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**Original Research Article**

# Diosmetin alleviates periodontitis by inhibiting oxidative stress and pyroptosis through Nrf2/NF-κB/NLRP3 axis

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## Abstract

***Purpose:*** *To investigate the therapeutic role of diosmetin in periodontitis and its probable mechanism of action.*
***Methods:*** *Lipopolysaccharide (LPS) was used to induce periodontitis in periodontal cells. Cell viability and apoptosis in response to LPS and diosmetin were evaluated using MTT and TUNEL assays. The oxidative stress and inflammatory responses in LPS-induced periodontitis and diosmetin effects in periodontal cells were detected using enzyme-linked immunosorbent assay (ELISA). In addition, the roles of diosmetin in pyroptosis and Nrf2/NF-kappa B/NLRP3 pathway were analyzed by immunoblot assays.*
***Results:*** *Diosmetin increased the viability of LPS-induced periodontal cells ($p < 0.01$). Diosmetin also alleviated the oxidative stress of periodontal cells ($p < 0.01$), reduced the secretion of pro-inflammatory factors in periodontal cells, and inhibited cell pyroptosis ($p < 0.01$). Diosmetin also mediated Nrf2/NF-kappa B/NLRP3 pathway ($p < 0.01$).*
***Conclusion:*** *Diosmetin alleviates periodontitis by inhibiting oxidative stress and pyroptosis through Nrf2/NF-κB/NLRP3 axis. However, in vivo studies are required to validate this finding*

***Keywords:*** *Periodontitis, Diosmetin, Oxidative stress, Pyrosis, Nrf2/NF-kappa B/NLRP3 pathway*



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## INTRODUCTION

Periodontitis is known as an inflammatory disease caused by bacteria; it leads to the loss of periodontal tissues and is the main cause of tooth loss in adults [1]. A long-term chronic inflammatory environment leads to changes in the epigenetic characteristics of cells and reduces the regeneration ability of periodontal tissues [2,3]. Periodontal tissue consists of the gingiva, periodontal membrane, cementum, and alveolar bone. The periodontal membrane plays an important role in periodontal tissue homeostasis, repair, and nutrition [4]. Periodontal pathogens induce inflammation and immune responses in periodontal tissues and promote the expressions of various cytokines, such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and nuclear factor κB receptor activator [5,6]. Recent studies have shown that pyroptosis,

which plays an important role in innate immunity, was closely related to periodontitis [7]. In contrast to apoptosis, pyroptosis involves caspase-1–dependent inflammatory programmed cell death characterized by cell swelling, perforation, lysis, and release of cell contents [8].

Currently, treatments for periodontitis include nonsurgical methods such as curettage plus root flattening, and medications such as ornidazole, levofloxacin, and azithromycin. However, dependence on antibiotics greatly limits drug therapy. Therefore, it is necessary to continue to develop new drugs to effectively treat this disease.

Diosmetin belongs to a family of flavonoids found in citrus plants and olive leaves [9]. It is reported to have a variety of biological properties, such as anti-cancer, antioxidant, and anti-inflammatory activities. Diosmetin protects LPS-induced mouse lung tissues in acute lung injury by activating the Nrf2/ HO-1 pathway and inhibiting NLRP3/caspase-1. Diosmetin also produces synergistic effects in combination with 5-fluorouracil in colorectal cancer cells [10]. Diosmetin protects against cardiac hypertrophy via the p62/Nrf2 pathway. In addition, diosmetin alleviates cerebral ischemia-reperfusion injury (CI-RI) by activation of the Keap1/Nrf2 pathway and the inhibition of NLRP3 inflammasomes [11]. However, whether diosmetin has therapeutic effects on periodontitis has not yet been reported. Herein, the therapeutic role of diosmetin on periodontitis, and the possible mechanism, were therefore investigated.

## EXPERIMENTAL

### Cell culture

Human periodontal ligament cells (HPDLCs) were obtained from ScienCell (ScienCell Research Laboratories, San Diego, CA, USA) and maintained in Dulbecco's Modified Eagle's Medium (Gibco, Grand Island, NY, USA) containing L-glutamine, glucose, 15 mM Hepes, 200 U/mL penicillin, 270 µg/mL streptomycin, and 10 % (v/v) fetal bovine serum at 37 °C in a humidified atmosphere of 5 % $CO_2$. For LPS and diosmetin stimulations, cells were treated with the indicated concentrations of LPS and/or diosmetin.

### Cell viability

The HPDLCs were plated at the density of 3 × $10^3$ cells/well in 96-well plates. Cell viability was assessed with the addition of MTT solution. Cells were incubated for another 4 h and the insoluble formazan pellets were dissolved with dimethylsulfoxide before the measurement of absorbance values at 450 nm wavelength.

### TUNEL staining

The HPDLCs were fixed with formaldehyde, rinsed with phosphate-buffered saline, and then stained using a cell apoptosis detection kit (Roche Molecular Biochemicals, Mannheim, Germany). The degree of apoptosis was measured using a microscope (Olympus, Tokyo, Japan). The apoptotic cells were manually counted.

### Determination of superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), and myeloperoxidase (MPO)

The HPDLCs were collected for detection of MDA, SOD, GSH, and MPO using relevant commercial kits (Jiancheng Bioengineering Institute of Nanjing, Nanjing, China). The cells were homogenized and centrifuged (1,000×*g*) for 20 min, and the supernatant was collected. Then, the HPDLCs were added. The samples were gently shaken, mixed, and covered for a reaction at 37 °C for 2 h. A microplate reader was then used to detect the absorbance values of each well at a wavelength of 450 nm. The experiment was repeated three times.

### Enzyme-linked immunosorbent assay (ELISA)

The concentrations of TNF-α, IL-1β, and IL-6 in the cell lysates were measured with ELISA kits. Briefly, HPDLCs were added to wells. Biotin-conjugated primary antibodies were then added to each well before the addition of avidin-conjugated horseradish peroxidase. Then, an enzyme substrate was added for color development. The intensity was measured with a microplate reader at a wavelength of 450 nm (R & D Systems, Minneapolis, MN, USA).

### Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Cellular total RNA was extracted with TRIzol reagent (Thermo, Rockford, IL, USA). Total RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The cDNA was amplified using the primers presented in Table 1.

### Western blotting

Proteins were extracted with RIPA buffer (Beyotime, Shanghai, China). Then, the total proteins were collected and electrophoresed

using 10 % SDS-PAGE, transferred to PVDF membranes, and blocked with 5 % fat-free milk. Subsequently, the membranes were incubated with primary antibodies for 1 h at room temperature.

Finally, the membranes were conjugated with anti-mouse IgG and/or anti-rabbit IgG (Abcam, Cambridge, UK) for 1 h. Specific proteins were visualized with an enhanced chemiluminescence detection kit (Thermo Fisher, Waltham, MA, USA).

| Gene | Forward (5´-3´) | Reverse (5´-3´) |
|---|---|---|
| TNF-α | GGTGCCTATGTCTCAGCCTCTT | GCCATAGAACTGATGAGAGGGAG |
| IL-1β | ACAAGGAGAAGAAAGTAATGAC | GCTGTAGAGTGGGCTTAT |
| IL-6 | AGACAGCCACTCACC | TTCTGCCAGTGCCTCTT |
| Caspase-1 | ATGGCCGACAAGGTCCTGA | TTTAATGTCCTGGGAAGAGGTAGA |
| GAPDH | AGAAGGCTGGGGCTCATTTG | AGGGGCCATCCACAGTCTTC |

### Statistical analysis

GraphPad 6.0 was used for statistical analysis. Three replicates were performed for each experiment. One-way analysis of variance and Student's *t*-test were used for statistical comparisons. A value of $p < 0.05$ represented significance.

## RESULTS

### Diosmetin enhances the cell viability of HPDLCs

Diosmetin addition improved cell viability when cells were stimulated with LPS (Figure 1 A and B). In addition, LPS significantly induced apoptosis. Furthermore, diosmetin treatment relieved the decrease in viability and the increase of cell apoptosis (Figure 1 C and D). Collectively, the results showed that diosmetin promoted cell viability in LPS-induced HPDLCs.

### Diosmetin relieves oxidative stress in HPDLCs

The inductions of malondialdehyde (MDA) and MPO, and reductions of SOD and GSH were found in the LPS group. Treatment with diosmetin reversed the SOD, MDA, GSH, and MPO effects (Fig. 2). These results suggested that diosmetin was associated with reduced oxidative stress in HPDLCs.

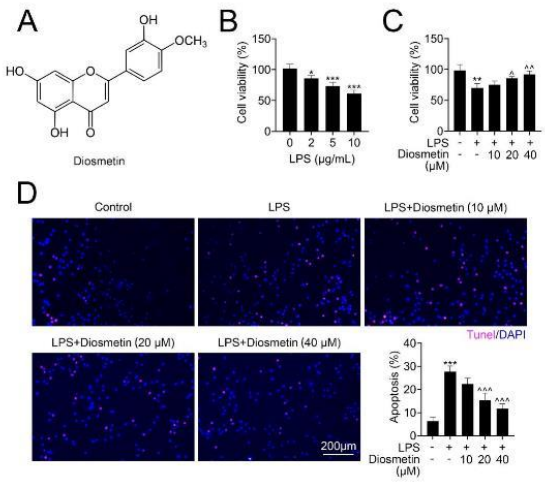


**Figure 1:** Diosmetin increased cell viability in human periodontal ligament cells (HPDLCs) (A) The structure of diosmetin. (B) The cell viability of HPDLCs exposed to lipopolysaccharide (LPS). (C) Cell viability of HPDLCs exposed to LPS and diosmetin. (D) Cell apoptosis as detected using TUNEL staining after exposure to LPS and diosmetin. ***$P < 0.001$ vs. control; ^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$ vs. LPS

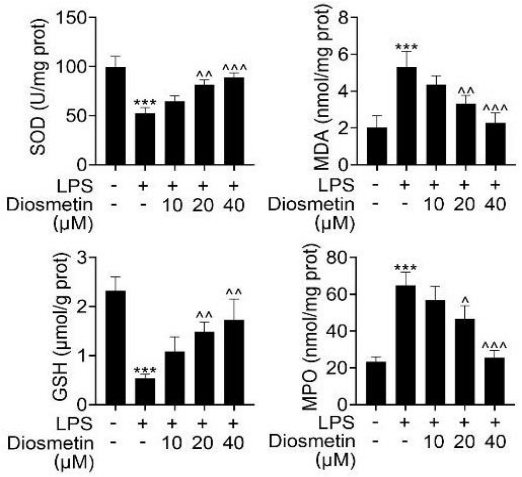


**Figure 2:** Diosmetin relieved oxidative stress in human periodontal ligament cells. The levels of superoxide dismutase (SOD), glutathione (GSH), malondialdehyde (MDA), and myeloperoxidase (MPO) in control, lipopolysaccharide (LPS), and diosmetin + LPS treated cells. ***$P < 0.001$ vs. control; ^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$ vs. LPS

### Diosmetin relieves LPS-induced HPDLC inflammation

The LPS increased the mRNA levels of IL-6, IL-1β, and TNF-α (Figure 3 A). Diosmetin treatment relieved LPS-induced cellular inflammation as indicated by a reduction of these cytokine levels (Figure 3 A). The levels of IL-6, IL-1β, and TNF-α proteins after diosmetin treatment showed similar results (Figure 3 B).

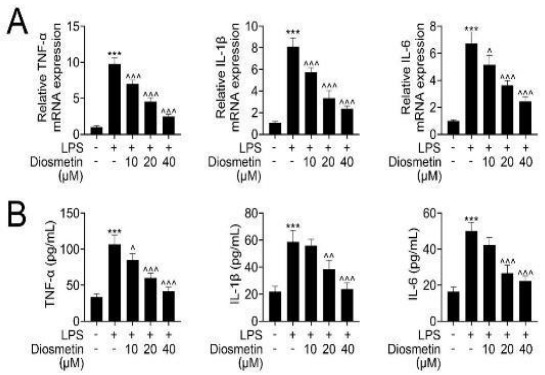


**Figure 3:** Diosmetin improved lipopolysaccharide (LPS)-induced human periodontal ligament cell inflammation. (A) The mRNA levels of TNF-a, IL-1b, and IL-6 in control, LPS, and diosmetin + LPS cells. (B) The protein levels of TNF-a, IL-1b, and IL-6 in control, LPS, diosmetin + LPS cells. ***$p < 0.001$ vs. control; ^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$ vs. LPS

### Diosmetin inhibits cell pyroptosis in HPDLCs

The LPS stimulated the expression of caspase-1, which was inhibited by diosmetin treatment (Figure 4 A), GSDMD-N, and cleaved-caspase-1, as well as inhibited the levels of gasdermin D (GSDMD). Diosmetin alleviated these changes (Figures 4 B and C). Together, these results showed that diosmetin inhibited cell pyroptosis in HPDLCs.

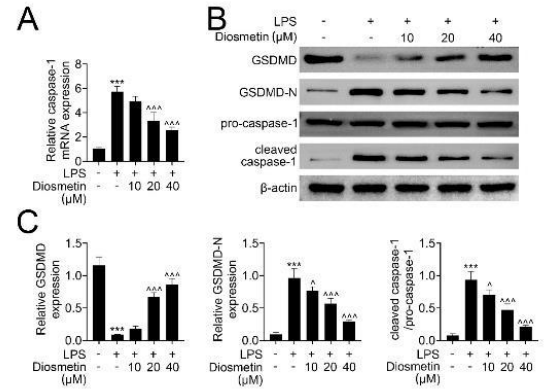


**Figure 4:** Diosmetin inhibited cell pyroptosis in human periodontal ligament cells. (A) Caspase-1 mRNA levels in control, lipopolysaccharide (LPS), and diosmetin + LPS cells. (B and C) The protein levels of gasdermin A (GSDMA), GSDMD-N, pro-caspase-1, and cleaved-caspase-1 in each group (B). The quantification is shown in panel C. ***$P < 0.001$ vs. control; ^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$ vs. LPS

### Diosmetin mediates Nrf2/NF-kappa B/NLRP3 pathway

The LPS upregulated the levels of p-p65, p-IκBα, and NLRP3, and reduced the levels of Nrf2 and HO-1. The effects of LPS were abrogated by diosmetin treatment (Figure 5). Diosmetin, therefore, alleviated periodontitis by suppressing oxidative stress and pyroptosis by regulating the Nrf2/NF-κB/NLRP3 pathway.

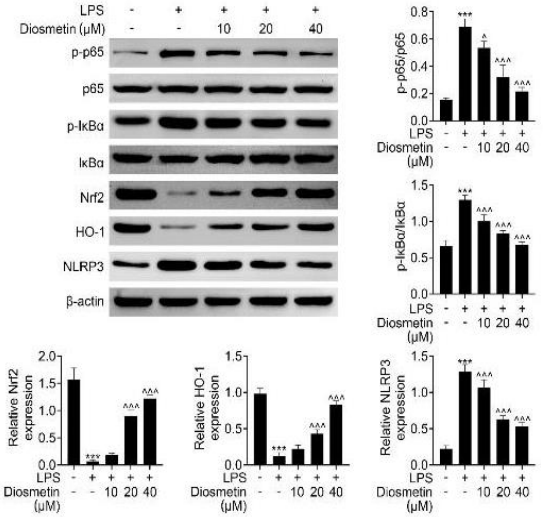


**Figure 5:** Diosmetin mediated the Nrf2/NF-kappa B/NLRP3 pathway of LCN1. The levels of p-p65, p-IkBa, Nrf2, HO-1, and NLRP3 in control, lipopolysaccharide (LPS)-treated, and diosmetin + LPS treated cells. ***$P < 0.001$ vs. control; ^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$ vs. LPS

## DISCUSSION

Periodontitis involves chronic inflammation of periodontal-supporting tissues and is mainly caused by local factors [12]. If gingivitis is not promptly treated, the inflammation may spread from the gums to the periodontal membrane, alveolar bone, and cementum, leading to periodontitis [13]. To further improve the therapeutic effects of periodontitis, it is necessary to further understand the mechanism of its progression, so that more effective therapeutic drugs can be developed [14]. In this study, diosmetin increased the activity of LPS-induced periodontal cells, alleviated oxidative stress, reduced the secretion of inflammatory factors, activated Nrf2 activity, and inhibited the NF-κB/NLRP3 pathway.

Based on the results of CCK-8 and TUNEL assays, diosmetin increased the viability of LPS-induced periodontal cells. Diosmetin also alleviated the oxidative stress of periodontal cells and reduced the secretion of proinflammatory factors, as determined by ELISA assays. Furthermore, using immunoblot assays, diosmetin inhibited cell pyrosis in periodontal cells. Diosmetin, therefore, served as a drug to treat periodontitis. The biological activities of

diosmetin have been widely studied. It has multiple activities such as anti-cancer, antioxidant, and anti-inflammatory effects, and experimental results have shown that it had good therapeutic effects on cardiac muscle injury, non-alcoholic steatohepatitis, colitis, and other diseases [15].

Diosmetin protects LPS-induced mouse lung tissue from acute lung injury by activating the Nrf2/ HO-1 pathway and inhibiting NLRP3/ caspase-1 [16]. In this study, an LPS-induced cell model was constructed, and the effects of diosmetin on inflammation and oxidative stress of LPS-stressed periodontal cells were assessed. Diosmetin also protected against cardiac hypertrophy via the p62/Nrf2 pathway. In addition, diosmetin alleviated cerebral ischemia-reperfusion injury (CI-RI) via the activation of the Keap1/Nrf2 pathway and inhibition of NLRP3 inflammasomes [17]. Similarly, diosmetin mediated Nrf2 activity, and inhibited the NF-κB/NLRP3 pathway, to alleviate periodontitis.

Other studies also revealed that diosmetin suppressed the growth and proliferation of HCC cells by regulating the cell cycle and lipid metabolism. Diosmetin also inhibits apoptosis and activates AMPK-induced autophagy during myocardial damage upon hypoxia treatment [18]. Diosmetin ameliorated vascular dysfunction by regulation of the Nrf2/HO-1 pathway in hypertensive rats, which was similar to the findings in this study. Diosmetin has therapeutic efficacy for the treatment of inflammation and oxidative stress, so these studies confirmed that diosmetin was an effective treatment for multiple types of diseases.

Pyroptosis is caspase-1–dependent inflammatory programmed cell death characterized by cell swelling, perforation, lysis, and release of cell contents, which differs from apoptosis. Pyrotopia also contributes to the development of many diseases, including periodontitis [19]. However, the precise molecular mechanism of this process remains unknown. In this study, diosmetin suppressed pyroptosis in LPS-induced periodontal cells.

## CONCLUSION

Diosmetin increases the activities of LPS-induced periodontal membrane cells, alleviates oxidative stress, reduces the secretion of inflammatory factors, and inhibits pyroptosis via Nrf2/NF-κB/NLRP3 pathway. Therefore, diosmetin may serve as a drug of choice for periodontitis.

## 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. Mingxuan Cheng, Pu Wang, and Di Wu designed the experiments and conducted them, analyzed and interpreted the data, and prepared the manuscript with contributions from all co-authors.



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