

Original Research Article

Embelin enhances the osteogenic potential of LPS-induced periodontal ligament stem cells by activating AMPK and SIRT1

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Abstract

Purpose: To investigate the role of embelin in periodontal ligament stem cells (PDLSCs) in an in vitro model of periodontitis.

Methods: Lipopolysaccharide (LPS)-stimulated PDLSCs was used to construct a periodontitis cell model. PDLSCs in the treatment group were pretreated with different concentrations of Embelin, and CCK-8 and TUNEL staining were used to analyze cell viability and apoptosis. Enzyme-linked immunosorbent assay (ELISA) kits were used to evaluate the levels of inflammatory cytokines (TNF- α , IL-1 β , IL-6, and MCP-1) while reactive oxygen species levels were assessed by 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining. Subsequently, osteogenic marker, ALP activity and protein expression levels of Runx2, OCN and BMP-2 in PDLSCs were evaluated by western-blot assay; AMPK and SIRT1 levels were also determined using Western blot assay.

Results: Embelin pretreatment inhibited PDLSCs apoptosis, inflammatory factors, and oxidative stress, but up-regulated ALP, Runx2, OCN, and BMP-2 levels ($p < 0.05$). In addition, AMPK phosphorylation and SIRT1 protein levels were regulated by embelin ($p < 0.05$).

Conclusion: Embelin exerts anti-inflammatory, anti-oxidative and osteogenic differentiation effects in LPS-induced PDLSCs cells in vitro by activating AMPK/SIRT1 signaling. Therefore, the compound has potentials for use in the management of periodontitis

Keywords: Periodontitis, PDLSCs, Embelin, Osteogenic differentiation, AMPK, SIRT1

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INTRODUCTION

Periodontitis a chronic inflammatory disease with a high incidence, affects 3.5 billion adults worldwide [1]. Periodontitis is characterized by inflammation of the gingival soft tissue accompanied by irreversible loss of alveolar bone, periodontal ligament and gingival tissue,

culminating in tooth loss [2]. Inflammation and dysregulated immune response are important factors contributing to periodontitis [3]. In addition, oxidative stress is thought to be a major factor in several chronic inflammatory diseases, including periodontitis.

Embelin, the main component of *Embelia ribes* Burm, has a wide range of pharmacological properties, including anti-convulsant, anti-diabetic, anti-cancer, neuroprotective, cardioprotective, antioxidant and anti-inflammatory effects [4]. Embelin treatment alleviated severe airway inflammation and immune cell infiltration in asthmatic rats [5]. In a thioacetamide-induced liver injury mouse model, embelin can alleviate acute liver injury with inflammatory cell infiltration and liver fibrosis [6]. Furthermore, studies have shown that embelin attenuates mitochondrial biogenic dysfunction and apoptosis of dopaminergic cells in rats with Parkinson's disease by activating SIRT1/AMPK [7]. The role of embelin in periodontitis is unclear.

In this study, a periodontitis cell model was designed to investigate the effects of embelin on inflammation, oxidative stress, and osteogenesis in lipopolysaccharide (LPS)-stimulated PDLSCs, and its mechanism of action.

EXPERIMENTAL

Cell culture

Periodontal ligament stem cells (PDLSCs LMAI Bio, Shanghai, China) were cultured in complete α -MEM medium, supplemented with 10 % fetal bovine serum (Gibco, USA) and 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco, Grand Island, NY, USA), and placed in 37 °C humidification incubator containing 5 % CO₂.

CCK8 assay

The cells (approximately 2000 cells/well) were inoculated in 96-well plates and cultured at 37 °C for 24 h. After the specified treatment, the cells were incubated with Cell Counting Kit-8 (Beyotime, Wuhan, China). Absorbance was measured spectrophotometrically at a wavelength of 450 nm.

TUNEL assay

The treated cells were immobilized at room temperature with 4 % paraformaldehyde for 15 min, and incubated with 50 μ l TUNEL at 37 °C in the dark. The nuclei were stained with DAPI for 2 - 3 min. The cells were then analyzed from three visual fields using an optical microscope (Carl Zeiss, Jena, Germany).

Enzyme-linked immunosorbent assay

After washing with phosphate buffered saline (PBS), the PDLSCs were centrifuged at 3000 rev/min for 10 min to remove dead cells and

debris, and the supernatant was obtained. TNF- α , IL-6, MCP-1, IL-1 β (Neobioscience, China) were determined by ELISA kits according to the manufacturer's instructions.

Assessment of oxidative stress levels

SOD activity in PDLSCs was measured using Total SOD Assay Kit (Beyotime) while MDA levels of PDLSCs were measured using Beyotime lipid peroxidation MDA assay kit. Reactive oxygen species levels were determined by collecting PDLSCs after different treatments, and incubated with DCFH-DA (10 μ mol/L, Beyotime Biotechnology, China) for 20 min. Fluorescence intensity of ROS observed under fluorescence microscope (Carl Zeiss, Germany).

Evaluation of ALP activity

PDLSCs were cultured in 6-well plates. After different treatments, the medium was changed to osteogenic induction medium (10 mol/L sodium glycerophosphate, 10 mol/L dexamethasone, 50 g/mL ascorbic acid and 100 ml/L fetal bovine medium). After 7 days, ALP assay kit (Beyotime) was used to determine ALP activity. The absorbance of PDLSCs was assessed spectrophotometrically at 405 nm.

Western blot assay

The treated PDLSCs were washed with PBS. Protein in the cells was subsequently cleaved and extracted in RIPA lysis buffer (Beyotime Biotechnology, China). The protein concentration of cell lysates was determined by the Bradford method using the Bradford method (Bio-Rad), and the protein was separated with SDS-PAGE, and then transferred to a 0.45 μ m PVDF membrane (Millipore, USA). It was sealed at room temperature with 5 % skimmed milk for 1 h. Overnight at 4 °C, the PVDF membrane was treated with primary antibody. After rinsing, incubated PVDF membrane with secondary antibody for 2 h at room temperature. Target protein bands were detected using an enhanced chemiluminescence system (P0018AS, Beyotime). Table 1 displays information about antibodies.

Statistical analysis

The data obtained are presented as mean \pm standard deviation (SD). Medical statistical software SPSS 20.0 was used for univariate analysis of variance between groups, followed by Tukey multiple comparisons. $P < 0.05$ was considered statistically significant.

Table 1: Antibodies used

Antibody	Manufacturer	Item number	Dilution
Runx2	Abcam	ab23981	1:1000
OCN	Abcam	ab133612	1:1000
BMP-2	Abcam	ab284387	1:2000
p-AMPK	Abcam	ab133448	1:1000
AMPK	Abcam	ab207442	1:1000
SIRT1	Abcam	ab110304	1:1000
β -actin	Abcam	ab8226	1:1000
Secondary Antibody	Abcam	ab288151	1:2000

RESULTS

Effect of embelin and LPS on the activity of PDLSCs

There was no cytotoxicity at 2.5 - 25 μ M embelin (Figure 1a). However, when PDLSCs were incubated with LPS (5 μ g/mL) to create the periodontitis cell model, and treated with 2.5 μ M-25 μ M embelin, LPS treatment decreased the viability of PDLSCs and increased TUNEL-positive cell rate (Figure 1 b - d). Embelin pretreatment significantly increased the viability but reduced the apoptosis of PDLSCs. There was no significant difference in therapeutic effect between 10 and 25 μ M embelin ($p < 0.05$), and so 10 μ M was selected as the optimum therapeutic concentration of embelin in the follow-up experiment.

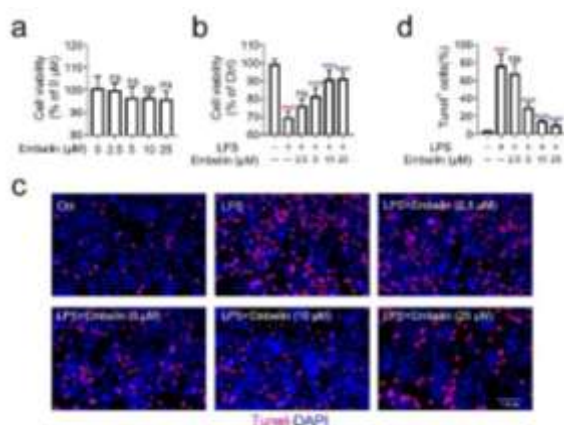


Figure 1: Embelin increased LPS-induced decrease in PDLSCs cell viability. (a) Effect of different concentrations of Embelin on cell viability. (b) Embelin increased LPS induced cell viability. (c) Representative images of TUNEL staining. (d) Percentage of TUNE positive cells. *** $P < 0.001$ compared with the control group; ^^ $p < 0.001$ compared with LPS group

Embelin reduced LPS-induced inflammation in PDLSCs

LPS induced higher levels of TNF- α , IL-1 β , IL-6, and MCP-1 when compared to the control

(Figure 2). Embelin preconditioning dose-dependently reduced inflammatory cytokine production in LPS induced PDLSCs.

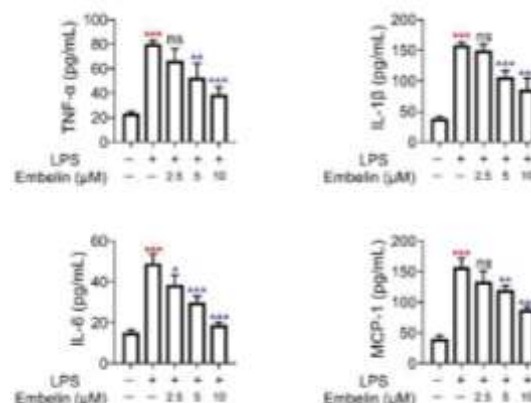


Figure 2: Embelin reduced the expression levels of TNF- α , IL-1 β , IL-6 and MCP-1 in LPS-induced PDLSCs. *** $P < 0.001$ compared with control group; $\wedge p < 0.05$ compared with LPS group; $\wedge\wedge p < 0.01$ compared with LPS group; $\wedge\wedge\wedge p < 0.001$ compared with t LPS group

Embelin alleviated LPS-induced oxidative stress in PDLSCs

DCFH-DA staining and immunofluorescence results showed that LPS induced decreases in SOD levels of PDLSCs and increases in MDA levels ($p < 0.05$; Figure 3 a). However, treatment with 5 and 10 μ M embelin significantly increased SOD level but decreased MDA accumulation. In addition, immunofluorescence results showed that ROS accumulation in LPS-treated PDLSCs increased significantly. The intracellular ROS levels in LPS and embelin groups were significantly lower than those in LPS group ($p < 0.05$; Figure 3 b).

Embelin promotes LPS-induced osteogenic differentiation of HPDLCs cells

Osteogenic differentiation was induced by osteogenic induction medium. As expected, ALP activity was inhibited by LPS stimulation, but reversed by Embelin preconditioning (Figure 4 a). Western blot analysis showed that the protein expressions of Runx2, OCN and BMP-2 in the LPS group were significantly down-regulated (Figure 4b and c). Embelin therapy reversed LPS inhibition of osteoblast expression. These results suggest that Embelin protects the osteogenic potential of PDLSCs.

Embelin up-regulated AMPK and SIRT1 activities

In order to study the effect of Embelin on the

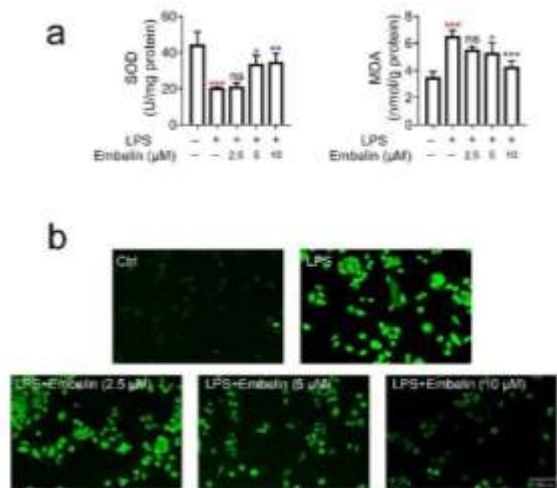


Figure 3: Embelin reduced oxidative stress in PDLSCs induced by LPS. (a) MDA and SOD levels in PDLSCs. (b) ROS levels in PDLSCs as determined by immunofluorescence. $***P < 0.001$ compared with the control group; $^{\wedge}p < 0.05$ compared with LPS group; $^{\wedge\wedge}p < 0.01$ compared with LPS group; $^{\wedge\wedge\wedge}p < 0.001$ compared with LPS group

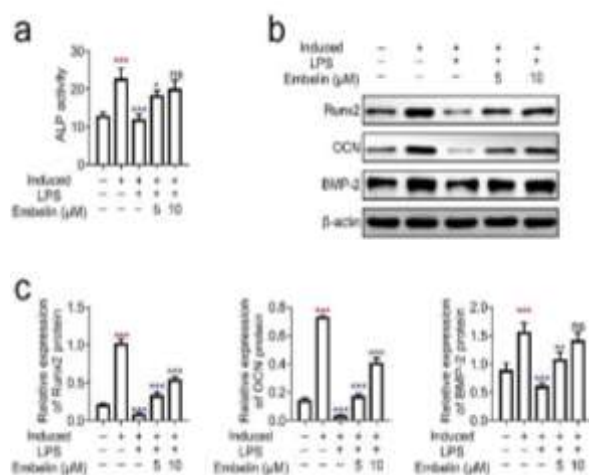


Figure 4: Embelin increased ALP activity and up-regulated the protein levels of Runx2, OCN and BMP-2. (a) ALP activity. (b) Protein levels of Runx2, OCN and BMP-2. $***P < 0.001$ compared with the control group; $^{\wedge}p < 0.05$ compared with the LPS group; $^{\wedge\wedge}p < 0.01$ compared with the LPS group; $^{\wedge\wedge\wedge}p < 0.001$ compared with LPS group

signaling cascade of periodontitis cells, the effects of embelin on the activities of AMPK and SIRT1 were studied using western blot assay (Figure 5). The results showed that the levels of AMPK phosphorylation and SIRT1 protein were significantly down-regulated after LPS treatment. Embelin increased p-AMPK/AMPK and SIRT1 levels in a concentration-dependent manner. These results suggest that embelin increased the activities of AMPK and SIRT1 in periodontitis model.

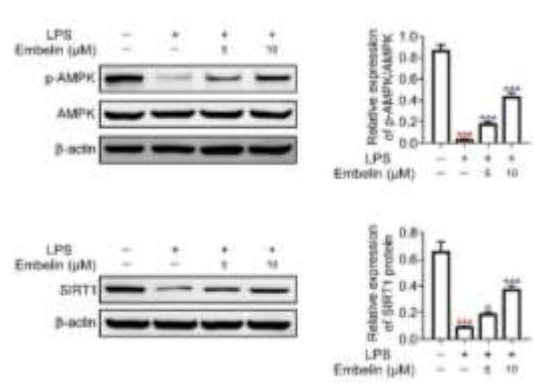


Figure 5: Embelin up-regulated activities of AMPK and SIRT1. $***P < 0.001$ compared with t control group; $^{\wedge}p < 0.05$ compared with LPS group; $^{\wedge\wedge}p < 0.001$ compared with LPS group

DISCUSSION

Periodontitis is a complex infectious oral disease, and is the sixth largest epidemic in the world. It mainly affects adults and occasionally affects children [8]. At present, there is no effective treatment for periodontitis. Periodontal ligament stem cells (PDLSCs) are derived from periodontal ligament tissue, and increasing evidence shows that PDLSCs are very effective in repairing periodontal bone defects and periodontal ligament injuries. Besides, PDLSCs promote the regeneration and repair of periodontal attachment tissue [9]. However, PDLSCs are easily obtained in clinical practice [10], and so they are widely used as potential "seed cells" for periodontal tissue and alveolar bone regeneration.

Periodontitis is caused by bacteria, especially Gram-negative bacteria. Imbalanced host antimicrobial immune response leads to an abnormal microenvironment, resulting in excessive inflammation and further PDLSCs dysfunction. Most published data suggest that damaged PDLSCs may lead to perturbations in periodontal homeostasis [11]. Lipopolysaccharide (LPS) is an important component of the gram-negative biological extracellular membrane, which induces the expression of pro-inflammatory cytokines and persistent inflammation. In addition, LPS reduces the proliferation rate of PDLSCs by inducing apoptosis [12]. In experiments, LPS is often used to induce periodontitis models *in vitro* and *in vivo*. LPS induced the decrease in cell viability of PDLSCs, caused cell apoptosis, and induced high levels of TNF- α , IL-1 β , IL-6, MCP-1 and ROS.

Embelia ribes Burm's bitter fruit has been used in traditional medicine for nearly a thousand years

to treat gastroenteritis, fever, and various inflammatory diseases. Its pharmacological properties are attributed to Embelin, a naturally occurring benzoquinone [4]. Embelin has received attention for its extensive pharmacological properties. It reduces intestinal inflammatory cytokines and improves intestinal barrier function [13]. Embelin has also shown therapeutic potential in cisplatin-induced nephrotoxicity by blocking NF- κ B and up-regulating Nrf2/HO-1 signaling pathway, thus increasing antioxidant enzyme activity, lowering MDA levels, and inhibiting inflammation [14].

The anti-inflammatory and antioxidant functions of embelin have been demonstrated in the present study. However, the effect of embelin in periodontitis remains unclear. Since the inflammatory environment and oxidative stress caused by periodontitis can inhibit the physiological function of PDLSCs, LPS-treated PDLSCs were treated with a safe concentration of embelin. The results showed that the levels of TNF- α , IL-1 β , IL-6, MCP-1 and ROS were significantly inhibited in response to LPS and apoptosis was alleviated.

Bone formation is the basis of bone development, and is related to osteogenic differentiation. Recent studies have shown that BMP-2 effectively accelerates osteoblast differentiation of PDLSCs, and promotes bone formation by increasing the expressions of osteogenic differentiation-related proteins ALP, RUNX2 and OCN [15]. Previous studies have shown that LPS inhibits Runx2, OCN levels, and ALP activity [11]. To investigate the role of LPS and Embelin in bone differentiation of PDLSCs, western blotting was performed and ALP activity was detected. The results showed that ALP activity and Runx2, OCN, and BMP-2 protein levels down-regulated by LPS, were up-regulated in a dose-dependent manner under Embelin treatment.

Furthermore, this study also investigated the potential mechanism of embelin's protective effect on PDLSCs. The results showed that embelin induced up-regulation of SIRT1 protein levels and AMPK phosphorylation levels in PDLSCs *in vitro*. AMPK is the most important energy sensor in cells, and when it is activated by phosphorylation, AMPK can maintain cell energy, help repair cell damage and reduce inflammatory response and periodontal tissue destruction [16]. SIRT1 is a member of the histone deacetylase family [17]. Previous studies have shown that resveratrol reduces oxidative stress and prevents periodontitis by activating SIRT1/AMPK/ antioxidant defense pathways,

alleviate alveolar bone resorption, and regulate osteogenic differentiation [18]. Therefore, embelin may be involved in the regulation of antioxidants, as well as anti-inflammatory and osteogenic differentiation in periodontitis through SIRT1/AMPK signal activation.

CONCLUSION

The findings of this study have established the important role of embelin in alleviating LPS-induced apoptosis, oxidative stress, inflammation, and osteogenic differentiation disorders in PDLSCs. It also protects PDLSCs by restoring lost periodontal ligaments and osteoblasts, and is a potential agent for the management of periodontitis. However, further studies are needed to determine the role of embelin in the body.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Lijian Wang, Weidong Wu and Xiaoying Wei designed the study and carried them out, supervised the data collection, analyzed and interpreted the data; Gang Zhang and Weidong Wu prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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