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Original Article

Resveratrol attenuates advanced glycation end product-induced senescence and inflammation in human gingival fibroblasts

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Abstract *Background/purpose:* The accumulation of advanced glycation end products (AGEs) lead to a series of immune responses such as: increased oxidative stress and inflammation which contribute to the development of diabetic complications and periodontal disease. Resveratrol is a natural compound that has anti-oxidant and anti-inflammatory effects. Studies have found that diabetes-induced periodontitis is mainly caused by oxidative stress, aging and increased inflammation. In view of resveratrol has been proposed to have the ability in anti-oxidant and anti-inflammation in a variety of tissues. However, the role of resveratrol in diabetic periodontitis remains to be investigated. In this study, we aimed to investigate the role of resveratrol in preventing and treating diabetic periodontitis.
Materials and methods: First, cell proliferation was measured in AGEs-treated human gingival fibroblast with or without resveratrol. We examined the reactive oxygen species (ROS) generation, senescence-associated beta-galactosidase (SA-β-gal) and senescence marker p16 in

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human gingival fibroblasts (HGFs) stimulated with AGEs with or without the treatment of resveratrol. To determine whether resveratrol has the potential to regulate inflammaging which is mediated via the NF-κB signaling pathway and, the expression of p65 and p-IκB were also investigated. Furthermore, the concentration of interleukin (IL)-6 and IL-8 were also measured in AGEs-stimulated HGFs treated with or without resveratrol.

Results: ROS generation, cell senescence, and the secretion of IL-6 and IL-8 were significantly upregulated following the treatment of AGEs. However, the administration of resveratrol suppresses the generation of IL-6 and IL-8 and cell senescence via inhibiting NF-κB signaling pathway. Our results revealed that resveratrol inhibits inflammaging by downregulating NF-κB signaling pathway.

Conclusion: According to our findings, AGEs increase senescence and the production of proinflammatory cytokines in the gingiva, while the administration of resveratrol impedes inflammaging via suppressing NF-κB signaling pathway.

Introduction

Diabetes mellitus (DM) is a metabolic disease attributed to hyperglycemia and insulin resistance which leads to a variety of complications such as cardiovascular disease, kidney disease, neuropathy, macrovascular and microvascular complications[1,2] virtually increase huge medical burden. Periodontitis is also a chronic inflammatory disease that occurs due to the pathogen's infiltration and series of immune responses, leading to the destruction of the periodontium. Numerous researchers pointed out that periodontitis is associated with various systemic diseases such as cardiovascular, respiratory and endocrine diseases.[3,4] Periodontitis is known to be the sixth major complication of diabetes, and accumulating evidence shows that diabetes is also a risk factor for periodontitis. Compared with non-diabetic subjects, diabetes can increase the risk of having periodontal disease by three folds.[5] Diabetes can cause the formation of advanced glycation end products (AGEs), which increase vascular permeability, degrade collagen fibers, and hasten the breakdown of non-mineralized connective tissue and alveolar bone under long-term high blood sugar conditions. These effects can ultimately result in periodontitis.[6] In addition, high concentrations of AGEs in serum, gingival tissue and gingival crevicular fluid were also found more commonly in type 2 diabetic patients with periodontitis.[7—9]

In general, high blood sugar can lead to the accumulation of advanced glycation end products (AGEs), which activate their receptor of AGEs (RAGE). This triggers chronic inflammation, oxidative stress, apoptosis, and reduced bone formation. Nuclear factor kappa B (NF-κB) is a key regulator of inflammatory pathways in periodontitis, which contributes to tissue damage and vascular dysfunction.[10] After applying AGEs to human gingival fibroblast (HGFs), AGEs bind to RAGE which activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to induce ROS generation. In addition, AGEs also activates NF-κB downstream signal transmission by regulating JNK, ERK1/2 or p38/MAPK signaling pathway, increasing the production of inflammatory cytokines,[11] which ultimately contributes to the exacerbation of periodontitis.

Aging accompanied by a low-grade inflammatory response is called inflammaging. Studies have found that senescence-associated secretory phenotype (SASP) is related to aging and type 2 diabetes.[12] Senescent cells accumulate in tissues and induce SASP, which triggers the secretion of pro-inflammatory cytokines, chemokines, and growth factors.[13] Ikegami et al. proposed that aging periodontal ligament cells produce SASP, which promotes the secretion of pro-inflammatory factors, and aggravates the occurrence of periodontitis.[14]

Resveratrol (RES) is a polyphenol that is abundant in grapes, berries and red wine.[15] Resveratrol, an activator of sirtuin1 (SIRT1) plays a pivotal role in extending life span.[16] Evidences showed multiple biological properties of resveratrol including anti-oxidation,[17] anti-aging,[18] anti-inflammatory,[19] anti-bacterial[20] and anti-cancer.[21] As shown in diabetic rats, resveratrol attenuated renal injury via regulating oxidative stress and inflammation.[22] According to various studies, resveratrol accelerates oxidative processes, which can lead to cellular senescence or death in cancer cells by modulating signaling cascades. It is reported that resveratrol decreased pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-8 and IL-6 in mice with inflammatory bowel disease.[23] Resveratrol has also been shown to diminish inflammation and oxidative stress by inhibiting PI3K/Akt and WNT/β-catenin signaling pathway in LPS-induced HGFs.[24] Therefore, we further investigated whether resveratrol has the potential to decrease AGEs-mediated senescence in gingival fibroblasts. This study aimed to explore the effect and molecular mechanisms of resveratrol on AGEs-induced inflammaging in HGFs.

Materials and methods

Cell culture

The study was approved by the Institutional Review Board at the Chung Shan Medical University Hospital (CSMUH No: CS1-22047). HGFs were extracted from two healthy persons during the crown lengthening procedure utilizing the previously reported approach.[25] In this study, HGFs were used between the third and eighth passages. Advanced Glycation End-Products (AGEs)-BSA was obtained from BioVision (Milpitas, CA, USA) and resveratrol from Sigma Chemical Co. (St. Louis, MO, USA). In the following tests, HGFs were exposed to advanced glycation end-products (AGEs)-BSA together with resveratrol at the given concentration for 24 h to investigate the impact of resveratrol.

Cell viability assay

HGFs were culture with AGEs in order to mimic diabetes in vitro. 10,000 cells/well of HGFs were seeded on 96-well plates for 24 h. Resveratrol was added at the serial doses (25, 50 and 100 μM) for a further 24 h of incubation after HGFs (500 μg/ml) were pretreated with AGEs for 24 h. Following the manufacturer's instructions, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] test was used to evaluate the cell proliferation rate of HGFs. All the cells were measured at the absorbance of 570 nm and the untreated cells (0 uM Resveratrol) were identified as 100% percentages relative to control.

Western blot

The Western blot analysis was utilized following the previously described protocol.[26] Primary antibodies against senescence marker p16 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), NF-κB signaling marker (p65) (Cell Signaling Technology, Beverly, MA, USA) and p-IκB (Cell Signaling Technology, Beverly, MA, USA) were used. Bound antibodies were detected using Enhanced chemiluminescence (ECL) and the image were captured by utilizing an ImageQuant LAS 4000 Mini (GE healthcare, North Richland Hills, TX, USA).

ELISA analysis

IL-6 and IL-8 concentration were determined using the ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions, and the absorbance was measured with a 450 nm filter by a microplate reader (MRX, Dynatech Laboratories, Chantilly, VA, USA). Each HGF sample was analyzed in triplicate.

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

Cellular senescence was measured by the activity of senescence-associated-β-gal (SA-β-Gal)using the Cellular Senescence Assay kit as described previously.[26] SA-β-Gal positive cells were observed by microscopy, and over 100 cells were counted in five independent fields.

Statistical analysis

Each experiment was replicated three times. One-way analysis of variance (ANOVA) was used for statistical analysis. Duncan's test was used to examine treatment differences, and a result of $P < 0.05$ was considered statistically significant.

Results

After administration of AGEs and RES on HGFs cell proliferation was investigated using MTT assay. It was shown that at the concentration of 25—100 μM of RES had no impact on cell proliferation rate in HGFs pre-treat with AGEs (Fig. 1). Studies have demonstrated that AGEs-induced oxidative stress leads to chronic inflammation[27,28] and RES has antioxidant activity,[24] thus we utilize DCFH-DA to investigate the formation of ROS. Results showed that AGEs significantly increased the production of ROS and administration of RES significantly suppressed ROS production (Fig. 2). To elucidate the effect of RES on AGEs induced senescence, SA-β-gal staining was examined. As shown in Fig. 3, RES significantly decreased SA-β-gal staining in HGFs treated with AGEs. p16 is the cellular senescence marker, result show that the protein level of p16 is significantly decreased

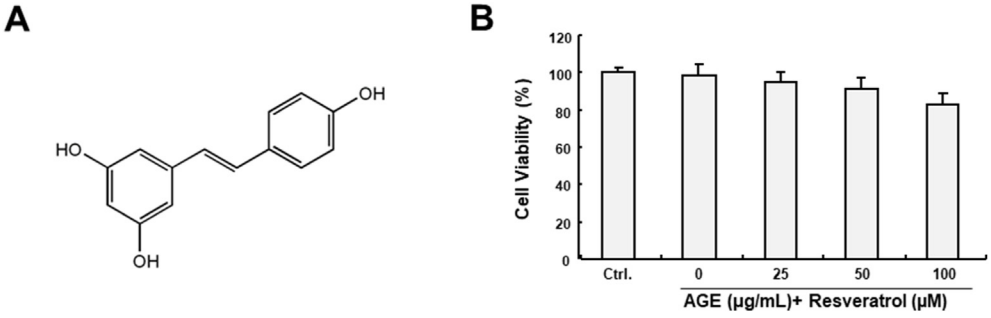

Figure 1 Effects of resveratrol on the cell proliferation rate in AGEs-treated HGFs Resveratrol ranging from 25 to 100 μM did not significantly affect the cell proliferation rate in HGFs treated with AGEs. Data represent the mean ± SD. *$P < 0.05$ compared to the group with 0 μM resveratrol.

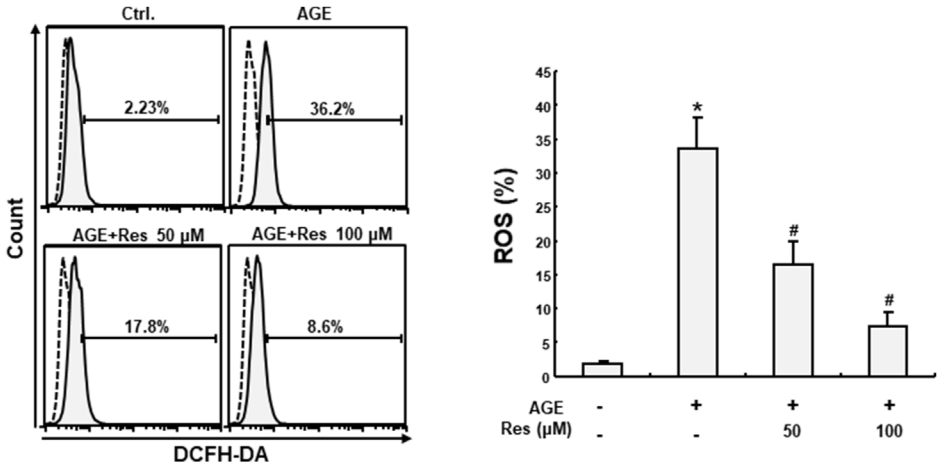

Figure 2 Effects of resveratrol on the production of ROS in the AGEs-treated HGFs DCFH-DA was utilized to investigate the effect of resveratrol on ROS production. AGEs-treated HGFs significantly increased the generation of ROS and administration of resveratrol reversed this phenomenon in a dose-dependent manner.

in a dose dependent manner (Fig. 3). In light of the fact that persistently high blood sugar will trigger pro-inflammatory cytokines and activate the NF-κB signaling pathway, ultimately leading to the development of diabetes. In order to investigate whether RES suppressed inflammation via NF-κB signaling pathways, HGF was

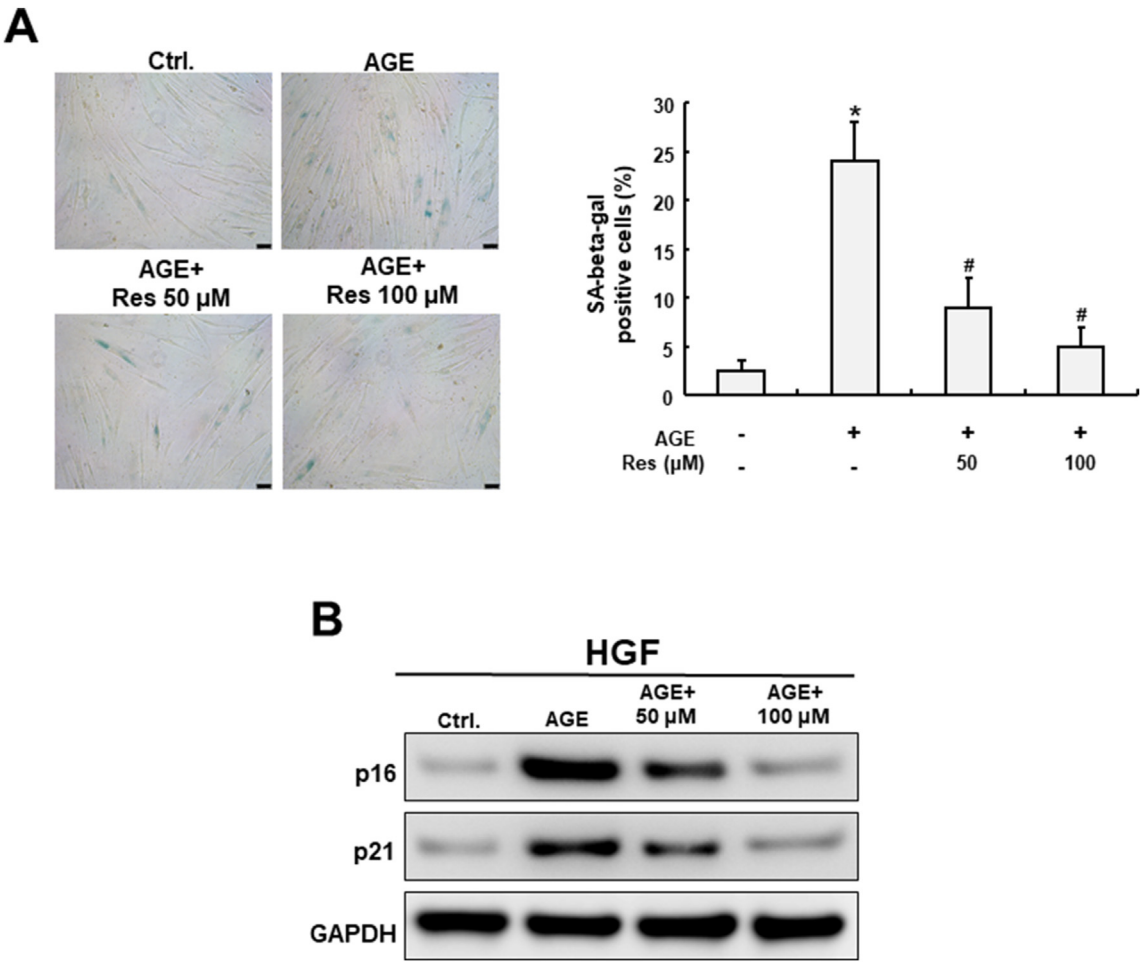

Figure 3 Effects of resveratrol on the cell senescence activity in the AGEs-treated HGFs. The expression of p16 and β-galactosidase (SA-β-Gal) staining, which are indicators of senescence activity, increased in the AGEs stimulated HGFs. The enhanced cellular senescence was then reversed by the administration of resveratrol.

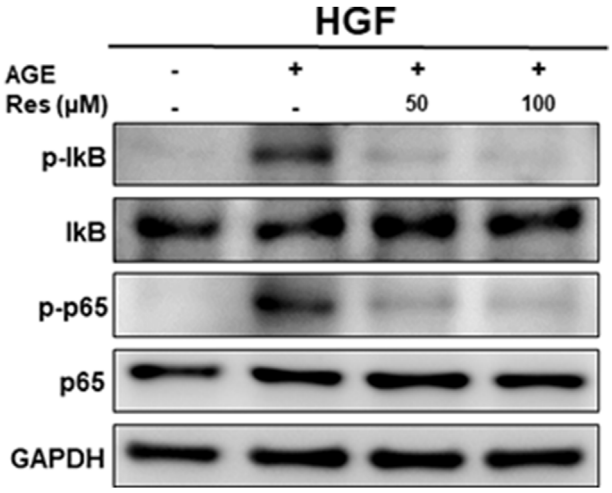

Figure 4 Effects of resveratrol on NF-κB signaling pathway in the AGEs-treated HGFs. Western blot was used in order to examine the expression of p65, a component of the NF-κB signaling pathway. The administration of resveratrol reduced the up-regulation of p65 caused by AGEs.

cultured with AGEs or AGEs with various concentrations of RES, respectively. Results have shown that AGEs significantly increased the protein expression of IκB, however, the administration of RES significantly diminished in a dose-dependent manner (Fig. 4). Last but not least, pro-inflammatory cytokines were examined, administration of AGEs significantly increased the secretion of IL-6 and IL-8 while RES suppressed the secretion of IL-6 and IL-8 (Fig. 5).

Discussion

Glycation caused by persistently high blood sugar leads to inflammatory reactions, which is a major cause of progressive aging of the body. Periodontitis is also characterized as an inflammatory disease that contributes to attachment loss or even tooth loss. Studies have shown that the accumulation of AGEs in diabetes can accelerate the inflammation in the periodontium, which in turn leads to exacerbation of periodontitis. Therefore, finding alternative ways to control diabetic periodontitis is of paramount importance. In our study, we found that AGEs can increase oxidative stress in HGFs, promote the secretion of cytokines IL-6 and IL-8 as well as accelerate cell senescence, which is similar to previous studies.[10,29] However, administration of resveratrol can successfully supress the NF-κB signaling pathway in HGFs triggered by AGEs and reduce inflammaging in a dose-dependent manner.

According to numerous studies, the common pathological characteristics of type 2 DM patients are primarily caused by AGEs which leads to the production of reactive oxygen species (ROS), activating the receptor of AGEs (RAGE), inflammation as well as apoptosis of pancreatic β cells. An excessive accumulation of AGEs increases periodontal inflammation and then aggravates the pathogenesis of periodontal disease.[28] Our study discovered that resveratrol decreased oxidative stress, inflammation, and cell senescence in HGFs primarily via modulating the NF-κB pathway. Ohtsu et al. discovered that resveratrol suppressed the production of inflammatory cytokines by modulating SIRT1 and AMPK and blocking the NF-κB signaling pathway.[30] According to studies, subcutaneous injection of resveratrol in rats with periodontitis for two weeks showed less alveolar bone loss and alleviated periodontal damage via suppressing the TLR4 signaling pathway.[31,32] *db/db* mice minimizing the incidence of periodontitis through reduction of alveolar bone loss and inflammation-related cytokines such as IL-1β, IL-6, IL-8, and TNF-α in the gingiva.[33]

Subsequently, p16 is a biomarker of tumor suppressor gene and cellular senescence,[34–36] p16 is also a cell cycle regulator, which is involved in mediating cell aging. Studies have shown that the expression of p16 is significantly upregulated in senescent cells. In addition, SA-β-gal is the most commonly used indicator to detect cell aging.[37,38] AGEs are known to be the main cause of aging. From our results, SA-β-gal and p16 were both abundantly expressed, however, these two markers are significantly decreased after the administration of resveratrol.

In general, inflammaging may account for a rise in the adverse consequences of diabetic periodontitis, making the treatment extremely elaborate. The results

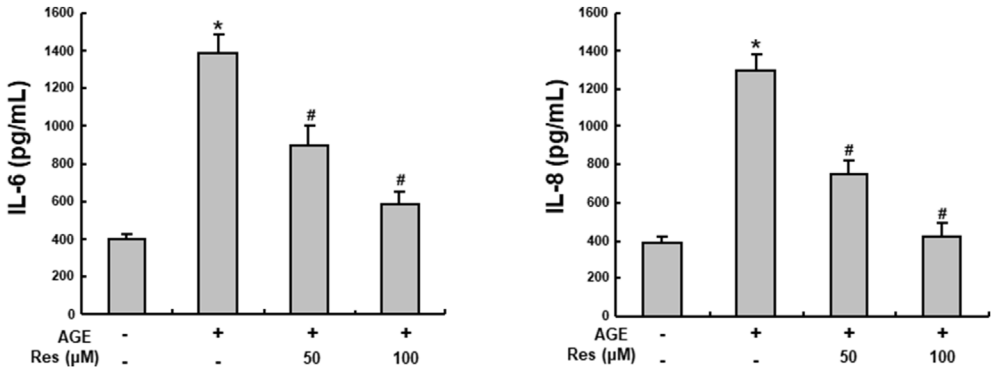

Figure 5 Effects of resveratrol on the production of pro-inflammatory cytokines in HGFs treated with AGEs. In HGFs, AGEs-induced IL-6 (A) and IL-8 (B) secretions were downregulated in a dose-dependent manner in response to the administration of resveratrol. The data are expressed as the mean SD. *$P < 0.05$ compared to the control group; #$P < 0.05$ compared to the group receiving AGEs.

show that AGEs promote the secretion of inflammatory cytokines IL-6 and IL-8, upregulating the expression of p16 and the NF-κB signaling pathway, leading to increased oxidative stress and cell senescence, which can be reversed after administration of resveratrol in HGFs. Our study illustrated that resveratrol attenuated AGEs-induced oxidative stress and inflammation via NF-κB signaling pathway in diabetic periodontitis. Future research needs to focus on exploring the molecular mechanism of resveratrol.

Declaration of competing interest

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

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