineralized nodules formation of hPDLCs with or without LPS and SAC (5 mM) treatment, as described above. hPDLCs were fixed with 4% paraformaldehyde in a 24-well plate. Then, hPDLCs were stained with Alizarin Red S solution (0.5%) for 30 min at room temperature. Evaluation of the osteogenic differentiation was performed using an inverted microscope (Leica DM IRM) at 200× magnification after washing several times.

Transfection

HPDLSCs were seeded in 6-well plates for 24 h and then transfected with either pcDNA3.1 or TLR4 plasmid (100 ng) carried by lipofectamine 2000 (Thermo Fisher, Madison, USA) for 12 h. After transfection, HPDLSCs were treated with SAC (5 mM) for 24 h. The effect of SAC on the TLR4/NF-kB pathway was evaluated by Western blotting and RT-qPCR analysis.

Statistical analysis

Statistical data are expressed as the mean±SEM with SPSS 19.0 and GraphPad Prism 5.0. The significance of differences was analyzed by analysis of variance (ANOVA) followed by Tukey-Kramer test in the multiple groups. Differences were considered statistically significant at P<0.05.
Results

Effects of SAC on inflammatory cytokines and oxidative stress in LPS-stimulated hPDLSCs

To determine the effect of SAC on proinflammatory cytokines and oxidative stress, hPDLSCs were cultured with or without 5 mM SAC in the presence of 1 μg/mL LPS for 24 h and examined by commercial test kits to detect the ROS, NO, iNOS production and the cytokines TNFα, IL-6 and IL-1β. We found the expression levels of TNFα, IL-6, and IL-1β were significantly suppressed by SAC treatment in a dose-dependent manner. Moreover, LPS-induced ROS, NO, and iNOS levels were also reduced in the presence of SAC, as shown in Figure 1. These data suggested that SAC can inhibit inflammation and oxidative stress with LPS stimulation.

SAC protected hPDLSCs from injury

To identify the role of SAC in hPDLSCs injury induced by LPS stimulation, CCK8 assay, flow cytometry, and Western blotting were employed to analyze hPDLSCs viability and apoptosis. CCK-8 assay results showed SAC promoted the proliferation of hPDLSCs after LPS stimulation (Figure 2A). In addition, flow cytometry results demonstrated SAC effectively attenuated cell apoptosis (Figure 3A, 3B) and increased the proportion of

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**Figure 2.** SAC protected hPDLSCs against injury. (A) hPDLSCs viability was analyzed by Cell Counting Kit-8 assay. (B, C) The cell cycle was assayed with flow cytometry. (D) Expressions levels of CyclinD1 and CDK2 proteins were evaluated by Western blotting. The data are expressed as means±SD; ** P<0.01, *** P<0.001 vs. Control. * P<0.05, ** P<0.01, *** P<0.001 vs. LPS.
cells in G2/M phase and S phase (Figure 2B, 2C). The expression levels of proteins related to cell cycle, such as CyclinD1 and CDK2, were downregulated by LPS treatment, and this effect was abolished by SAC (Figure 2D). These results were further confirmed by Western blotting, showing that the expression level of Bcl2 was decreased by LPS stimulation compared with the control group. Conversely, expression levels of Bax and cleaved caspase3 were increased in hPDLSCs in the LPS group, and the expression level of caspase3 exhibited no significant change as compared with the LPS treatment group. However, SAC treatment reversed the abnormal expression of proteins involved in apoptosis, as shown in Figure 3C, 3D.

**Effects of SAC on ALP activity, mineralization, and osteogenic differentiation ability of hPDLSCs**

ALP, identified as a marker of osteoblast differentiation, is an exoenzyme appearing in the early stages of cell mineralization...
Figure 4. Effects of SAC on ALP activity, mineralization, and osteogenic differentiation ability of hPDLSCs. Alkaline phosphatase (ALP) activity was checked by ALP assay kit (A) and ALP staining, 400× magnification (B). (C) The mineralized nodules formation of hPDLSCs was detected by Alizarin Red S staining, 200× magnification. (D) Expression levels of proteins involved in osteogenic differentiation, including bmp2, Oct4, Sox2, and Runx2, were detected by Western blotting. The data are expressed as means±SD; *** P<0.001 vs. Control. * P<0.05, ** P<0.01 vs. LPS.
induction in osteoblasts. ALP activity analysis showed SAC treatment significantly promoted the ALP activity of LPS-stimulated hPDLSCs (Figure 4A, 4B). To further verify that SAC can induce osteogenic differentiation of hPDLSCs, Alizarin Red S staining was performed. SAC treatment enhanced the ability of mineralization and osteogenic differentiation in hPDLSCs compared with the LPS group (Figure 4C). Moreover, the expression levels of proteins involved in osteogenic differentiation, includingbmp2, Oct4, Sox2, and Runx2, were greatly downregulated after incubation in LPS as measured by Western blotting, and expression of these proteins was significantly increased in the presence of SAC (Figure 4D). This further proves the hypothesis that SAC has a potential therapeutic effect on periodontitis.

**SAC attenuate LPS-induced inflammation via TLR4/NF-κB pathway in hPDLSCs**

Through Western blotting analysis, we demonstrated that phosphorylation of NF-κB-p65 and expression levels of MyD88 and TLR4 were increased by LPS stimulation as compared to the presence of SAC (Figure 4D). This further proves the hypothesis that SAC has a potential therapeutic effect on periodontitis.
control group, but SAC treatment suppressed phosphorylation of NF-κB-65 and expression levels of TLR4 and MyD88 (Figure 5A). To further evaluate the role of the TLR4/NF-κB pathway in periodontitis inflammation, hPDLSCs were transfected with TLR4 plasmid or shRNA-TLR4. The results indicated that the TLR4 overexpression reverses the inhibition effect of SAC on secretion of inflammatory cytokines in LPS-stimulated hPDLSCs. Conversely, interference of TLR4 expression strengthened the anti-inflammation effect of SAC. Finally, the cells were pre-treated with or without NF-κB inhibitor (20 μM) for 1 h in the presence of LPS. NF-κB inhibitor also had same synergistic effect on the anti-inflammatory action of SAC (Figure 5B–5F). This finding confirms that SAC exerts an anti-inflammation effect in LPS-stimulated hPDLSCs via the TLR4/NF-κB pathway.

Discussion

Periodontitis is a chronic inflammatory disease of the periodontium, primarily induced by microbial challenge and affecting tooth-supporting structures [22,23]. Immune reaction is the major player in the pathogenesis of periodontitis. hPDLSCs was reported to possess the capacity of regeneration in periodontal tissue [15]. Furthermore, it is reported that SAC has anti-inflammatory and antioxidative effects on LPS-induced inflammatory response via activation of AMPK/Nrf2 signaling [18]. Thus, LPS-induced hPDLSCs, as an in vitro model of periodontitis, were established to investigate the mechanisms underlying the therapeutic effects of SAC on periodontitis.

Periodontitis is an inflammatory disease mediated by immunization, and its initiation and progression involve a complex interaction between Gram-negative bacteria action and the innate immune response [24]. It has been reported that exposure to LPS leads to increased concentrations of TNF-α and IL-1β in hPDLSCs and inhibits cell proliferation and migration [8]. Moreover, LPS is a potent inducer of intracellular ROS and causes a notable increase of NO and iNOS levels in murine macrophage cells [25]. Consistent with our results, LPS stimulation induced increased levels of inflammatory cytokines and oxidative stress in hPDLSCs, which was suppressed by SAC treatment in a dose-dependent manner. A previous study demonstrated that LPS-induced activation of the TLR4 signaling pathway decreased cell viability and promoted cell apoptosis [8]. It is reported that mRNA expressions and protein levels of Bax and caspase-3 are upregulated and Bcl-2 expression is decreased by LPS treatment in osteoblast cells [26]. Hence, the caspase-mediated antiapoptotic effect of SAC can be indicated by the enhanced viability of hPDLSCs and increased expression levels of Bcl-2, as well as the decreased expression levels of Bax and cleaved caspase3 in cells. ALP, which is a marker of osteoblast differentiation, plays a pivotal role in connective tissue calcification and mineral deposition [27]. Previous studies have indicated that ALP activity and cell metabolism and viability were decreased by LPS treatment in osteoblast cells [26], which is consistent with our study. In the present study, SAC treatment reversed the down-regulated ALP activity and calcification ability induced by LPS. Moreover, the decreased expression levels of proteins involved in osteogenic differentiation, including bmp2, Oct4, Sox2, and Runx2, were elevated by SAC treatment in hPDLSCs. Taken together, our results show that SAC treatment enhances mineralization and osteogenic differentiation ability, further suggesting SAC may be a candidate for clinical therapy of periodontitis.

Toll-like receptor 4 (TLR4) recruits proinflammatory mediator receptors-associated kinase to take part in host inflammatory response to infection through interaction with MyD88, and then activates downstream signaling pathways, such as MAPKs and NF-κB. NF-κB signaling has a vital role in regulating the genes that encode inflammation-associated molecules and cytokines. It is reported that the activation of the TLR4/MyD88 complex induced by LPS stimulation triggers the release of cytokines cascade, including IL-8, IL-1α, and TNF-α and β [28]. In the present study, upregulation of the TLR4/NF-κB signaling pathway was induced by LPS stimulation in hPDLSCs, and was reversed by SAC treatment. However, TLR4 overexpression abolished the anti-inflammation and TLR4/NF-κB inhibition effects of SAC treatment in hPDLSCs, and interference of TLR4 expression and NF-κB inhibitor strengthened the anti-inflammatory effect of SAC. The above results indicate that SAC attenuates LPS-induced inflammation via the TLR4/NF-κB pathway in hPDLSCs. Thus, we conclude that SAC has a potential therapeutic effect on periodontitis and our results provide novel clinical insights into therapeutic strategies.

Conclusions

In summary, SAC treatment inhibited the secretion of inflammatory cytokines and altered the oxidative stress status induced by LPS. In addition, SAC protected hPDLSCs from injury and upregulated ALP activity. Moreover, SAC enhanced the ability of mineralization and osteogenic differentiation in hPDLSCs with inflammatory condition. Interestingly, SAC attenuated inflammation and apoptosis via the TLR4/NF-κB pathway in hPDLSCs with LPS stimulation, which was reversed by TLR4 overexpression. Further research is needed to investigate the mechanism underlying the therapeutic effect SAC on periodontitis.

Conflict of interests

None.
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