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Original Article

Flammulina velutipes polysaccharides exhibit potent antioxidant and anti-pyroptotic properties in diabetes-associated periodontitis: A preliminary in vitro study

Chun-Cheng Chen [a,b†], Ni-Yu Su [a,b†], Szu-Han Chen [a,c,d], Yi-Wen Liao [c,d], Chang-Wei Hsieh [e,f,g**], Cheng-Chia Yu [a,b,c*]

[a] School of Dentistry, Chung Shan Medical University, Taichung, Taiwan
[b] Department of Dentistry, Chung Shan Medical University Hospital, Taichung, Taiwan
[c] Institute of Oral Sciences, Chung Shan Medical University, Taichung, Taiwan
[d] Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan
[e] Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan
[f] Department of Food Science, National Ilan University, Yilan, Taiwan
[g] Department of Medical Research, China Medical University Hospital, Taichung, Taiwan

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Abstract *Background/purpose:* Diabetes-associated periodontitis (DAP) is a complicated illness characterized by elevated oxidative stress and a pro-inflammatory response. There is a bidirectional relationship between diabetes and periodontitis. Although *Flammulina velutipes* polysaccharides (FVP) has demonstrated anti-inflammatory properties, its specific role in DAP remains uncertain. The purpose of our study was to seek the protective effects and the underlying mechanism of FVP against advanced glycation end products (AGEs) and lipopolysaccharide (LPS)-induced damage in human gingival fibroblasts (HGFs).
Materials and methods: Our study used HGFs treated with AGEs and LPS to mimic the in vitro environment of DAP. MTT assay was utilized to seek the various concentrations of FVP (a component of *Flammulina velutipes*) affected cell survival, migration, reactive oxygen species (ROS) generation, and cell senescence. Western blotting was used to evaluate the expression of pyroptosis pathway-related proteins, while ELISA was used to detect proinflammatory cytokines interleukin (IL)-6 and IL-8 levels.

* Corresponding author. Institute of Oral Sciences, Chung Shan Medical University, South Dist., Taichung City 402, Taiwan.
** Corresponding author. Department of Food Science and Biotechnology, National Chung Hsing University, South Dist., Taichung City 402, Taiwan.
E-mail addresses: welson@nchu.edu.tw (C.-W. Hsieh), ccyu@csmu.edu.tw (C.-C. Yu).
† These two authors contributed equally to this study.

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Results: Our study discovered that FVP exhibited minimal cytotoxicity to HGFs at the dosages examined. Co-treatment with AGEs and LPS dramatically reduced HGFs cell survival and migratory capacity, while considerably increasing intracellular ROS levels and expression of the cell senescence marker p16. However, the treatment of FVP restore these AGE and LPS-induced adverse effects, as evidenced by the restoration of cell survival and wound healing capacity, ROS production, and decrease the protein expression of p16. FVP inhibits AGEs and LPS-induced cell pyroptosis by lowering pyroptosis markers (apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), NLR family pyrin domain containing 3 (NLRP3), caspase-1, gasdermin D (GSDMD), and IL-1β. Furthermore, FVP dramatically decreased proinflammatory cytokine production levels, including IL-6 and IL-8.
Conclusion: The results of our study demonstrated that FVP had a significant protective effect on human gingival fibroblast damage caused by AGEs and LPS. The addition of FVP can reverse pathological processes such as cellular oxidative stress, poor wound healing, inflammaging, and pyroptosis caused by AGEs and LPS. These findings suggest that FVP could be effective as a therapeutic or adjuvant treatment for DAP.
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Introduction

The main characteristics of diabetes mellitus (DM) are insulin resistance and persistent hyperglycemia, which lead to excessive accumulation of advanced glycation end products (AGEs) in tissues, thereby increasing oxidative stress and ultimately causing diabetic complications.[1] Periodontitis is an inflammatory disease which is triggered by infectious periodontal bacteria residing in dental plaque and has been considered as the sixth complications of DM.[2] Uncontrolled blood sugar increase the accumulation of AGEs and accelerates the risk of periodontal tissue destruction and alveolar bone loss.[3] Current research indicate a bidirectional association between diabetes and periodontitis, diabetes-induced AGEs raises the concentration of pro-inflammatory cytokines that exacerbate the impairment of periodontal tissue[4–6] and vice versa. Appropriate periodontal disease treatment can reduce glycosylated hemoglobin levels and improve blood sugar control. However, the underlying mechanism between diabetes and periodontitis remains to be elucidated.

Chronic inflammation[7] and elevated oxidative stress[8–10] are the primary causes of diabetes-associated periodontitis (DAP). AGEs bind to the receptor of AGEs (RAGE), leading to increased oxidative stress, inflammation, and production of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin (IL)-1β in periodontal tissues.[11] In DAP, AGEs stimulates mitogen activated protein kinase (MAPK) and nuclear transcription factor-κB (NF-κB) pathways by binding to receptor for AGEs (RAGE), enhancing IL-6 and intercellular adhesion molecule-1 (ICAM-1) expression in human gingival fibroblasts (HGFs).[12] Besides, elevated and sustained blood sugar levels exacerbate oxidative stress, which, in turn, is a significant driver of cell senescence.[13] Exposure to periodontal pathogens can induce senescence in periodontal ligament fibroblasts (PDLFs). These senescent PDLFs cells secrete senescence-associated secretory phenotype (SASP) such as IL-6, IL-8, IL-1β, TNF-α, which contributes to increased chronic inflammation and potentially accelerates alveolar bone destruction.[14]

Pyroptosis is an inflammatory programmed cell death that plays a vital role in the pathogenesis of DAP.[15,16] Upon pathogen activation, pathogen-associated molecular patterns (PAMPs) activate the NLR family pyrin domain containing 3 (NLRP3) inflammasome, which recruits apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase-1 to form an inflammasome complex. Next, pro-capsase-1 is converted to caspase-1 and in turn cleaves gasdermin D (GSDMD), causing the insertion of the GSDMD N-terminus into the cell membrane and release inflammatory cytokines such as IL-18 and IL-1β ultimately leading cell rupture.[17] Pyroptosis is caused by activation of the NLRP3 inflammasome, which has been found to be increased in periodontal ligament cells, gingival fibroblasts, epithelial cells, and macrophages in response to lipopolysaccharide (LPS) stimulation.[18,19] Recent investigations have found a link between DAP and pyroptosis, with greater cell death owing to pyroptosis detected in gingival tissue of DAP patients. Recent research has elucidated that hyperglycemia contributes to periodontitis by suppressing macrophage autophagy and ROS-inflammasome-mediated cell pyroptosis.[20]

Besides blood sugar control and conventional periodontal therapy, researchers are devoted to finding out whether there are adjunctive therapies that can be used for DAP. Studies have shown that many edible and medicinal fungi and their extracts such as polysaccharides have significant biological activities such as anti-inflammatory, antioxidant, and immunomodulatory.[21] *Flammulina velutipes* (FV) is a common edible fungus which contains a high content of polysaccharides which are the primary bioactive component of FV.[22] *Flammulina velutipes* polysaccharides (FVP) have gained attention for their remarkable health-promoting features particularly in minimizing oxidative stress and inflammation.[23] Studies have shown that yellow strain FVP have higher antioxidant and ROS-scavenging properties than white strain FVP, implying a greater

potential for therapeutic actions.[24,25] In addition, Ma et al. demonstrated that fermented FVP suppresses the NLRP3 signaling pathway, leading to downregulation of IL-1β, IL-6, IL-18, and TNF-α and thereby exerting anti-inflammatory and antioxidant effects that alleviate intestinal inflammation in mice.[26] While FVP has demonstrated anti-inflammatory and antioxidant effects, its ability to alter pyroptosis-mediated inflammasome activation or delay cellular senescence in the periodontal microenvironment under hyperglycemic conditions remains uninvestigated. As a result, the current study intends to evaluate the effects of FVP on both pyroptotic signaling and senescence markers in DAP, as well as to clarify the molecular pathways by which FVP may provide periodontal protection. The goal of this study was to provide preliminary evidence for the possible use of FVP as a novel therapeutic or supplementary intervention in DAP management.

The purpose of this study was to establish the foundational evidence for the potential use of FVP as a novel therapeutic or complementary intervention in managing DAP.

Materials and methods

Polysaccharide extraction from yellow strain of FVP

The method of preparing and isolating FVP was as described previously.[24] To remove lipids and pigments, the fruiting bodies were immersed in 80 % ethanol and extracted at 75 °C for 6 h. Following extraction, the residue was exposed to ultrasonic-assisted water extraction for 150 min at a solid-liquid ratio of 20 ml/g, an ultrasonic frequency of 65 Hz, and a temperature of 95 °C. The water extraction phase was done three times. After the extraction is finished, the mixture is centrifuged at 20 °C and 2000 times gravity for 10 min to separate the supernatant from the insoluble residue. Polysaccharides called FVP were then collected at ethanol concentrations of 80 % for fractional precipitation at 4 °C.

Cell culture

HGFs will be extracted from the gingiva of healthy patients undergone crown lengthening at Chung Shan Medical University Hospital. Gingival tissue from diabetic patients will be retrieved during surgical periodontal therapy. Primary HGFs will be cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10 % fetal bovine serum and 1 % penicillin-streptomycin. AGEs-BSA and LPS were obtained from Bio-Vision (BioVision, Inc., Milpitas, CA, USA).

Cell viability assay

1×10^4 cells/well of HGFs were seeded in 96-well plates (Corning, Acton, MA, USA) and allowed to attach for 24 h. HGFs were then treated with 200 μg/mL AGE-BSA and LPS for 24 h, followed by various concentrations of FVP (0—500 μg/mL) for another 24 h. Cell viability and proliferation were evaluated by adding 100 μL of 10-fold diluted Prestoblue reagent to each well and subsequently measuring absorbance at 570 nm. Control group defined 100 % cell viability, and results from other groups were expressed relative to the control.

Wound healing assay

The procedures will follow in accordance to our previous study. Cells were seeded onto 12-well plates and cultured to roughly at a confluence of 90 %. Next, sterile 200 μL pipette tip was used to create a wound region on the cell monolayer. Cell migration into the wound region was observed and photographed using a microscope at 0 and 24 h.

Reactive oxygen species analysis

Following the treatments mentioned above, HGFs cells were rinsed, fresh medium and 10 μM 2′,7′-dichlorodihydrofluorescein diacetate indicator were added, and cells were incubated for 1 h at 37 °C. Subsequently, cells were washed, detached with Trypsin-EDTA, collected by centrifugation, and rinsed again with PBS. Flow cytometry was then used to assess DCF fluorescence intensity, thereby quantifying intracellular ROS levels.

Western blot

The Western blot analysis was utilized following the previously described protocol.[27] Primary antibodies against cell senescence marker p16 and pyroptosis markers ASC (Cell Signaling Inc., Danvers, MA, USA), NLRP3 (Invitrogen Life Technologies, Carlsbad, CA, USA), pro-caspase-1 and cleaved caspase-1 (Abcam, Cambridge, UK), pro-GSDMD and cleaved GSDMD (Cell signaling), pro-IL-1β and IL-1β (Cell signaling), and GAPDH (Invitrogen) were used. Secondary antibodies against anti Mouse (Millipore, Merck, Darmstadt, Germany) and Polyclonal Rabbit Anti Human (Agilent Technologies, Santa Clara, CA, USA) were also used. Bound antibodies were detected using enhanced chemiluminescence (ECL), and images were captured using an ImageQuant LAS 4000 Mini.

Senescence-associated β-galactosidase (SA-β-gal) activity

Cellular senescence was detected based on a previously established procedure using a commercially available Cellular Senescence Assay kit to quantify the activity of SA-β-Gal (senescence marker β-galactosidase). SA-β-Gal positive cells will be observed and counted using an optical microscope.

ELISA analysis

IL-6 and IL-8 concentrations in cell culture supernatants were determined using ELISA kits, following the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader. Each HGFs sample was analyzed in triplicate.

Statistical analysis

All experiments were repeated three times independently. The data were analyzed using one-way analysis of variance (ANOVA), and the differences among the treatment groups were compared using Duncan's multiple range test. The result is considered statistically significant when *P*-value is less than 0.05.

Results

First of all, the effect of a variety of concentrations of FVP on the cell viability of HGFs was assessed. The results indicated that FVP at doses ranging from 0 to 500 μg/ml did not significantly impair cell viability (Fig. 1A). Therefore, concentrations of 250 and 500 μg/ml FVP were chosen for further studies. To imitate the conditions of DAP, HGFs were exposed to AGEs and LPS. Results showed that co-treatment with AGEs and LPS dramatically reduced HGFs viability, but the cell viability was significantly recovered by the addition of FVP (Fig. 1B).

The scratch assay was used to investigate the impact of FVP on the migration of HGFs treated with AGEs + LPS. The results demonstrated that cell migration drastically decreased while AGEs + LPS treatment. Nevertheless, both 250 μg/ml and 500 μg/ml FVP significantly restored cell migratory ability, indicating that FVP can counteract AGEs + LPS-induced impairment in HGFs cell migration (Fig. 1C). Intracellular ROS levels were measured in order to further investigate the therapeutic impact of FVP on HGFs exposed with AGEs and LPS. The findings showed that ROS generation in HGFs was elevated by AGEs + LPS stimulation. On the other hand, ROS production was considerably reduced in a dose-dependent manner by the administration of 250 and 500 μg/ml FVP (Fig. 2).

Senescence-associated β-galactosidase (SA-β-gal) staining was used to examine how FVP affected AGEs and LPS-induced senescence in HGFs. Fig. 3 demonstrated that AGE and LPS stimulation aggravated HGFs senescence, as indicated by elevated protein expression of the senescence marker p16, and greater SA-β-gal positive cells. In accordance with treatment of 250 μg/ml and 500 μg/ml FVP both dramatically decreased the percentage of senescent cells and inhibited the expression of p16.

Multiple studies reveal the elevation of the NLRP3 inflammasome proteins in patients with periodontitis and uncontrolled type 2 diabetes.[28,29] Furthermore, the study investigated the effect of FVP on AGEs and LPS-induced pyroptosis. Stimulation with AGEs and LPS activated the NLRP3 inflammasome, resulting in increased production of the adaptor protein ASC and NLRP3. This activation resulted

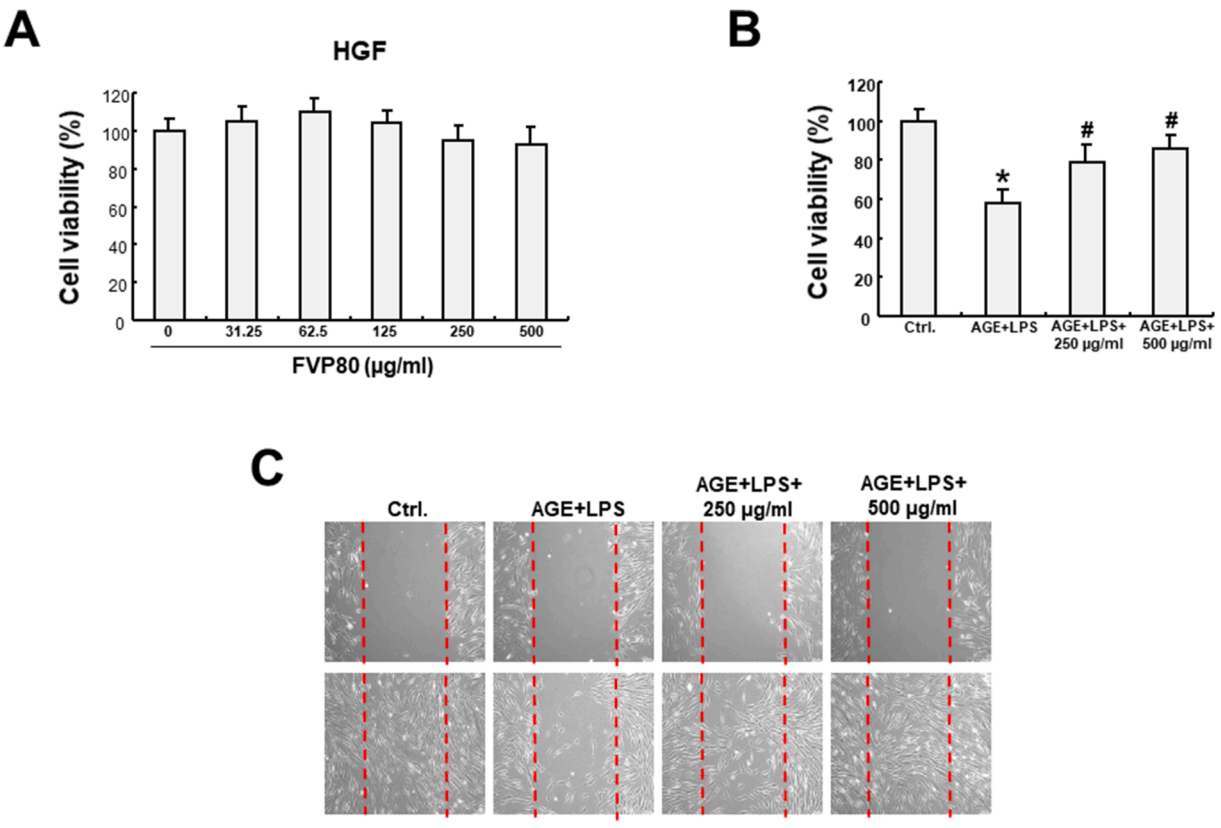

Figure 1 Flammulina velutipes polysaccharides (FVP) treatment restored cell viability and improved wound healing ability in human gingival fibroblasts (HGFs) induced by advanced glycation end products (AGEs) and lipopolysaccharide (LPS).
The cell viability was assessed using the MTT assay after 24 h of incubation with varying concentrations of FVP (A). HGFs were pretreated with AGEs and LPS for 24 h, followed by the addition of 250 and 500 μg/mL FVP to examine the cell viability (B) and wound healing capacity (C). Data represent the mean ± SD. *$P < 0.05$ compared to control group; #$P < 0.05$ compared to the AGEs + LPS group.

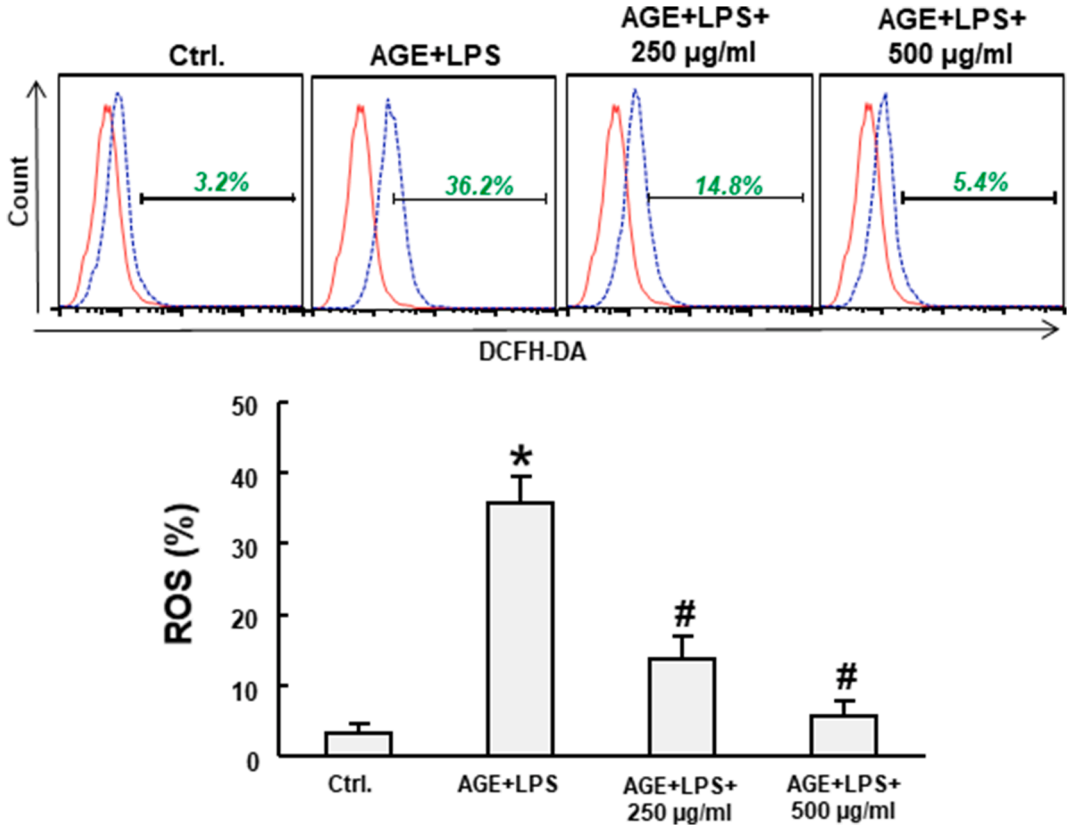

Figure 2 Flammulina velutipes polysaccharides (FVP) treatment elevated reactive oxygen species (ROS) production in advanced glycation end products (AGEs) and lipopolysaccharide (LPS)-treated human gingival fibroblasts (HGFs).
Cellular ROS production in HGFs pretreated with AGEs and LPS, followed by the addition of FVP, was examined using DCFH-DA staining and flow cytometry. Data represent the mean ± SD. *$P < 0.05$ indicates a significant difference compared to the control group, and #$P < 0.05$ indicates a significant difference compared to the AGEs-treated group.

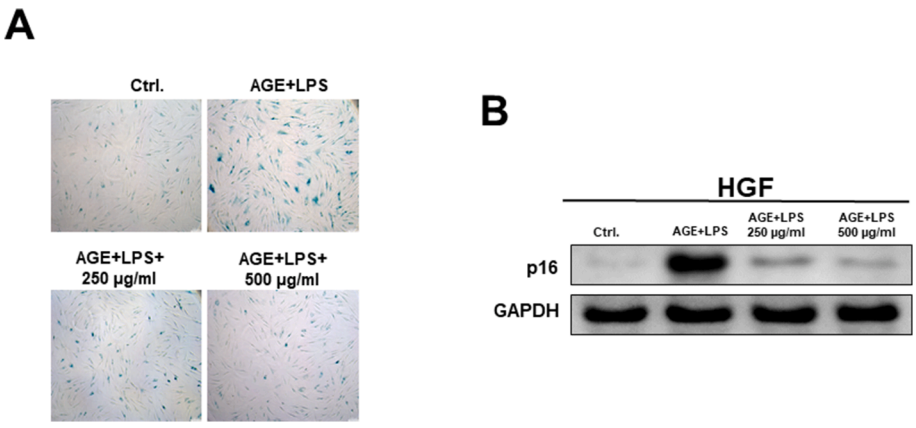

Figure 3 Flammulina velutipes polysaccharides (FVP) attenuated cell senescence triggered by advanced glycation end products (AGEs) and lipopolysaccharide (LPS)-treated human gingival fibroblasts (HGFs).
Senescence-associated β-galactosidase (SA-β-gal) activity was measured in HGFs pretreated with AGEs and LPS, followed by the addition of FVP (A). The protein level of senescence marker p16 was determined by Western blot under the same treatment conditions (B).

in the cleavage of pro-caspase-1, pro-GSDMD, and pro-interleukin (IL)-1β into their active forms (cleaved-caspase-1, cleaved-GSDMD, and cleaved-IL-1β). FVP administration significantly reduced the expression and activation of pyroptosis-related proteins, showing its ability to inhibit NLRP3-mediated pyroptosis (Fig. 4). Ultimately, AGEs and LPS increased the release of the pro-inflammatory cytokines IL-6 and IL-8 in HGFs, but FVP effectively decreased

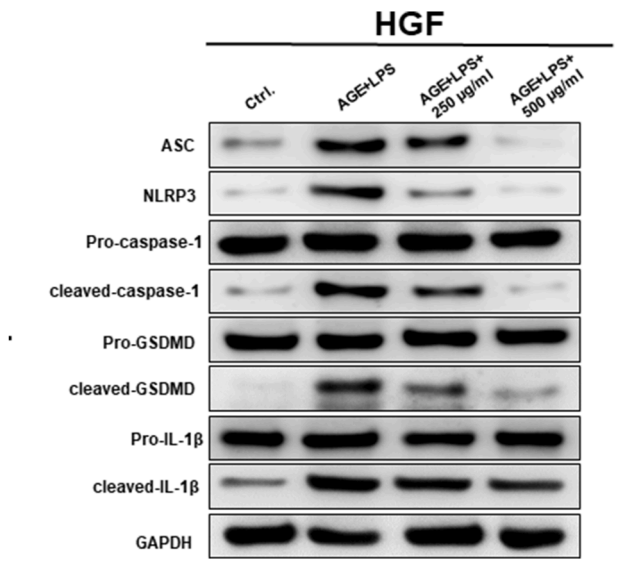

Figure 4 ***Flammulina velutipes*** **polysaccharides (FVP) suppressed the protein expression of pyroptosis markers induced by advanced glycation end products (AGEs) and lipopolysaccharide (LPS)-treated human gingival fibroblasts (HGFs).**
Protein levels of NLRP3, pro-caspase-1, cleaved caspase-1, pro-GSDMD, cleaved-GSDMD, pro-IL-1β, and cleaved IL-1β were measured by Western blot in HGFs pretreated with AGEs and LPS, followed by the addition of FVP. GAPDH was used as a loading control. Data represent the mean ± SD. $^{*}P < 0.05$ compared to control group; $\#P < 0.05$ compared to the AGEs + LPS group.

the levels of these pro-inflammatory cytokines, indicating its anti-inflammatory properties (Fig. 5).

Discussion

Our study initially demonstrates that FVP effectively protected HGFs from AGEs/LPS-induced damage through scavenging ROS and inhibiting NLRP3 inflammasome pathway, accompanied by multiple effects such as anti-inflammation and promoting cell repair. First, our study demonstrate that the administration of FVP does not exhibit cytotoxicity. Furthermore, pretreatment with AGEs and LPS reduced cell viability in HGFs; however, the subsequent addition of FVP was observed to enhance cell survival (Fig. 1). Besides, our results also demonstrated that FVP could significantly reduce the intracellular ROS level of HGFs induced by AGEs/LPS, which is consistent with previous reports on the antioxidant activity of edible and medicinal fungal polysaccharides.[23]

Flammulina velutipes (FV), especially the yellow strain often known as Jinhua mushroom in Taiwan consist of a high concentration of vital amino acids, proteins, dietary fiber, and polysaccharides[30] which has been reported to have liver-protective,[31] anti-cancer,[32] and immunomodulatory[33] activities in various studies. The research results clearly show that FVP not only effectively improves the cell survival rate and migration and repair potential of HGFs under the co-stimulation of AGEs and LPS, but more importantly, it exhibits strong antioxidant capacity and significantly inhibits the excessive generation of intracellular ROS. ROS is not only a direct executor of cell damage, but also one of the important activation signals of NLRP3 inflammasome. The pathological process of DAP is closely related to persistent oxidative stress and inflammatory response. Pyroptosis, as a programmed proinflammatory cell death mode, has been considered to play a core role in tissue damage and inflammation amplification in recent years. Indeed, FVP particularly from certain strains, exhibit superior antioxidant and ROS-scavenging capabilities compared to those from the white strain.[24,25] Furthermore, FVP has demonstrated significant anti-pyroptotic potential by effectively reducing the expression of key pyroptosis mediators, including NLRP3, caspase-1, and IL-1β, across various experimental models.[26,30] This anti-pyroptotic activity is highly pertinent to DAP, where increased pyroptotic cell death and NLRP3 inflammasome activation are evident in gingival tissues[34,35] and correlate with greater disease severity and inflammation.[28]

In diabetic patients, an increased burden of advanced glycation end-products (AGEs) triggers inflammatory cascades that accelerate periodontal tissue destruction.[36] Conversely, periodontitis exacerbates systemic inflammation, which can impair insulin sensitivity and complicate diabetes management.[37] NLRP3 inflammasome is a critical mediator and its activation elevated levels of both NLRP3 and pyroptotic markers are detected in the gingival tissues

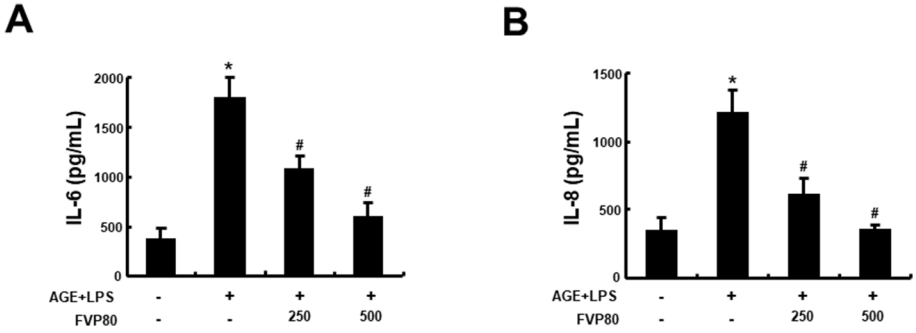

Figure 5 ***Flammulina velutipes*** **polysaccharides (FVP) decreased the production of pro-inflammatory cytokines induced by advanced glycation end products (AGEs) and lipopolysaccharide (LPS)-treated human gingival fibroblasts (HGFs).**
The production of IL-6 (A) andIL-8 (B) was evaluated in HGFs pretreated with AGEs and LPS, followed by treatment with 250 and 500 μg/mL FVP. Data represent the mean ± SD. $^{*}P < 0.05$ compared to control group; $\#P < 0.05$ compared to the AGEs + LPS group.

of patients with DAP.[34,35] Notably, recent studies have revealed that FVP possess significant anti-pyroptotic properties,

The core highlight of this study is that it revealed that FVP has a significant inhibitory effect on NLRP3 inflammasome-mediated cell pyroptosis. The classical pathway of pyroptosis involves the NLRP3 protein sensing danger signals, recruiting ASC and activating pro-caspase-1. Activated caspase-1 further cleaves GSDMD, releasing the GSDMD-N-terminal fragment with pore-forming activity, which punches holes in the cell membrane, causing cell swelling, rupture, and release of a large amount of proinflammatory contents, including mature IL-1β and IL-18. The data of this study showed that FVP significantly downregulated the levels of NLRP3, ASC, activated caspase-1 and GSDMD-N, and also inhibited the release of mature IL-1β. This finding echoes reports in the literature that other natural polysaccharides such as *Poria cocos* polysaccharides and *Lycium barbarum* polysaccharides exert anti-inflammatory effects by inhibiting NLRP3 inflammasome activation,[38,39] suggesting that FVP may intervene in the pyroptosis pathway through similar or unique mechanisms. For example, the antioxidant effect of FVP may reduce the upstream triggering signals of NLRP3 inflammasome activation; in addition, it is not ruled out that FVP may directly or indirectly affect the expression of NLRP3 protein, ASC oligomerization, or caspase-1 activation. Currently, the development of therapeutic strategies targeting NLRP3 inflammasome or cell pyroptosis for diabetes or periodontitis has become a research hotspot, and this activity of FVP provides strong theoretical support for its application. In addition to its core anti-pyroptotic effect, FVP also exhibits a wide range of cytoprotective effects. Its ability to reduce the secretion of proinflammatory cytokines IL-6 and IL-8 further enhances its anti-inflammatory potential, which is consistent with the results of multiple research reports that polysaccharides inhibit inflammatory cytokines such as NF-κB. Furthermore, the inhibitory effect of FVP on cell senescence markers p16 and senescence-associated β-galactosidase (SA-β-gal)-positive cells also suggests that it has the potential to delay cell function degeneration in DAP, because cell senescence has been shown to aggravate inflammatory responses and weaken tissue repair capacity. These multifaceted protective effects—antioxidant, anti-pyroptotic, anti-inflammatory, and anti-aging—together constitute the molecular basis for FVP to combat cellular damage in simulated DAP.

In conclusion, our study provides strong evidence indicate that FVP can effectively protect HGFs from multiple insults under conditions simulated with DAP. FVP showed antioxidant and anti-pyroptosis properties coupled with its anti-inflammaging effects, making it a natural candidate with great development potential, providing important perspectives for the future development of an effective treatment strategies for inflammatory diseases such as DAP.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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