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Protective effect of acacetin in human periodontal ligament cells *via* regulation of autophagy and inflammation

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Received March 25, 2020, accepted April 24, 2020

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Pharmazie 75: 436-439 (2020) *doi: 10.1691/ph.2020.0435*

Our study investigated the effects of acacetin, a natural flavonoid compound, on the survival and expression of inflammatory related cytokines in lipopolysaccharide (LPS)-stimulated human periodontal ligament (PDL) cells. Treatment with acacetin significantly promoted survival and suppressed apoptosis in LPS-stimulated PDL cells in a dose-dependent manner, as shown by CCK-8 and flow cytometry assays, respectively. Moreover, ELISA assay showed that acacetin dose-dependently attenuated LPS-induced increases of TNF-α, IL-6 and IL-1β in PDL cells. Western blot analysis showed that administration of acacetin dose-dependently increased the ratio of LC3II/LC3I, as well as the expression of beclin-1, as compared to LPS-stimulated PDL cells. Inhibition of autophagy by rapamycin, an autophagy inhibitor, increased the production of pro-inflammatory cytokines and decreased survival, abolishing the beneficial role of acacetin in LPS-stimulated PDL cells. In addition, the expression of GSK-3β, a regulator of autophagy, was suppressed by administration with acacetin in a dose-dependent manner. Acacetin treatment promotes survival and suppresses inflammation in LPS-stimulated PDL cells via regulating autophagy and GSK-3β signal in PDL cells, suggesting that acacetin may be a potential novel agent for the treatment of chronic periodontitis.

1. Introduction

Periodontitis is a chronic multifactorial inflammatory disease with an adverse impact on human health. Accumulation of microorganisms e.g., Gram-negative bacteria, mainly contributes to the pathogenesis of periodontitis, leading to periodontal tissue destruction and tooth loss (Cobb 2017; Graziani et al. 2017). Lipopoiysaccharide (LPS), a component existing in the cell walls of Gram-negative bacteria, has been shown to induce the secretion of various inflammatory cytokines and suppress cell survival. The periodontium plays supporting roles in the tooth and is composed of the mineralized bone-like cementum, the alveolar bone and the periodontal ligament, which is a connective tissue between the cementum and alveolar bone (Mombelli et al. 2018). Human periodontal ligament (PDL) cells play critical roles in periodontitis progression and regeneration of periodontal tissues. Upon stimulation with LPS, PDL cells produce a variety of cytokines, such as Tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), matrix metalloproteinase-2 (MMP-1) and MMP9, which are responsible for activation of circulating immune cells, resorption of alveolar bone, and destruction of collagen (Liu et al. 2019; Onizuka and Iwata 2019). The traditional therapies for periodontitis are not effective in severe cases of periodontitis, thus the development of novel therapy options is necessary.

Acacetin, a flavonoid compound naturally present in plants such as chrysanthemum and safflower, has been widely reported to possess immunoregulatory properties (Jaganathan and Mandal 2009). It has been reported that acacetin prevents sepsis-induced acute lung injury *via* its anti-inflammatory and antioxidative activities. In addition, the suppressive role of acacetin in LPS-induced up-expression of induced NO synthase (iNOS) and cyclo-oxygenase-2 (COX-2) in murine macrophages has been previously reported (Pan et al. 2006). Autophagy plays an essential role in the pathogenesis of multiple inflammatory diseases such as periodontitis (Wei et al. 2018; Song et al. 2017). The functional association between autophagy and acacetin has been reported in human acute leukemia Jurkat T cells and breast cancer cells (Lee et al. 2017). However, the protective role of acacetin on periodontitis-related cells has not been investigated. Therefore, our study investigated the protective effect and the associated mechanism of acacetin in PDL.

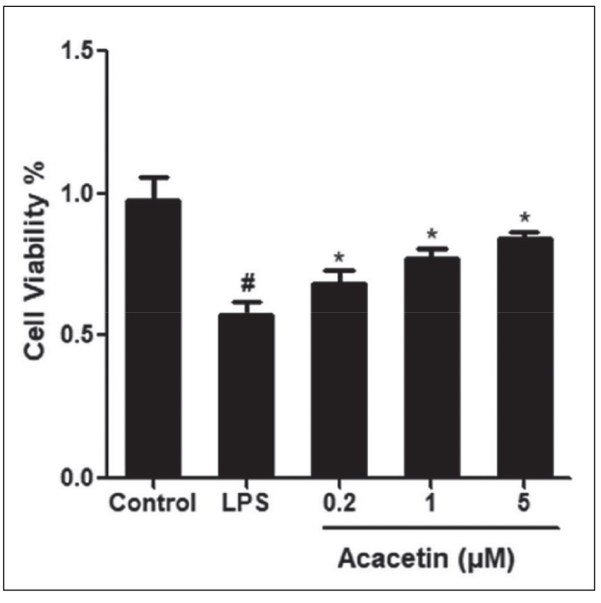

Fig. 1: Acacetin treatment promotes survival in human PDL cells exposed to LPS. Human PDL cells were exposed to LPS (1 μg/mL) and acacetin at different concentrations (0.5, 1, and 5 μM) for 24 h. Cell proliferation was measured using the CCK-8 assay. Error bars indicate mean ± SD. # $p<0.05$, versus the control group; * $p<0.05$, versus the LPS group. Statistical analysis was performed using One-way ANOVA and Tukey's post hoc test.

2. Investigations and results

2.1. Acacetin treatment promotes survival and reduces apoptosis in human PDL cells exposed to LPS

Firstly, PDL cells were exposed to LPS at the concentration of 1 μg/mL to simulate the inflammatory microenvironment of the body. As was shown in Fig. 1, LPS stimulation significantly suppressed PDL cell survival after 24 h treatment. Meanwhile, the effect of acacetin on PDL cell viability was measured using CCK-8 assay. Compared with the LPS-treated group, treatment with acacetin obviously elevated viability in LPS-stimulated PDL cells in a dose-dependent manner (Fig. 1). Moreover, the efects of LPS and acacetin on PDL cell apoptosis was measured by flow cytometry. As a result, LPS stimulation significantly induced apoptosis in PDL cells, whereas administration with acacetin obviously reversed LPS-induced apoptosis (Fig. 2). Taken together, these results revealed that acacetin treatment could suppress LPS-induced cellular injury in PDL cells.

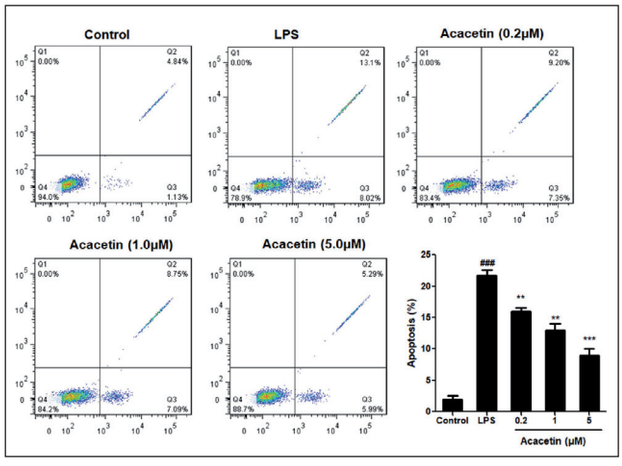

Fig. 2: Acacetin treatment reduces apoptosis in human PDL cells exposed to LPS. Human PDL cells were exposed to LPS (1 μg/mL) and acacetin at different concentrations (0.5, 1, and 5 μM) for 24 h. Cell apoptosis was measured using the flow cytometry assay. Error bars indicate mean±SD. ### p<0.001, versus the control group; ** p<0.01, *** p<0.001, versus the LPS group. Statistical analysis was performed using One-way ANOVA and Tukey's post hoc test.

2.2. Acacetin treatment alleviates LPS-induced inflammatory response in human PDL cells

Given that acacetin has been reported to exhibit anti-inflammatory effects, we next investigated the effects of acacetin on the expression of inflammatory mediators, such as TNF-α, IL-6, and IL-1β. ELISA assay showed that the expression of TNF-α, IL-6, and IL-1β significantly increased in PDL cells exposed to LPS. Conversely, treatment with acacetin remarkably suppressed LPS-induced upregulation of inflammatory cytokines including TNF-α, IL-6, and IL-1β, at a dose-dependent manner (Fig. 3).

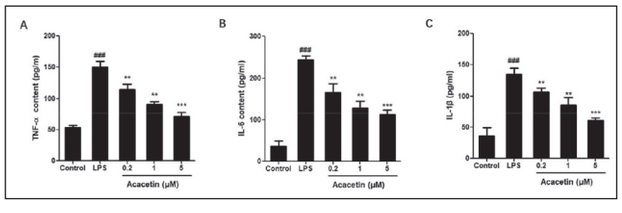

Fig. 3: Acacetin treatment alleviates LPS-induced inflammatory response in human PDL cells. Human PDL cells were exposed to LPS (1 μg/mL) and acacetin at different concentrations (0.5, 1, and 5 μM) for 24 h. ELISA assay was performed to detect the production of TNF-α (A), IL-6 (B), and IL-1β (C) in human PDL cells. Error bars indicate mean ± SD. ### p<0.001, versus the control group; ** p<0.01, *** p<0.001, versus the LPS group. Statistical analysis was performed using One-way ANOVA and Tukey's post hoc test.

2.3. Acacetin treatment activates autophagy in human PDL cells exposed to LPS

It is well known that autophagy plays an important role in cell death and inflammation. We thus explored whether acacetin treatment could regulate PDL cell viability and inflammatory response *via* autophagy. Interestingly, western blot analysis showed that administration with acacetin dose-dependently increased the ratio of LC3II/LC3I, as well as the expression of beclin-1, as compared to LPS-stimulated PDL cells (Fig. 4). These data indicated that administration with acacetin could regulate cell survival and inflammatory response *via* activation of autophagy in PDL cells.

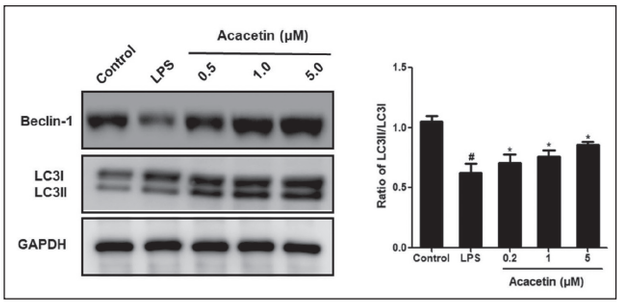

Fig. 4: Acacetin treatment activates autophagy in human PDL cells exposed to LPS. Human PDL cells were exposed to LPS (1 μg/mL) and acacetin at different concentrations (0.5, 1, and 5 μM) for 24 h. The protein expression of beclin-1 and LC3I/II as well as LC3II/LC3I ratio were measured using western blot and quantitative analysis. Error bars indicate mean±SD. ### p<0.001, versus the control group; ** p<0.01, *** p<0.001, versus the LPS group. Statistical analysis was performed using One-way ANOVA and Tukey's post hoc test.

2.4. Acacetin treatment suppresses GSK-3β signal in LPS-stimulated PDL cells

To elucidate the underlying mechanism by which acacetin regulates PDL cell autophagy, we detected the expression of GSK-3β, a regulator of autophagy. Compared to LPS-stimulated PDL cells, administration of acacetin significantly suppressed the expression of GSK-3β in a dose-dependent manner (Fig. 5), indicating that inhibition of GSK-3β pathway may contribute to the regulatory role of acacetin on PDL cellular behaviors.

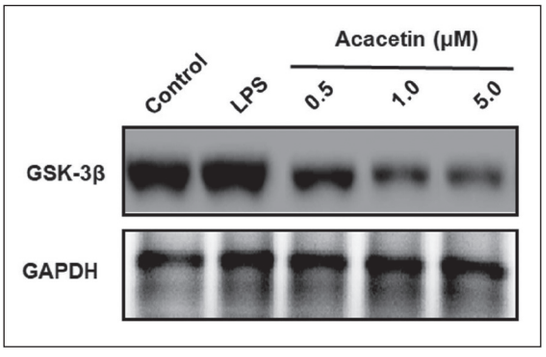

Fig. 5: Acacetin treatment suppresses GSK-3β signal in LPS-stimulated PDL cells. Human PDL cells were exposed to LPS (1 μg/mL) and acacetin at different concentrations (0.5, 1, and 5 μM) for 24 h. The protein expression of GSK-3β was measured using western blot analysis.

2.5. Autophagy-mediated role of acacetin on PDL cells exposed to LPS

To ascertain the role of autophagy in acacetin-induced survival and anti-inflammatory effects in PDL cells, the autophagy inhibitor rapamycin was then employed in PDL cells exposed to LPS. Results showed that rapamycin treatment obviously decreased PDL cell survival and promoted apoptosis, reversing the protective role of acacetin on LPS-stimulated cells. (Figs. 6A and B). Moreover,

inhibition of autophagy by rapamycin increased the production of pro-inflammatory cytokines, thus abolishing the suppressive role of inflammation induced by LPS in PDL cells (Fig. 6C and D). Taken together, these results verified that acacetin elevated cell survival and suppressed inflammation dependent on autophagy in PDL cells.

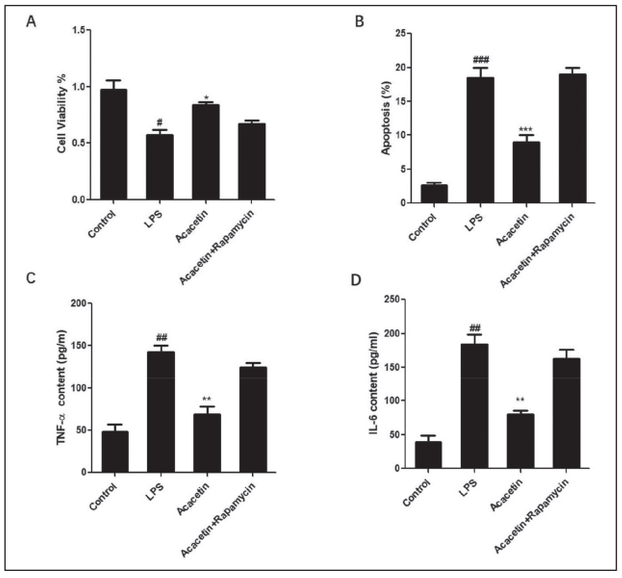

Fig. 6: Autophagy-mediated role of acacetin on PDL cells exposed to LPS Human PDL cells were exposed to LPS (1 μg/mL), acacetin (5 μM) or rapamycin for 24h. (A) Cell proliferation was measured using the CCK-8 assay. (B) Cell apoptosis was measured using the flow cytometry assay. ELISA assay was performed to detect the production of TNF-α (C) and IL-6 (D) in human PDL cells. Error bars indicate mean ± SD. ## $p<0.01$, ### $p<0.001$, versus the control group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, versus the LPS group. Statistical analysis was performed using One-way ANOVA and Tukey's post hoc test.

3. Discussion

Periodontitis is prevalent in the Chinese population and may occur in up to 50% of adults in the US (Eke et al. 2015). In this study, the effects and mechanism of acacetin were explored on human PDL cells, which is a cellular model widely used in periodontitis. Results showed that treatment with acacetin obviously promoted survival, inhibited apoptosis and inflammation in PDL cells exposed to LPS. Furthermore, acacetin treatment could activate cell autophagy and autophagy related to the GSK-3β signal. Therefore, these data revealed that acacetin could regulate cell survival and inflammation mediating the autophagy pathway, possibly providing a supplement for treatment of periodontitis.

During the pathogenesis of periodontitis, bacteria-induced inflammation is believed to be a crucial pathological factor. Therefore, numerous studies have focused on the role of human PDL in periodontitis due to their ability to secret inflammatory mediators in response to bacterial stimulation (Chen et al. 2016; de Jong et al. 2017). In addition, the critical role of inflammatory mediators in periodontitis has been widely reported. During the development of periodontitis, elevated production of TNF-α, IL-6 and IL-1β has been found in gingival crevicular fluid from patients with periodontitis, and they significantly contribute to the inflammatory response, leading to the periodontal tissue destruction and loss of periodontal attachment (Murakami et al. 2018; Yoshihara-Hirata et al. 2018). Application of IL-1 and TNF-α antagonists effectively suppresses the inflammatory response and bone destruction in experimental periodontitis (Delima et al. 2001; Fu et al. 2019).

Acacetin is an active flavonoid compound, isolated from a variety of plants. Numerous studies have reported that acacetin possesses anti-inflammatory, anti-cancer, anti-oxidative properties (Liu et al. 2018; Wu et al. 2018). However, its beneficial role has never been investigated in periodontitis. In the current study, we determined the protective role on LPS-stimulated human PDL cells. results showed that treatment with acacetin significantly improved survival and suppressed apoptosis in LPS-stimulated PDL cells in a dose-dependent manner. In addition, acacetin dose-dependently suppressed LPS-induced upregulation of inflammatory cytokines including TNF-α, IL-6, and IL-1β. Taken together, these data validated the beneficial effects of acacetin on the in vitro model of periodontitis.

The autophagy pathway is a homeostatic process with multiple effects on innate and adaptive immunity. One of the pivotal contributions of autophagy in immunity is the cell autonomous control of cellular viability, organ damage and the production of pro-inflammatory mediations (Deretic and Levine 2018; Zhong et al. 2016). However, autophagy is a double-edged sword in regulating cell death and inflammation dependent on the cellular context. Some studies point out that autophagy contributes to cell damage and inflammation, whereas a contradictory conclusion is made by that autophagy inhibits LPS-induced cell death and promotes the production of pro-inflammatory mediators in microglial cells (Ye et al. 2017; Bussi et al. 2017). In the current study, administration with acacetin dose-dependently increased the ratio of LC3II/LC3I, as well as the expression of beclin-1, as compared to LPS-stimulated PDL cells. Moreover, inhibition of autophagy by rapamycin increased the production of pro-inflammatory cytokines and decreased survival, thus abolishing the beneficial role of acacetin in LPS-stimulated PDL cells. GSK-3β is a multi-functional protein kinase involved in various cellular processes, such as proliferation, apoptosis, migration and autophagy. Studies reported that inhibition GSK-3β regulates autophagy activation (Mancinelli et al. 2017). Gavilán et al. (2013) demonstrated that GSK-3β inhibition triggers a profound autophagic response in human breast cancer cells. Sun et al. (2016) showed that GSK-3β plays an important role in controlling autophagy by mediating cytoplasmic LKB1 translocation and LKB1-AMPK interaction. In our study, administration of acacetin significantly suppressed the expression of GSK-3β in a dose-dependent manner, indicating that inhibition of GSK-3β pathway may contribute to the regulatory role of acacetin on PDL cellular behaviors.

In summary, the current study demonstrated that acacetin exhibits pro-proliferative and anti-inflammatory effects in LPS-stimulated human PDL cells. Mechanically, the beneficial role of acacetin on survival and apoptosis is mediated by cell autophagy and GSK-3β signal in PDL cells. these findings imply that acacetin may be developed as a supplement to current therapeutic regimens for periodontitis.

4. Experimental

4.1. Cell isolation and culture

PDL cells were isolated from the middle third of the root surface of premolars from healthy donors. This study was approved by the Ethics Committee of affiliated hospital of Inner Mongolia Medical University and obtained informed consent from all participants. The PDL cells were cultured at 37 °C with 5% CO_2 in DMEM supplemented with 10% FBS plus 1% penicillin/streptomycin.

4.2. CCK-8 assay

The effect of acacetin on PDL cells survival was determined using CCK-8 assay. PDL cells at the density of 3 x 10^3 cells/well were seeded into 96-well plates. Then, cells were stimulated with LPS for 24 h, and then treated with different concentrations of acacetin for 24 h. Subsequently, cells were incubated with CCK-8 solution (Sigma-Aldrich, USA). The optical density (OD) of 570 nm was measured by using a microplate reader (Molecular Devices, California, USA).

4.3. Apoptosis

The effects of acacetin on cell apoptosis were analyzed using annexin V/PI apoptosis assay (BD, USA) according to the manufacturer's instruction. In brief, PDL cells at the density of 3 x 10^3 cells/well were seeded into 96-well plates. Then, cells were stimulated with LPS for 24 h, and then treated with different concentrations of acacetin for 24 h. The cells from each group were washed with ice-cold PBS, and were resuspended in 100 μl binding buffer. Annexin V-FITC and PI were added to cell suspension, and incubated for 15 min in the dark at room temperature. Samples were then analyzed by flow cytometry.

4.4. Western blot

The effects of acacetin on signaling transduction were determined using western blot. The samples were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gels

and then transferred onto nitrocellulose membranes (Merck Millipore; Darmstadt, Germany). After being blocked with 5% skim milk, the membranes were incubated with primary antibodies against beclin-1, LC3 and GSK-3β at 4 °C overnight. The membranes were then incubated with corresponding secondary antibodies for 2 h at room temperature. Images were obtained using electrochemiluminescence (ECL).

4.5. Statistical analysis

Data were expressed as means±SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. A P-value < 0.05 was considered significant.

Funding: This study is supported by Doctoral Fund Project of affiliated hospital of Inner Mongolia Medical University :" Application of Cone-Beam CT in the study of the position and angle of the upper middle incisors with Class II division 1 malocclusion "(project number : NYFY BS 2018) and Inner Mongolia natural science foundation project: "Application of Cone-Beam CT in the study of the position and angle of the upper middle incisors with Class II division 1 malocclusion"(project number : 2018MS08103)

Conflict of interest: The authors declare that they have no competing interests.

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