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Original Article

Quercetin ameliorates advanced glycation end product-induced wound healing impairment and inflammaging in human gingival fibroblasts

Chao-Yen Huang [a,b,c], Min Yee Ng [d†], Taichen Lin [d,e†], Yi-Wen Liao [a,f], Wei-Shiuan Huang [a], Chang-Wei Hsieh [g], Cheng-Chia Yu [a,d,e*], Chun-Jung Chen [d,h**]

[a] *Institute of Oral Sciences, Chung Shan Medical University, Taichung, Taiwan*
[b] *Department of Emergency Medicine, Chung Shan Medical University Hospital, Taichung, Taiwan*
[c] *School of Medicine, Chung Shan Medical University, Taichung, Taiwan*
[d] *School of Dentistry, Chung Shan Medical University, Taichung, Taiwan*
[e] *Department of Dentistry, Chung Shan Medical University Hospital, Taichung, Taiwan*
[f] *Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan*
[g] *Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan*
[h] *Division of Periodontics, Department of Dentistry, Chi Mei Medical Center, Tainan, Taiwan*

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Abstract *Background/purpose:* Diabetes mellitus (DM) and periodontal disease are both prevalent and chronic inflammatory disorders that have significant health impact. Many studies have pointed out that advanced glycation end-products (AGEs) in DM induces inflammaging, which is a pre-aging and hyperinflammatory condition, and it has been linked to a greater likelihood in developing periodontitis. Inflammaging in DM has been shown to be driven by AGEs-induced cell senescence, inflammatory cytokines, and oxidative stress, resulting in the degradation of periodontium. Quercetin has shown abilities to decrease inflammation and oxidative stress in a variety of tissues, however, the effect in diabetic periodontitis remains uncertain. Thus, the aim of this study was to investigate its impacts on inflammaging in diabetic periodontitis.
Materials and methods: We examined cell proliferation in human gingival fibroblasts (HGF), wound healing, IL-6 and IL-8 secretions, cellular senescence expression, and the formation of

* Corresponding author. Institute of Oral Sciences, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Rd., Taichung 40201, Taiwan. Fax: 886-4-24759065.
** Corresponding author. Division of Periodontics, Department of Dentistry, Chi Mei Medical Center, No. 901, Zhonghua Rd., Yongkang Dist., Tainan 71004, Taiwan.
E-mail addresses: ccyu@csmu.edu.tw (C.-C. Yu), markb0111@yahoo.com.tw (C.-J. Chen).
† These two authors contributed equally to this study.

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reactive oxygen species (ROS) in response to AGE stimulation with and without Quercetin intervention. Following that, we looked into NF-κβ activity to see if Quercetin mediate its effects via this pro-inflammatory signaling.
Results: Quercetin at 20 μM and below did not have any impact on HGFs' cell proliferation rate. Quercetin intervention improved the AGEs-impaired wound healing, in addition to the attenuation of AGEs-induced ROS in a dose-dependent pattern. Moreover, Quercetin therapy dose-dependently inhibited AGEs-induced cell senescence activity along with its senescence associated secretion phenotype (SASP) secretions such as IL-6 and IL-8. Western blot analysis indicated that Quercetin was able to reverse the phosphorylation of p65 and Iκβ in AGEs-stimulated HGFs, demonstrating it can modulate NF-κβ pathway.
Conclusion: Accumulation of AGEs can elicit inflammaging in HGFs, as seen by increased pro-inflammatory cytokines, cell senescence expression and oxidative stress. The results proposed that Quercetin is able to ameliorate inflammaging in diabetic periodontitis and improve wound healing via the suppression of NF-κβ pathway and hence, may be a promising approach for treatment of diabetes-associated periodontitis.

Introduction

Diabetes mellitus is a metabolic illness caused by long-term elevated blood glucose levels. DM poses a severe global health and financial burden due to its high prevalence[1] and association with a variety of comorbidities. These include retinopathy, peripheral arterial diseases, neuropathy,[2] as well as periodontitis, which is a chronic inflammatory disease of the underlying supporting tissues of teeth.[3] Periodontitis can cause tooth exfoliation and have a detrimental impact on patient's quality of life if it is not addressed.[4–6] It is established that accumulation of advanced glycation end-products (AGEs) in DM induces inflammaging which is a state of pre-aging associated inflammation.[7] Inflammaging in diabetes can aggravate the host immune response towards periodontal pathogens, leading to more severe periodontal tissue damage.[8,9] Hallmarks of AGEs-induced inflammaging include the upregulation of oxidative stress and cellular senescence along with its pro-inflammatory secretions, also acknowledged as the senescence-associated secretory phenotype (SASP).[10]

"Oxidative stress" refers to a breakdown in the redox signaling regulation mediated by either a surplus of ROS, or a loss of control mechanisms. Several investigations have established that AGEs promotes the buildup of oxidative stress in the gingiva.[11,12] AGEs have been demonstrated to promote generation of reactive oxygen species (ROS) via stimulating lipid peroxidation and glycoxidation.[13] The build-up of oxidative stress can ultimately lead to an increase in pro-inflammatory interleukins activity[14] and hinder tissue healing.[11]

Besides that, when AGEs interact with their receptors (RAGE), they can generate prolonged endoplasmic reticulum stress, prompting the cells to undergo premature aging or senescence.[15] These senescent cells, on the other hand are capable in expressing and producing a range of inflammatory modulators such as cytokines, which is referred to as the SASP.[16] It has been revealed that periodontal cells in mice with DM displayed heightened expression of cellular senescence and SASP secretion.[17] As IL-6 and IL-8 are the most abundantly expressed SASP cytokines,[18] it was not surprising that DM individuals who suffers from periodontitis had significantly higher levels of periodontal interleukin (IL)-6[19] and systemic interleukin (IL)-8[20] compared to healthy individuals with periodontitis alone. In short, the accumulation of cell senescence coupled with its pro-inflammatory secretions in inflammaging could further accelerate the degradation of periodontal tissue in the course of DP.[8,9,21] As a result, a treatment strategy designed to target inflammaging in diabetes associated periodontitis would be essential.

Quercetin is a dietary flavonoid,[22] which can be found in various fruits, vegetables and herbs such as cherries, broccoli, apples, onions, or mangoes, and red thorax root.[23] Numerous therapeutic traits have been reported in Quercetin which includes antioxidant, anti-inflammatory, antibacterial,[24,25] anti-diabetic[26] and anti-tumor activities.[27] It has been noted that supplementation of Quercetin helps with metabolic control in DM as well as some of its associated complications.[26] For example, in mice models with type 2 DM, Quercetin group showed better glycemic control[26] and improved renal functions,[28] whereas other studies have demonstrated its inhibitory effect on alveolar bone loss in periodontitis.[27,29,30] It was thought that Quercetin suppresses the inflammatory bone resorption via the inhibition of NF-κb signaling. Although Quercetin was observed to have favorable outcomes in DM and periodontitis separately, its effect on both comorbidities have not been elucidated so far. Consequently, we would like to explore whether Quercetin has the potential to inhibit AGEs-induced inflammaging in human gingival fibroblasts (HGFs) and its underlying mechanism.

Materials and methods

Cell culture

All steps were carried out in compliance with the authorized guidelines from the Institutional Review Board at the

Chung Shan Medical University Hospital. HGFs from two healthy individuals were obtained after crown lengthening procedure using an explant technique as previously mentioned.[31] In this investigation, cell cultures of two individual HGFs between the third and eighth passages were employed. Advanced Glycation End-Products (AGEs)-BSA was purchased from BioVision (Milpitas, CA, USA) and Quercetin was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell viability assay

10,000 cells/well of HGFs were planted on 96-well plates (Corning Inc., Rochester, NY, USA) for 48 h. After the cells achieved good adherence, Quercetin was added at determined concentrations (0, 5, 10, 20, 40, 80 μM) for further 24 h incubation. Cells viability was then evaluated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's manual. The 570-nm absorbance of untreated cells (0 μM Quercetin) was set to 100%, and data were calculated as percentage of control.

Wound healing assay

Once the seeded HGFs have reached 80% confluence, a sterile 200-L pipette tip will be used to scrape the monolayer across the center of the well on a 12-well culture plate. The cells then were left to grow for another 48 h. Cell migration towards the denuded region was imaged under a microscope at 0 and 48 h.

Western blot

The methods outlined previously will be employed in the Western blot analysis.[31] The primary antibodies against cellular senescence marker (p16, Invitrogen Inc., Waltham, MA, USA) markers, NF-κB signaling marker (Iκβ, p- Iκβ (Abcam, Cambridge, UK), p65 and p-p65 (Invitrogen Inc.)) were used.

ROS analysis

Flow cytometry was used to measure the ROS production. HGFs cells were incubated with 10 μM DCFH-DA (Merck sigma-aldrich, Burlington, MA, USA) for 30 min at 37 °C. After PBS washing, the cells were trypsinized. DCF fluorescence of 10,000 cells would be analyzed by BD FACSCalibur (BectoneDickinson, CA, USA) at excitation and emission wavelengths of 404 and 524 nm, respectively.

ELISA analysis

HGFs were exposed to AGEs-BSA simultaneously with quercetin at indicated concentration for a period of 48 h for collecting conditioned medium. The conditioned medium was collected and mixed with protease inhibitor cocktail. Through ELISA kit (R&D Systems, Minneapolis, MN), the secretion levels of IL-6 and IL-8 were measured. The absorbance was examined with a 450 nm filter on a microplate reader (MRX, Dynatech Laboratories, Chantilly, VA, USA). Each sample was analyzed in triplicate.

Senescence activity detection

By using a Cellular Senescence Assay kit (Merck Millipore, Burlington, MA, USA), the activity of senescence-associated-gal (SA-Gal) was assessed to estimate the level of cellular senescence. HGFs were grown in a 6-well plate for 24 h. On the next day, cells were washed with PBS, then added 1× Fixing Solution and 1× SA-β-gal Detection Solution in sequence. Finally, the blue stained cells were counted with microscopy. Microscopically, SA-Gal positive cells were identified.

Statistical analysis

Each experiment was replicated three times. One-way analysis of variance was used for the statistical analysis (ANOVA). Tests of differences in the treatments were analyzed by Duncan's test and a value of $P < 0.05$ was regarded as statistically significant.

Results

First, it was revealed that the cell proliferation rate in HGFs was unaffected by quercetin of 5—20 μg/mL (Fig. 1). In simulating diabetes periodontitis, HGFs were exposed to advanced glycation end-products (AGEs) in the subsequent studies. Quercetin of 5, 10 and 20 μg concentration were simultaneously introduced to examine its therapeutic potential. When Quercetin was introduced, it was revealed that it improved the AGE-induced wound healing impairment markedly (Fig. 2). To explore its anti-inflammaging effect, we looked into the levels of ROS production, cell senescence activities and SASP secretions. It was observed that AGEs stimulated ROS production in HGFs and Quercetin intervention managed to suppress them dose-dependently (Fig. 3). Moreover, as shown in Fig. 4, AGEs noticeably enhanced the senescence activity and protein levels of p16, but the addition of Quercetin counteracted this phenomenon. Quercetin was also shown to suppress AGEs-elicited SASP secretions including IL-6 and IL-8 in a dose-dependent manner, indicating its anti-inflammaging potential (Fig. 5).

To better understand how Quercetin exhibits its anti-inflammaging potential, we evaluated the levels of proteins associated with NF-κB signaling pathways. When the proteins levels and phosphorylation activities of Iκβ and p65 were measured, it was observed that the greater concentrations of Quercetin were able to inhibit the activation of NF-κB (Fig. 6). Our research proposed that Quercetin possesses anti-inflammaging traits and they were mediated via the suppression of NF-κB signaling pathways.

Discussion

Persistent hyperglycemia in DM has been shown to contribute to inflammaging, which is a state of a pre-aging and pro-inflammatory state.[7] Inflammaging has been

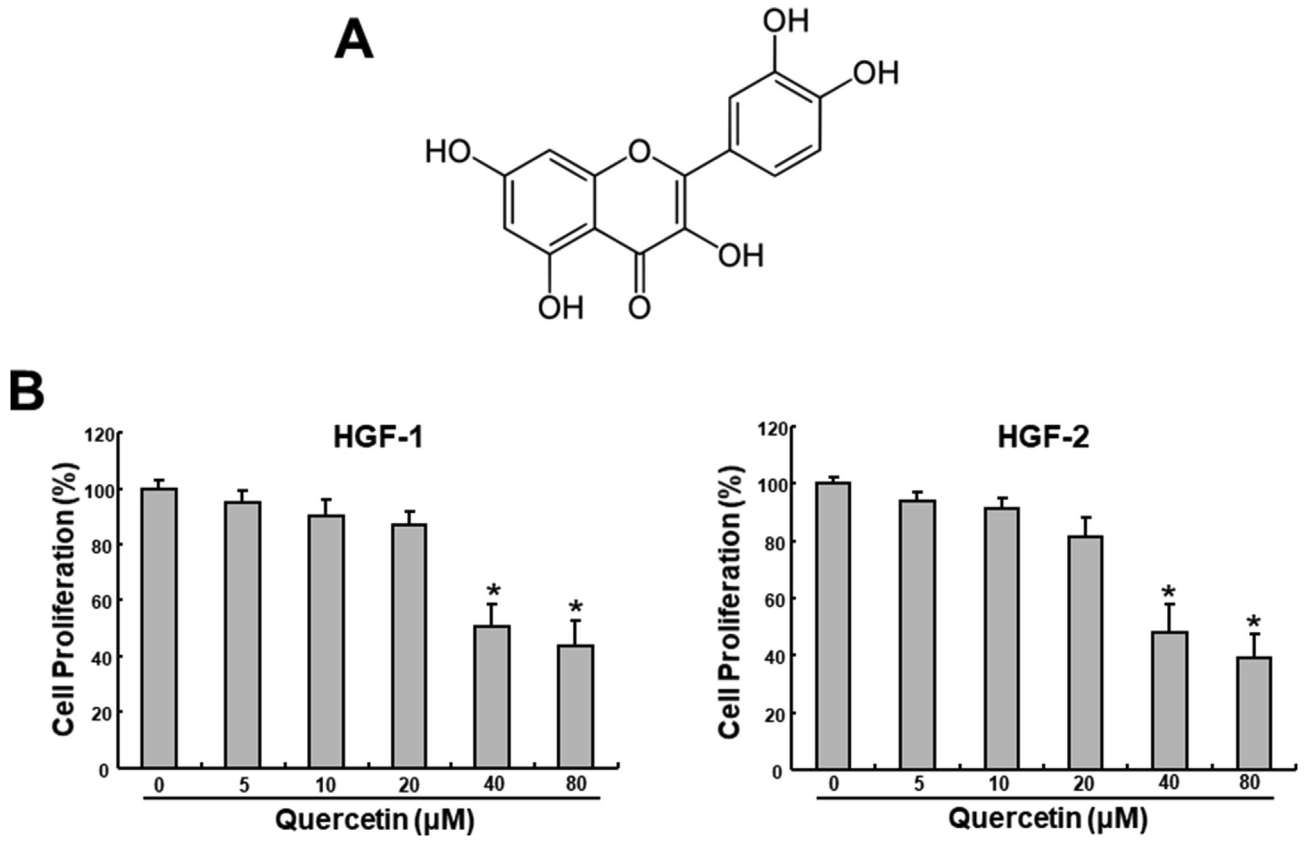

Figure 1 Quercetin's impact on the cell proliferation in the AGEs-stimulated HGFs. MTT was utilized to examine the cell survival/proliferation rate of Quercetin on HGFs. 1 × 104 cells/well of HGFs were seeded in 96 well and treated with 0, 5, 10, 20, 40, 80 μM of Quercetin; Quercetin at 20 μM and below did not have any significant impact on HGFs' cell proliferation rate. Data represent the mean ± SD. *$P < 0.05$ compared to control group; #$P < 0.05$ compared to the control.

demonstrated to raise the likelihood and severity of periodontitis.[7–10,32] In this study, when HGFs were stimulated with AGEs, the cells exhibited signs of inflammaging. These include the upregulation of oxidative stress and cellular senescence along with increased production of pro-inflammatory cytokines (shown in Figs. 2 and 3 respectively), which were in line with prior observations.[10,17,32,33]

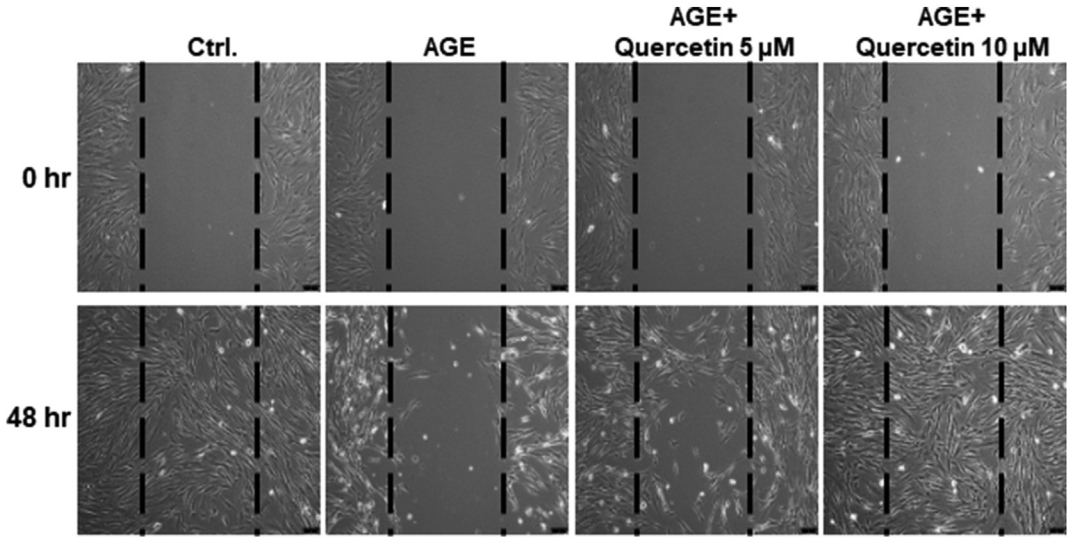

Figure 2 Impact of Quercetin on the wound healing ability in HGFs treated with AGEs/LPS. The AGEs-induced poor wound healing was reversed in HGFs treated with Quercetin treatment. Data from three independent experiments, each in triplicate.

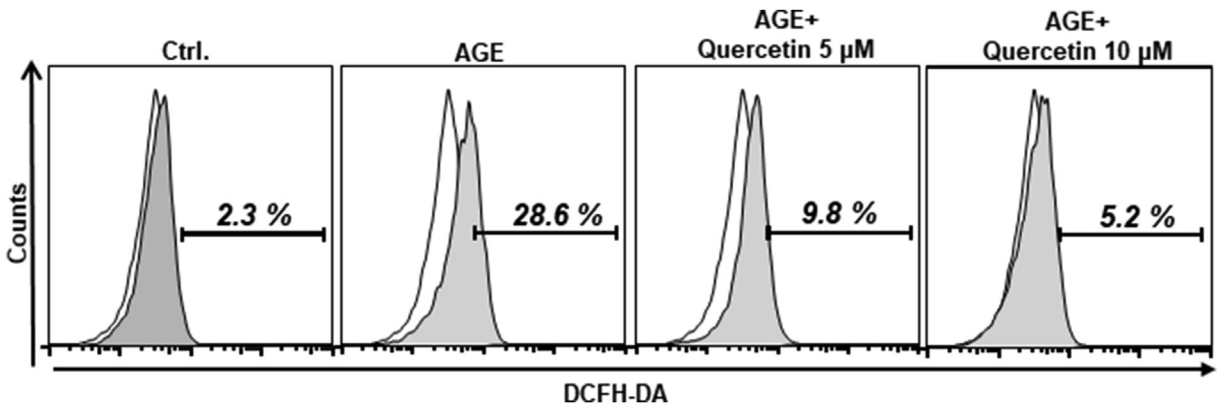

Figure 3 Effects of Quercetin on the production of ROS in the AGEs-stimulated HGFs. The AGEs-induced ROS in HGFs was downregulated in response to Quercetin treatment (0—10 μM) in a dose-dependent manner. *$P < 0.05$ compared to no treatment control group.

When Quercetin was added to the cells as intervention, it was shown to be effective in combating inflammaging, characterized by reduced oxidative stress, cell senescence and SASP secretions. In aspects of oxidative stress, Quercetin was revealed to be a potent antioxidant which can neutralize ROS and limit subsequent oxidative injury in diverse range of cells, namely human periodontal ligament (PDL) cells,[34] human dermal fibroblasts[35] and retinal pigment epithelial cells.[36] In addition, Quercetin supplementation in animal models were shown to boost antioxidant defences and protect against oxidative stress-related liver and brain injuries[37] as well as renal dysfunction.[38]

The current data shown that Quercetin was able to suppress AGEs-induced senescence activity and SASP secretions such as pro-inflammatory IL-6 and IL-8 in HGFs, which are consistent with several prior studies.[39—41] Apart from these interleukins, Quercetin was also discovered to inhibit other SASP factors[42] such as IL-1β, MMPs, COX-2, and PGE2.[43] Quercetin was thought to exert its anti-inflammatory properties by modulating NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) system.[39] NF-κB pathway is an important transcription factor known to regulate the body's response to inflammation and oxidative stress[44,45] and its activation can trigger the production of

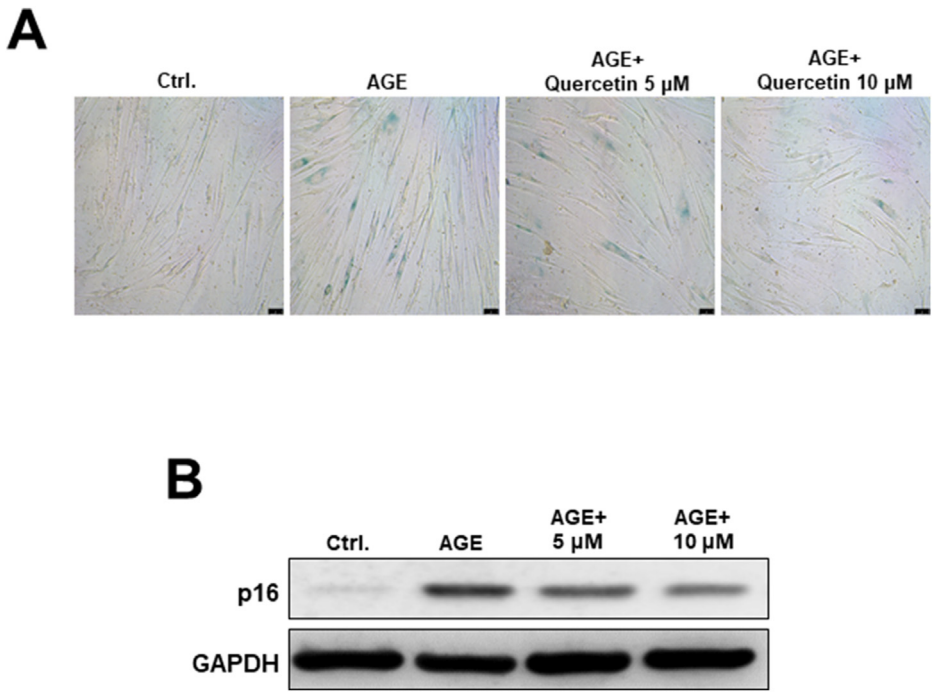

Figure 4 Effects of Quercetin on AGEs-induced (A) cellular senescence and (B) its marker p16 (A) Quercetin dose-dependently repressed the AGEs-induced cell senescence in HGFs. (B) The protein level of p16 is upregulated in the AGEs-stimulated cells but suppressed following treatment of Quercetin in a dose-dependent fashion. *$P < 0.05$ compared to no treatment control group.

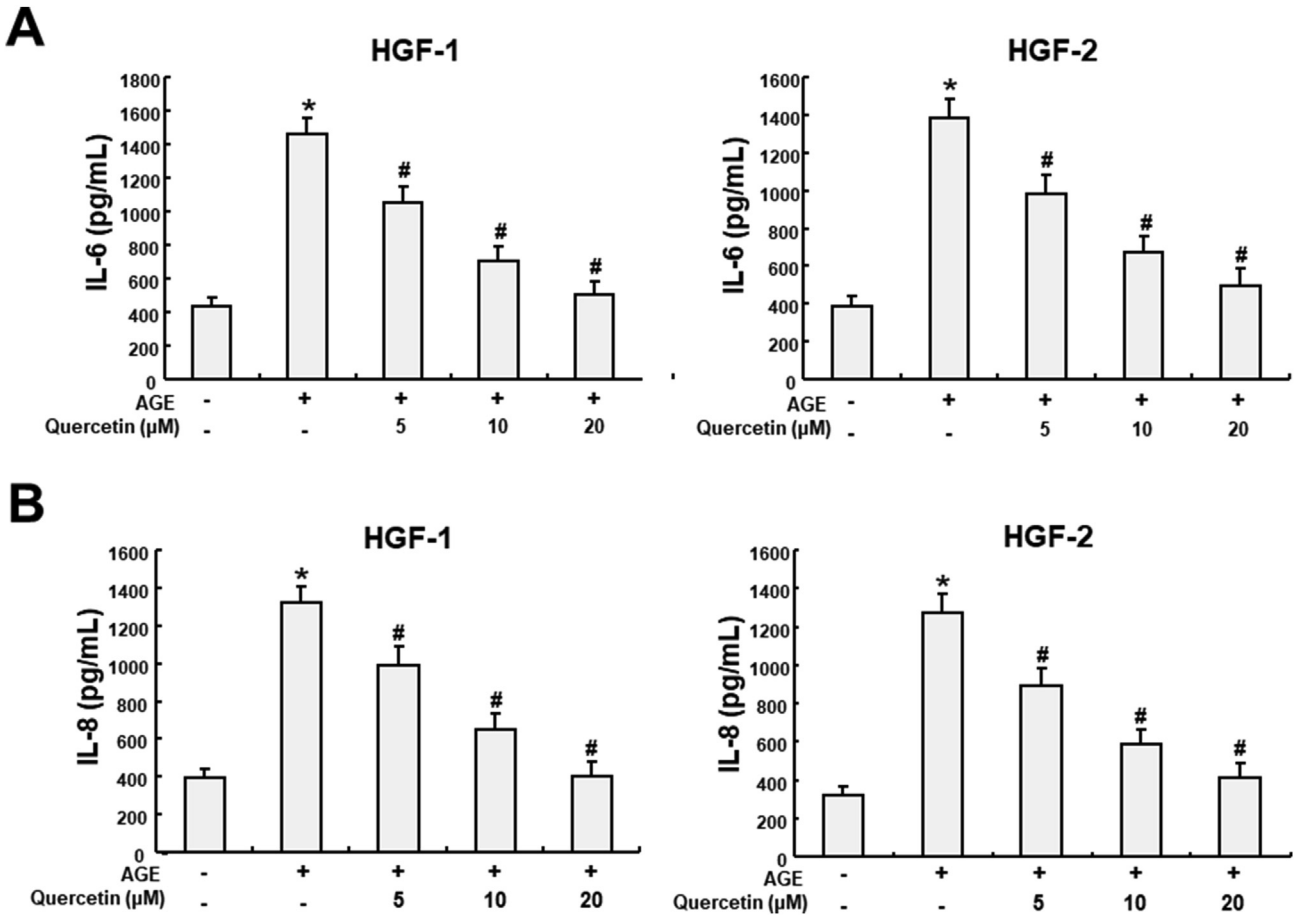

Figure 5 The secretion of (A) IL-6 and (B) IL-8 in the AGE-cultured HGFs with or without Quercetin. ELISA assay was applied to examine the concentration of IL-6 (A) and IL-8 (B) with AGEs and indicated concentration of Quercetin. The expression levels of IL-6 (A) and IL-8 (B) were elevated in the AGEs-stimulated cells but suppressed following treatment of Quercetin in a dose-dependent fashion. Data represent the mean ± SD. *$P < 0.05$ compared to no treatment control group; #$P < 0.05$ compared to AGE only group.

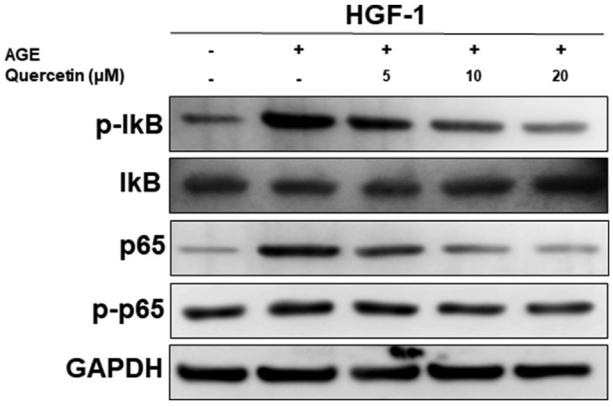

Figure 6 Impact of Quercetin on NF-κβ pathway in the AGE-stimulated HGFs. The protein levels of Iκβ, p-Iκβ, p65 and p-p65 were induced in the AGEs-stimulated cells but suppressed following treatment of Quercetin in a dose-dependent fashion. Data represent the mean ± SD. *$P < 0.05$ compared to control group.

pro-inflammatory cytokines and other signaling molecules, thereby fueling the inflammatory response.[46]

As such, it was worth investigating whether Quercetin imposes its anti-inflammaging qualities through the modulation of this signaling. AGEs accumulation in the present was observed to amplify the levels of protein kinases associated with NF-κB signaling and to stimulate their phosphorylation. Quercetin, on the contrary, was revealed to inhibit the activity of NF-κB signaling. These results were in line with previous studies where Quercetin inhibited lipopolysaccharide (LPS)-stimulated NF-κβ activation in RAW 264.7 macrophage[47,48] and in bone marrow-derived macrophage.[49]

In a nutshell, Quercetin in this study has demonstrated its anti-inflammaging trait in diabetic periodontitis models in-vitro. Quercetin was able to suppress oxidative stress, cellular senescence and IL-6 and IL-8 and these impacts might be mediated via the suppression of NF-κB pathways and therefore, could hold a novel therapeutic approach in DM patients with periodontitis.

Declaration of competing interest

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

Acknowledgments

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