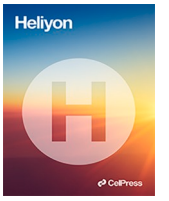
Heliyon 10 (2024) e27478

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Metformin alleviates junctional epithelium senescence via the AMPK/SIRT1/autophagy pathway in periodontitis induced by hyperglycemia

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ARTICLE INFO

Keywords:
Periodontitis
Junctional epithelium
Hyperglycemia
Metformin
Senescence
Oxidative stress

ABSTRACT

The junctional epithelium (JE) serves a crucial protective role in the periodontium. High glucose-related aging results in accelerated barrier dysfunction of the gingival epithelium, which may be associated with diabetic periodontitis. Metformin, an oral hypoglycemic therapeutic, has been proposed as a anti-aging agent. This study aimed to clarify the effect of metformin on diabetic periodontitis and explore its mechanism in ameliorating senescence of JE during hyperglycemia. The db/db mice was used as a diabetic model mice and alterations in the periodontium were observed by hematoxylin-eosin staining and immunohistochemistry. An ameloblast-like cell line (ALC) was cultured with high glucose to induce senescence. Cellular senescence and oxidative stress were evaluated by SA-β-gal staining and Intracellular reactive oxygen species (ROS) levels. Senescence biomarkers, P21 and P53, and autophagy markers, LC3-II/LC3-I, were measured by western blotting and quantitative real-time PCR. To construct a stable SIRT1 (Sirtuin 1) over-expression cell line, we transfected ALCs with lentiviral vectors overexpressing the mouse SIRT1 gene. Cellular senescence was increased in the JE of db/db mice and the periodontium was destroyed, which could be alleviated by metformin. Moreover, oxidative stress and cellular senescence in a high glucose environment were reduced by metformin in *in-vitro* assays. The autophagy inhibitor 3-MA and SIRT1 inhibitor EX-527 could dampen the effects of metformin. Overexpression of SIRT1 resulted in increased autophagy and decreased oxidative stress and cellular senescence. Meanwhile, AMPK (AMP-activated protein kinase) inhibition reversed the anti-senescence effects of metformin. Overall, these results suggest that metformin alleviates periodontal damage in db/db mice and cellular senescence in ALCs under high glucose conditions via the AMPK/SIRT1/autophagy pathway.

1. Introduction

Periodontitis is an inflammatory disease caused by the progressive destruction of tooth support tissues due to long-term invasion of periodontal tissue by dental plaque [1,2]. As the sixth commonest complication of diabetes, the susceptibility and severity of

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https://doi.org/10.1016/j.heliyon.2024.e27478
Received 22 May 2023; Received in revised form 21 February 2024; Accepted 29 February 2024
Available online 8 March 2024

periodontitis is reported to be increased by diabetes [3–6]. Diabetes is defined as a group of metabolic diseases characterized by hyperglycemia and mounting evidence indicates that cellular senescence induced by hyperglycemia may be associated with the pathogenesis of diabetic periodontitis [7–9].

The junctional epithelium (JE), a nonkeratinized epithelium, is a specialized epithelial structure that is in contact with the tooth surface to prevent the invasion of bacteria into the underlying hard and soft tooth-supporting tissues [10,11]. As an epithelial barrier, the JE plays an important role in maintaining gingival and periodontal health [12–14]. Accumulation of senescent cells results in damage to the epithelial barrier by increasing expression of inflammatory cytokines directly affecting barrier integrity and epithelial permeability [15]. High glucose-related aging and oxidative stress leads to accelerated physical, chemical and immune barrier dysfunction of the gingival epithelium, which may be a key cause of diabetic periodontitis [16,17]. Recent evidence suggests that hyperglycemia is associated with a decrease in epithelial barrier function [18,19]. However, little research focus has been applied to dysfunction of the JE barrier in periodontitis after hyperglycemia.

Metformin is a biguanide and currently one of the most commonly utilised medications for the treatment of type 2 diabetes [20]. It has been reported that locally delivered metformin, particularly in gel form, can markedly improve the effect of mechanical periodontal therapy for periodontitis [21,22]. Clinical trials revealed that metformin has been applied locally to relieve clinical symptoms of periodontitis [23,24]. However, the underlying mechanisms by which metformin treatment functions in periodontitis in the JE has not been clearly resolved. Significant evidence does exist that reveals metformin can delay ageing, inhibit age-related pathological changes and reduce oxidative stress damage [25,26]. As previously shown, metformin can alleviate oxidative stress induced-senescence by activating AMPK and promoting autophagy in human lens epithelial cells [27]. Additional studies have reported that metformin can modulate hyperglycemia-induced endothelial senescence by activating silent mating type information regulation 2 homolog-1 (sirtuin-1, SIRT1) [28]. Moreover, it was reported that metformin attenuates epithelial cell senescence by activating autophagy in MLE-12 cells [29]. Therefore, we speculate that metformin may protect the JE from senescence through the AMPK/SIRT/autophagy pathway in hyperglycemia.

In the present study, we focused on the JE and explored the mechanism of metformin in periodontitis secondary to pre-diabetic hyperglycemia. *In vivo*, db/db diabetic mice were used to study the effect of metformin on the periodontium in hyperglycemia. *In vitro*, we established a high-glucose induced cellular senescence model to explore the molecular mechanisms by which metformin alleviates cellular senescence in JE.

2. Materials and methods

2.1. Animal experiments

Sixteen four-week-old male diabetic BKS-db/db mice (C57BLKS/J-leprdb/leprdb*) and eight age-matched BKS mice were purchased from Changzhou Cavens Experimental Animal Co., Ltd. The mice were maintained at 23 ± 2 °C and 55 ± 5% humidity with 12h light/dark cycles and free access to food and water. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals. The present study was approved by the Laboratory Animal Ethics Committee of the Institute of Medicinal Plant Development, Binzhou Medical University (2022-391).

Based on changes in blood glucose value at different periods, mice were divided into two batches. The first batch of mice were kept until 6 weeks of age and the second batch of mice were maintained until their 14th week. Animals were randomly divided into the following groups: control mice (C), db/db mice (D), and db/db mice that were intraperitoneally injected with metformin (DM) at a dose of 250 mg/kg daily. The 6-week-old groups were treated with metformin at 4 weeks of age for 2 weeks, and mice in the 14-week-old group were treated with metformin at 8 weeks of age for 6 weeks. Mice in the C, D, DM groups were treated with a equivalent sterile water.

2.2. Histological and immunohistochemical analysis

The mandibular bone of each mouse was harvested and fixed in 4% paraformaldehyde at 4 °C overnight. After decalcification in 10% ethylene diamine tetraacetic acid (EDTA) solution and changing of the solution every two days for one month, tissues were embedded in paraffin and sliced (5 μm). Sections containing the interalveolar crest between the first and second molars were collected and stained with hematoxylin and eosin (HE). For immunohistochemistry (IHC), the sections containing JE between the first and second molars were incubated with E-cadherin (1:200; proteintech, Cat No: 20874-1-AP), P21 (1:200; proteintech, Cat No: 10355-1-AP) and P53 (1:600; proteintech, Cat No: 10442-1-AP) antibodies, and a secondary antibody (1: 1000) against rabbit IgG (Proteintech, Cat No:SA00004-2) was utilised prior to staining with 3, 3′-diaminobenzidine (DAB). Images of stained tissue were acquired using a light microscope with an attached digital camera (BX53F; Olympus. Tokyo, Japan).

2.3. Cell culture

The ameloblast-lineage cell (ALC) line (donation from Professor Souichi Koyota) was maintained in normal glucose (NG, 5.5 mM) Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12; Meilunbio, MA0214) supplemented with 10% fetal bovine serum (FBS; EVERY GREEN, 13011-8611). Cells were maintained at 37 °C in a humidified 5% CO_2 incubator. To establish the cellular senescence model, ALCs were treated with high glucose (HG, 45 mM) for 72 h. In addition, some ALCs were treated with metformin (2 mmol/L) for 2 h after serum starvation for 24 h, prior to incubation with normal or high glucose media for 72 h. ALCs were divided into

the following groups: the normal glucose group (NG), the normal glucose treated with metformin group (Met), the high glucose group (HG) and the high glucose treated with metformin group (HG + Met).

2.4. Establishment of SIRT1 stable cell lines

To construct a stable SIRT1 overexpression cell line, we transfected ALCs with lentiviral vectors overexpressing the mouse SIRT1 gene; these vectors were constructed and purchased from GenePharma (Shanghai, China). The cells were seeded in 24-well plates, and transfection was performed when 40% confluency was reached. Polybrene (5 μg/ml) was used to facilitate transfection of ALC cells with lentivirus. The stable cell line was selected for using puromycin. Overexpression of SIRT1 was confirmed by western blotting analysis.

2.5. Western blotting assay

Total protein was extracted from ALCs by incubation with RIPA lysis buffer (Beyotime, Cat No: P0013K) for 30 min on ice. The samples were next centrifuged at 12,000 g at 4 °C for 20 min. Protein concentrations were determined using a BCA protein assay kit (solarbio, catalog no. PC0020) before being separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% or 12% acrylamide resolving gel. Separated protein bands were further transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were next incubated in TBST (0.05% Tween-20) with 5% nonfat milk for 2 h at room temperature, followed by an incubation for 14 h with primary antibodies against (polyclone) LC3 (1:2000; proteintech, Cat No: 14600-1-AP), P21 (1:2000; proteintech, Cat No: 10355-1-AP), ac-P53 (1:2000; Affinity biosciences, Cat No: AF-3744), P53 (1:2000; proteintech, Cat No: 10442-1-AP), and β-actin (1:2000; proteintech, Cat No: 81115-1-RR). The membranes were subsequently incubated with the appropriate corresponding horseradish peroxidase-conjugated secondary antibody (Beyotime, Cat No: A0208) for 1 h. The bands were detected using electrochemiluminescence (Tanon, Shanghai, China). The relative integrated density values (IDVs) were calculated using ImageJ software with β-actin utilised as an internal control.

2.6. Real-time PCR

Total RNA was extracted from ALCs using Trizol RNA isolating regents (TaKaRa Biotechnology, Japan). The concentration and quality of RNA were determined using the Nanodrop 2000 spectrophotometer (NanoDrop Products). Next, RNA was reverse transcribed to cDNA using a Prime Script RT reagent Kit (TaKaRa Biotechnology, Japan). Real-time PCR was performed by SYBR® Premix ExTaq™ II kits (TaKaRa Biotechnology, Japan) and RNA primers as follows: P21–F: 5′-GTCGCTGTCTTGCACTCTGG-3′, P21-R: 5′-CCAATCTGCGCTTGGAGTGATA-3’; P53–F: 5′-AACTTACCAGGGCAACTATGGCTTC-3′, P53-R: 5′-AACTGCACAGGGCACGTCTTC-3’; GAPDH-F: 5′-TGTGTCCGTCGTGGATCTGA-3′, GAPDH-R: 5′-TTGCTGTTGAAGTCGCAGGAG-3’. Amplification and quantitative polymerase chain reaction (PCR) measurements were carried out using the LightCycler® 96 Instrument (Roche Biochemicals). The mRNA expression was normalized to that of GAPDH. Relative quantification calculations were performed using the $2^{-\Delta\Delta Ct}$ relative quantification method.

2.7. SA-β-gal staining

SA-β-gal activity was evaluated using a Senescence-Associated β-Galactosidase Staining kit (Solabio, Peking, China) according to the manufacturer’s instructions. ALCs seeded in 6-well plates were washed with phosphate-buffered saline (PBS), fixed for 15 min, and washed 3 times. Cells were incubated with β-galactosidase staining solution at 37 °C overnight. Cells were further washed twice with PBS before being observed and photographed with an inverted microscope (invitrogen EVOS M5000). Finally, cells in each group were counted using ImageJ software.

2.8. Intracellular reactive oxygen species (ROS) analysis

Intracellular ROS levels were determined using an ROS Assay kit (Beyotime, China). After treatment with high glucose in 24-well plates, cells were washed with PBS. Subsequently, cells incubated with 1% 2,7-dichlorofluorescin diacetate (DCFH-DA) diluted in DMEM/F12 at 37 °C for 20 min followed by washing three times with PBS. Green fluorescence levels were observed using a fluorescence microscope (Invitrogen EVOS M5000).

2.9. Statistical analysis

GraphPad Prism V8.0 was used to analyze the data. The data were presented as the mean ± standard deviation (SD) in all groups. Unpaired Student’s t-tests were used for comparisons between two groups. Differences among groups were analyzed using one-way ANOVA. Statistical significance was defined as P values < 0.05.

3. Results

3.1. Metformin alleviates cellular senescence of the junctional epithelium and periodontal destruction in db/db mice

First, we detected the effect of hyperglycemia on the periodontium. As illustrated in Fig. 1Aa, at week 14 in group D, the elongation of rete ridges invaded the underlying connective tissue (black arrow), causing the fascicules of the collagen fibers to break and disarrange. Moreover, inflammatory cell infiltration was observed inside the epithelium (yellow arrow). Unclear boundaries between the epithelium and connective tissue were also observed. After treatment with metformin, the damage to periodontal tissue was reduced, as shown by the regular arrangement of fibers in a horizontal direction. E-cadherin, as a critical junction protein, plays an important role in the epithelial barrier [30]. IHC staining of the JE reveals that the percentage contribution of E-cadherin-positive cells in group D was significantly decreased compared with group C (Fig. 1Ab). Interestingly, metformin alleviated this damage as shown in

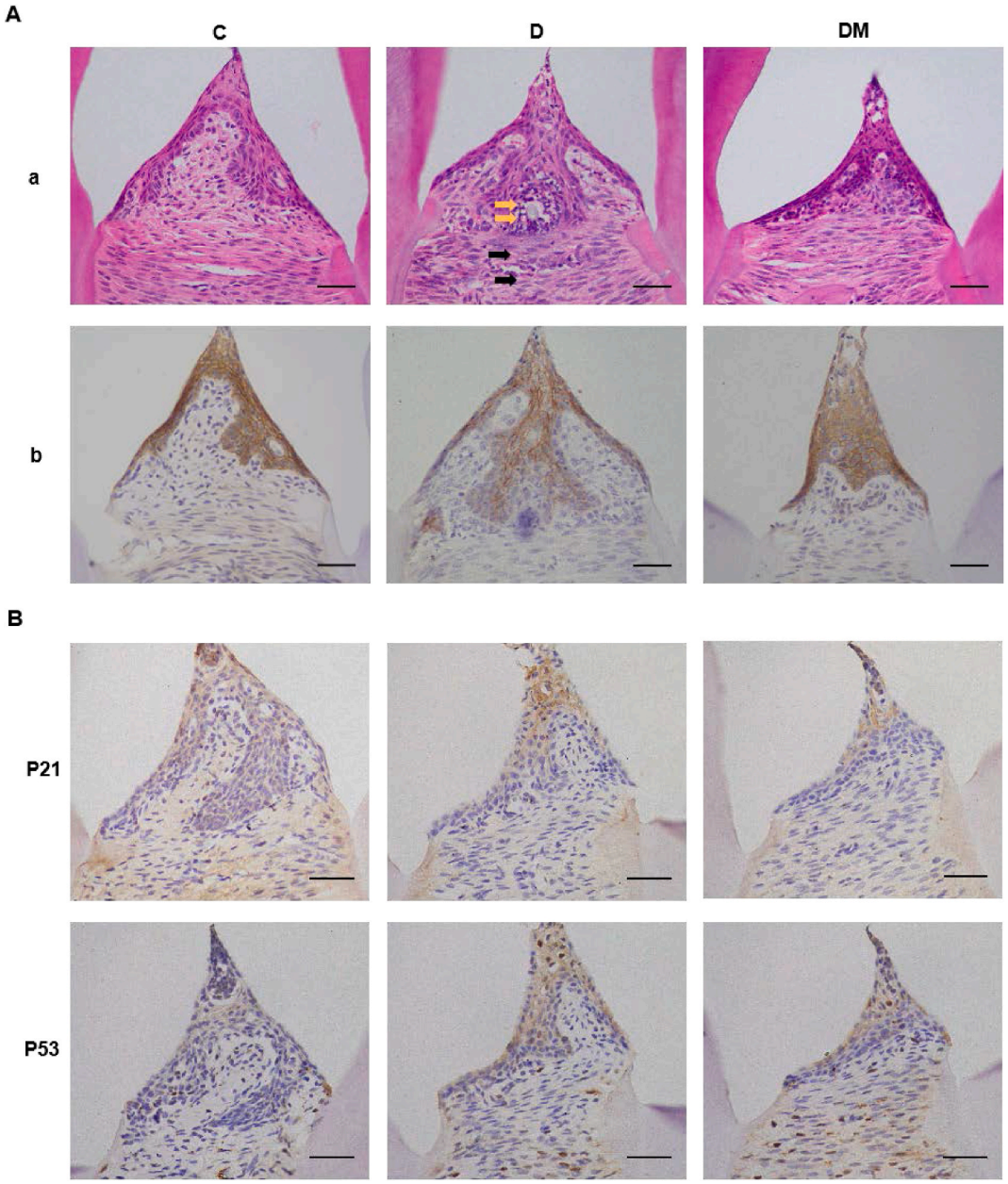

Fig. 1. Metformin alleviates periodontium destruction and cellular senescence of the JE in db/db mice. (A) HE staining (a) and IHC staining of E-cadherin (b) of JE between M1 and M2 at 14-week-old. Original magnification × 40. Scale bar, 40 μm. (B) IHC staining of P21, P53 in JE between M1 and M2 at 6-week-old. Original magnification × 40. Scale bar, 40 μm. M1, first molar, M2, second molar.

group DM wherein E-cadherin accumulated in JE. Next, we investigated the senescent phenotype caused by hyperglycemia in periodontal tissue in situ and we validated our mouse model at different ages using IHC demonstrating presence of senescence biomarkers P21 and P53. As shown in Fig. 1B, the percentage of P21 and P53 positive cells was significantly increased in 6-week-old db/db mice in the JE. Meanwhile, the percentage contribution of P21 and P53 was dramatically decreased in the JE after daily treatment with metformin for 2 weeks. Altogether, these data demonstrate that senescent cell burden and periodontal damage in mice is caused by hyperglycemia, and that metformin could alleviate these pathologic changes.

3.2. High glucose (HG) induces cellular senescence and oxidative stress in ALCs

Firstly, we constructed a cellular senescence model wherein ALCs were treated with HG at different time-points. After HG treatment for 48, 60, and 72h, cellular senescence was indicated by two markers: accumulation of SA-β-Gal activity, and the expression of P21 and P53. At 48 and 60 h, there was no statistically significant difference between the HG group and the NG group with regards to P21 and P53 expression. However, at 72 h, the expression levels of P21 and P53 were significantly higher in the HG group than in the NG group (Fig. 2A). Accordingly, there was no change in the level of SA-β-Gal staining senescence in ALCs after HG treatment for 48h and

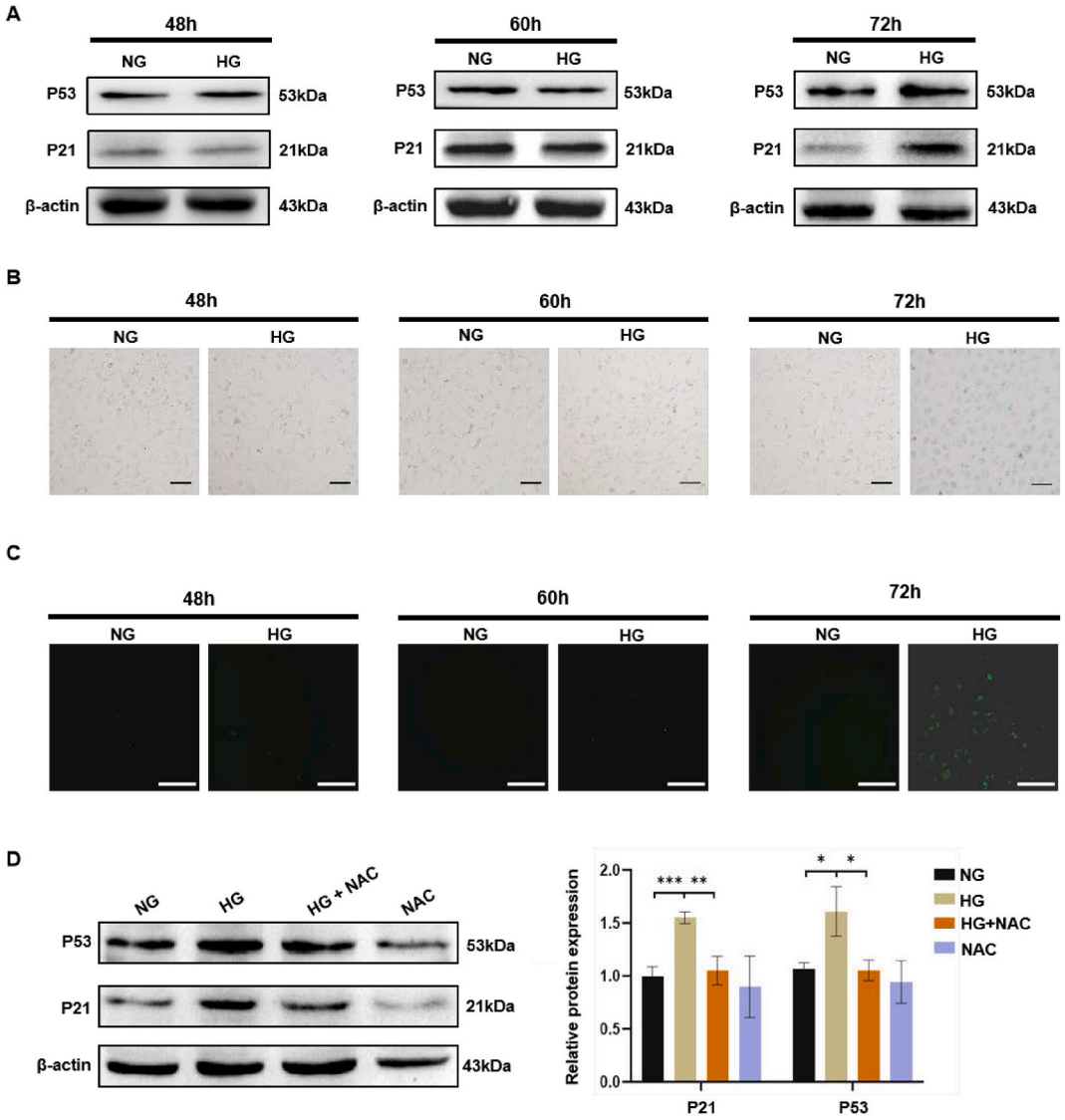

Fig. 2. High glucose induces senescence and oxidative stress in ALCs. (A) Western blotting detected the expression of P21 and P53 in ALCs that were treated with NG and HG at different time-points. (B) The percentage of SA-β-gal-positive cells was detected in ALCs that were exposed to NG and HG at different times. Scale bar, 100 μm. (C) Effect of HG on intracellular ROS production in ALCs. ROS was stained with a dichloro-dihydro-fluorescein diacetate fluorescent probe. Scale bar, 300 μm. (D) Western blotting detected the expression of P21 and P53 in ALCs that were treated with NAC. Data shown represent means ± SD; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. All results are representative of at least three independent experiments.

60h. Similarly, the SA-β-gal positive staining rate was significantly higher in the HG group than in the NG group at 72 h (Fig. 2B). Together, these results suggest that treatment with HG for 72 h was adequate to induce cellular senescence in ALCs and was applied in the subsequent studies.

The most feasible mechanism underpinning HG-induced senescence is oxidative stress [31]. Therefore, ROS accumulation was measured to determine the effect of HG on oxidative stress in ALCs. As such, results demonstrated that up-regulation of ROS was detectable at 72 h after induction of HG conditions (Fig. 2C). Next, we used the antioxidant NAC to investigate whether oxidative stress induces cellular senescence after HG in ALCs. NAC could reduce HG-induced cellular senescence as revealed by the reduced expression levels of P21 and P53 in Fig. 2D. These data suggest that HG induces cellular senescence by triggering oxidative stress.

3.3. Metformin alleviates oxidative stress and cellular senescence in ALCs in high glucose conditions

According to the results of our previous experiments, we observed that metformin had no effect on cell viability at a concentration of 2 mM, so this concentration was selected for further investigations. Firstly, we studied the effect of metformin on oxidative stress by detected intracellular ROS in ALCs after exposure HG conditions. The addition of metformin substantially decreased the ROS content in the HG group compared to in the NG group (Fig. 3A). Furthermore, as demonstrated by SA-β-Gal staining, metformin treatment reduced the percentage of SA-β-gal-positive cells (Fig. 3B). Meanwhile, as per western blotting and RT-PCR analysis, metformin inhibited the expression of P21 and P53 protein and mRNA (Fig. 3C and D). These results indicate that metformin alleviates HG-induced cellular senescence in ALCs.

3.4. Metformin attenuates cellular senescence by activating SIRT1-mediated autophagy in ALCs

To study the mechanism of anti-senescence during metformin treatment in ALCs under HG conditions, we firstly investigated the effect of metformin on autophagy in an HG environment in ALCs. We performed western blotting to detect the ratio of autophagy markers, LC3-II/LC3-I. As revealed in Fig. 4A, compared with the control group, the ratio of LC3-II/LC3-I was significantly increased after treatment with metformin. Moreover, inhibition of autophagy by 3-methyladenine (3-MA) significantly decreased the effect of metformin. The results demonstrated that 3-MA treatment caused increased intercellular ROS and expression of P21 and P53 proteins compared with the Met group (Fig. 4B and C). These findings suggest that metformin alleviates senescence primarily by the activation

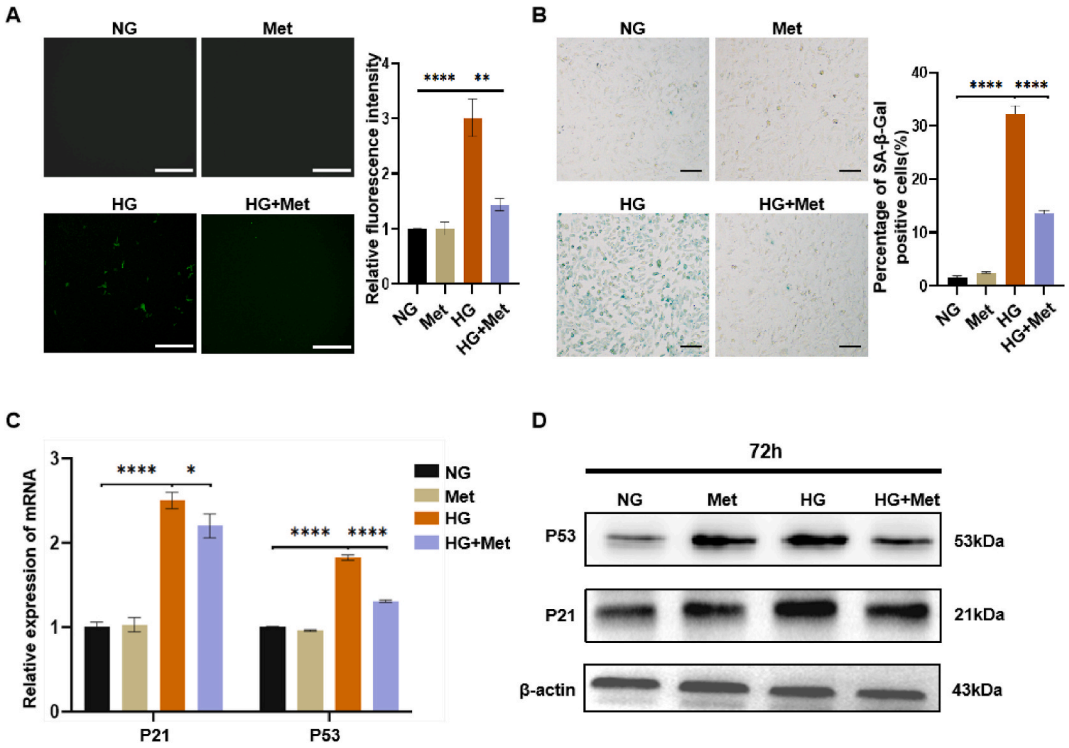

Fig. 3. Metformin treatment alleviates high glucose induced oxidative stress and senescence in ALCs. (A) After treatment with Met, the intracellular ROS production in ALCs was reduced. Scale bar, 300 μm. (B) SA-β-gal-positive cells were detected in ALCs that were exposed to NG, Met, HG, and HG + Met. Scale bar, 100 μm. (C and D) P21 and P53 mRNA and protein expression levels were detected by RT-PCR and western blotting in ALCs. Data shown represent means ± SD; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. All results are representative of at least three independent experiments.

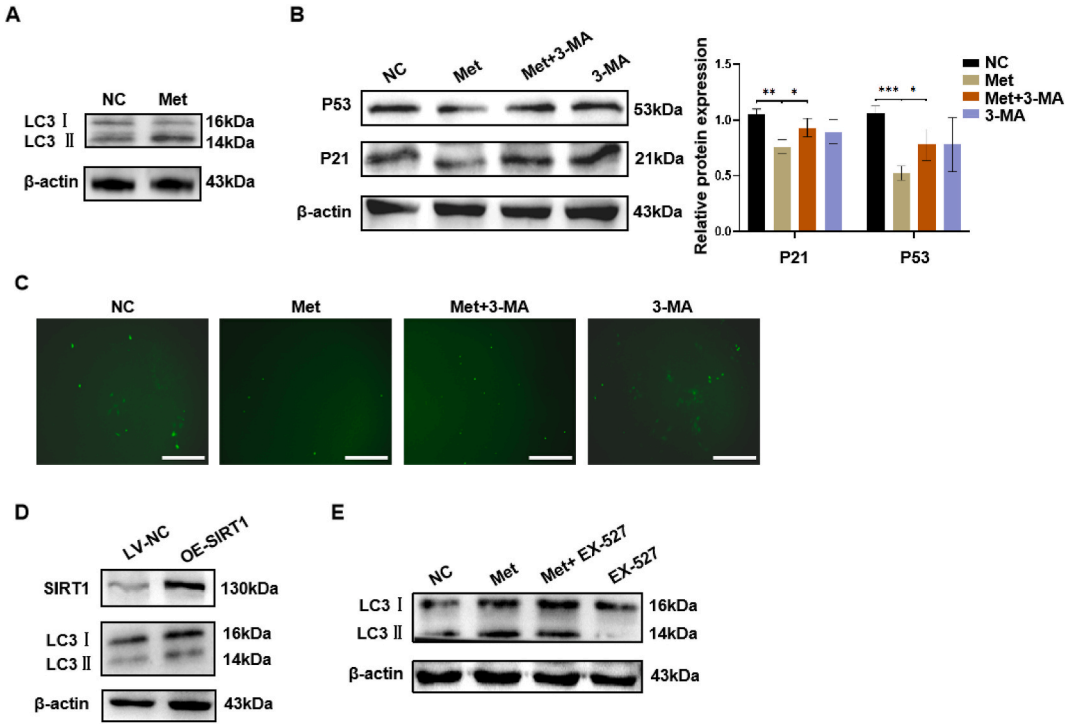

Fig. 4. Metformin attenuates cell senescence by activating the autophagy pathway through SIRT1 in ALCs. (A) Western blotting was used to detect the ratio of LC3-II/LC3-I in ALCs that were cultured in HG and HG + Met. (B and C) Intracellular ROS (B) and expression of P21 and P53 (C) were detected after pretreatment with 3-MA. Scale bar, 300 μm. (D) The expression of SIRT1 and the ratio of LC3-II/LC3-I were detected in ALCs overexpressing SIRT1 by western blotting. (E) Western blotting was further used to detect the ratio of LC3-II/LC3-I after pretreatment with EX527. Data shown represent means ± SD; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All results are representative of at least three independent experiments.

of autophagy. Additionally, SIRT1 is a well-established activator of autophagy [32]. Thus, to examine whether SIRT1 mediates the effects of metformin on autophagy in ALCs, we constructed a stable SIRT1 overexpressing cell line using lentiviral vectors. Compared with the LV-NC group, the expression of SIRT1 and the ratio of LC3-II/LC3-I were markedly increased in SIRT1 overexpressing cells (Fig. 4D). Meanwhile, pretreatment with the SIRT1 inhibitor Ex527 reduced the autophagy promoting effect of metformin, as detected by the ratio of LC3-II/LC3-I (Fig. 4E). Taken together, these data suggest that SIRT1 mediates the autophagy-promoting effects of metformin.

3.5. SIRT1 mediates the protective effects of metformin against oxidative stress and senescence

Given prior results, we decided to explore whether SIRT1 was responsible for mediating the effects of metformin on oxidative stress and senescence in ALCs. Firstly, we detected the effect of metformin on SIRT1 under HG conditions. Results revealed that the protein expression level of SIRT1 was increased in the Met group compared with the control group (Fig. 5A). Next, pretreatment of ALCs with the SIRT1 inhibitor Ex527 revealed the effect of metformin on intracellular ROS accumulation was reversed by SIRT1 inhibition (Fig. 5B). Meanwhile, levels of positive SA-β-Gal staining and of P21 and P53 expression were increased in the Met + Ex527 group compared with the Met group (Fig. 5C and D). Furthermore, to investigate the effect of SIRT1 on oxidative stress and cellular senescence under HG conditions, SIRT1-overexpressing lentivirus were stably transfected into ALCs. As shown in Fig. 5E, compared with the LV-NC group, intracellular ROS production was decreased suggesting that SIRT1-overexpression could dampen oxidative stress in ALCs during HG conditions. Meanwhile, results revealed that compared with the LV-NC group, SIRT1 protein expression levels were significantly increased, but the protein expression levels of ac-P53, P53 and P21 were evidently decreased; the same trends were demonstrated by SA-β-gal staining (Fig. 5F and G). These data suggest that SIRT1-induced deacetylation of P53 partially inactivates P53, and this partial loss of function can attenuate P53-dependent cellular senescence.

3.6. Metformin activates the SIRT1-autophagy pathway via AMPK

As is widely reported, metformin was revealed to alter biological functions in cells by activating AMPK. AMPK activation is conducive to cellular homeostasis and senescence prevention [33]. Therefore, to determine whether metformin exerts its protective role in an AMPK-dependent manner, we used an AMPK inhibitor (Compound C) to examine its role in the mechanisms induced by metformin. As revealed in Fig. 6A and B, Compound C attenuated the effect of metformin on oxidative stress and cellular senescence in

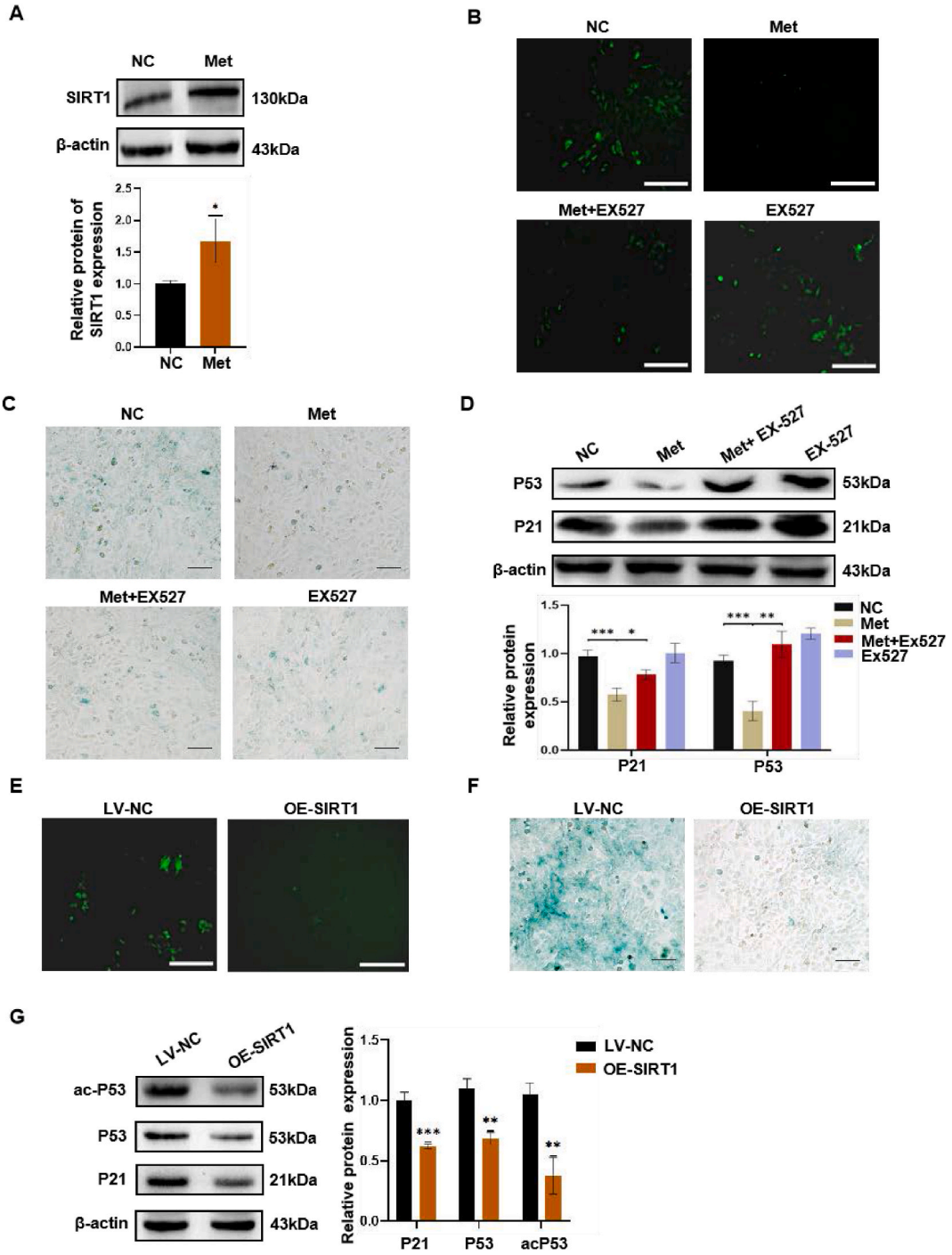

Fig. 5. SIRT1 mediates a metformin-induced reduction in cell senescence in ALCs. (A) Western blotting was used to detect protein expression levels of SIRT1 in ALCs that were cultured in HG and HG + Met. Pretreatment of ALCs with SIRT1 inhibitor Ex527. (B) The intracellular ROS production in ALCs. Scale bar, 300 μm. (C) The percentage of SA-β-gal-positive cells was detected in ALCs. Scale bar, 100 μm. (D) The protein expression levels of P21 and P53 in ALCs were detected by western blotting. (E) Intracellular ROS production in ALCs. Scale bar, 300 μm. (F) The percentage of SA-β-gal-positive cells was detected to determine the effect of SIRT1 on senescence in ALCs. Scale bar, 100 μm. (G) The protein expression levels of ac-P53, P53 and P21 were detected in ALCs overexpressing SIRT1 by western blotting. Data shown represent means ± SD; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. All results are representative of at least three independent experiments.

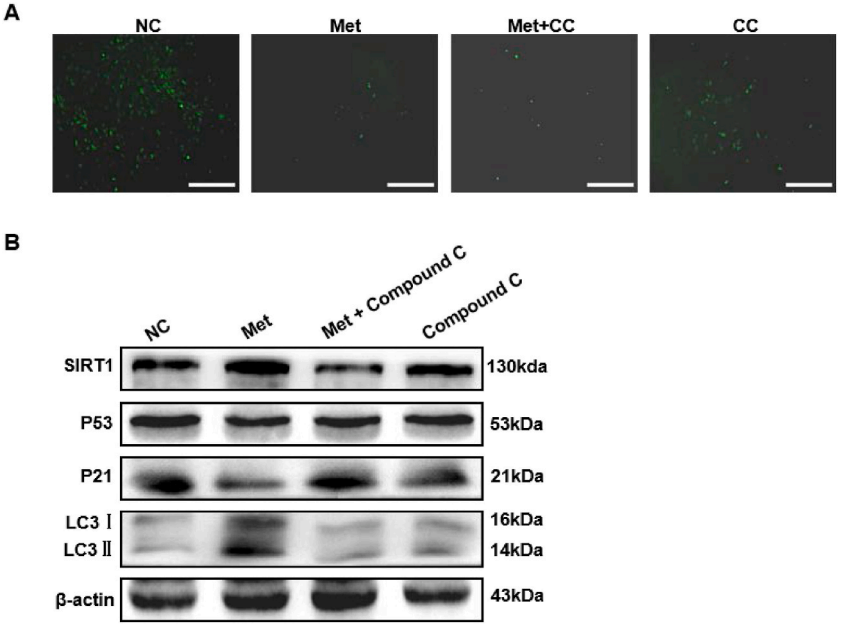

Fig. 6. Metformin activated the SIRT1-autophagy pathway via AMPK. (A) Intracellular ROS production in ALCs after pretreatment with Compound C. (B) P53, P21 and SIRT1 protein expression levels and the ratio of LC3-II/LC3-I were detected in ALCs that were exposed to Met, Compound C + Met and Compound C. All results are representative of at least three independent experiments.

ALCs in an HG environment. Furthermore, we explored whether Compound C could counteract the effect of metformin on the SIRT1-autophagy pathway. As expected, Compound C reduced the ability of metformin to active the SIRT1-autophagy pathway, as evidenced by protein expression levels of SIRT1 and the LC3-II/LC3-I ratio (Fig. 6B).

4. Discussion

In this study we identified the potential association between accumulated senescent cells in the JE and the destruction of the junctional epithelial barrier and periodontal tissue in db/db mice. Moreover, we demonstrated the protective effects of metformin in ameliorating cellular senescence in the JE induced by hyperglycemia via the AMPK/SIRT1/autophagy signaling pathway.

In the development of periodontitis, the JE is a critical tissue barrier and the permeability of the JE is essential for maintaining periodontal health. In this study, we demonstrated that the junctional epithelial barrier and periodontal tissue are damaged during hyperglycemia consistent with previous studies [16,34]. Age-dependent changes in cell adhesion complexes have been associated with an increase in barrier permeability [30,35,36]. Hyperglycemia can cause a high local burden of senescent cells in many tissues, such as adipose tissues, renal tubular, and vascular smooth muscle tissue amongst others [37,38]. Moreover, senescent cells constantly secret inflammatory factors, which have an adverse effect on the control of inflammation [39]. Senescent cells adopt several unique identifying characteristics, including accumulation of DNA damage sensors like P53, upregulation of cell cycle inhibitors such as P21, reactive oxygen species production, and SA-β-Gal activity [40,41]. In the present study, the elevated expression levels of P21 and P53 indicate that hyperglycemia aggravated the local burden of senescent cells in the JE of db/db mice. These results indicate that hyperglycemia may play an important role in the accumulation of senescent cells in the JE that results in barrier dysfunction driving the progression of periodontitis.

Metformin, an anti-diabetic drug, has been reported to alleviate the hyperglycemia-induced preaging state in periodontitis [34]. Our results indicate that metformin could decrease cellular senescence in the JE and alleviate damage to periodontal tissue in db/db mice. Meanwhile, metformin treatment could attenuate HG-induced senescence in ALCs. However, the molecular mechanism by which metformin attenuates hyperglycemia-induced cellular senescence in the JE is not clear. Previous reports suggest that the beneficial effects of metformin on aging and health are primarily indirect via its effects on cellular metabolism and reduction of oxidative stress [42].

Oxidative stress, defined as disturbances to the pro-/anti-oxidant balance, is harmful to cells due to the excessive generation of ROS and nitrogen species [43]. In a hyperglycemic environment, a set of metabolic abnormalities are initiated, including the polyol, AGE formation, PKC, and hexosamine pathways; these pathways augment ROS generation and subsequently facilitate oxidative stress [44,45]. It has been reported that augmented ROS directly results in damage to vital biomolecules, such as proteins, lipids and DNA, and the accumulation of molecular damage intracellularly induces cellular senescence [43]. Evidence has linked ROS to the pathological destruction of connective tissue in periodontal disease [46]. Interestingly, metformin was demonstrated to inhibit the process of oxidative stress and ameliorate periodontitis [25,47–49]. Similarly, in our study, metformin could dampen HG-induced oxidative

stress in ALCs.

Autophagy is an essential metabolic process that can remove damaged or senescent organelles and as a major sensor of redox signaling can decreases oxidative stress to maintain cellular homeostasis [50,51]. The SIRT1 mediated autophagy pathway is universally required to prevent a range of diseases, such as diabetic kidney disease, skin aging, and acute pancreatitis [52]. Previous study suggested that metformin alleviates hepatic steatosis through SIRT1-mediated effects upon the autophagy machinery [53]. Moreover, autophagy was demonstrated to mediate the anti-aging effects of metformin [54,55]. Therefore, we speculated that autophagy may be involved in the mechanisms of metformin and our investigating revealed that metformin could protect against oxidative stress -induced cellular senescence through the SIRT1-mediated autophagy pathway in ALCs exposed to a high glucose environment.

SIRT1 exerts an important role in both cellular defense against oxidative stress and cellular senescence; it can also be activated by metformin. SIRT1 prevents premature aging/cellular senescence by regulating FOXOs, P53, and P21, in addition to molecules involved in DNA damage and repair [56]. In the present study, the SIRT1 inhibitor EX527 reversed the effects of metformin on cellular oxidative stress and cellular senescence. P53 plays a pivotal role in the context of senescence; its activation can occur in a DDR-dependent or DDR-independent manner [57]. As a deacetylase, SIRT1 can deacetylate certain key proteins, such as P53, to delay cellular senescence, a finding that is consistent with our experimental results [58]. These results indicated that metformin may protect against cellular senescence by activating SIRT1 to deacetylate P53 in ALCs exposed to high glucose.

The generally accepted mechanism of metformin's mechanism of action is stimulation of AMPK. The role of AMPK in the prevention of aging is generally attributed to its effects on the activation of SIRT1 and FOXO1, as well as the suppression of mTOR [59]. Activation of AMPK increases cellular NAD + levels, which in turn activates SIRT1 activity and autophagy [60]. A previous study reported that metformin triggered AMPK-SIRT1 signaling and increased the deacetylation of P53 in HepG2 cells in HG conditions [61]. Additional studies have demonstrated that metformin could inhibit the process of oxidative stress, promote the induction autophagy, and protect diabetic renal injury by activating the AMPK/SIRT1 pathway [62]. In our study, an AMPK inhibitor alleviated the effects of metformin on antioxidant, anti-senescence and SIRT1-mediated autophagy in ALCs. These data suggest that metformin alleviates cellular senescence by activating SIRT1-mediated autophagy induction via an AMPK-independent pathway in ALCs.

Overall, our results suggest that hyperglycemia resulted in accelerated cellular senescence in the JE and induced the damage of the JE barrier and periodontal tissue simultaneously. Meanwhile, the present study further clarified the mechanism by which metformin treatment functions to ameliorate cellular senescence in the JE. Currently, few studies have investigated cellular senescence in the JE during diabetic periodontitis. Further research is required to deconvolute the potential mechanisms of diabetes and periodontitis to provide new avenues for their clinical diagnosis and treatment.

Funding

This study was supported by the National Natural Science Foundation of China (Grant No. 81870738).

Data availability statement

The dataset supporting the conclusions of this article is included within this article and is available from the corresponding author upon request.

Ethics approval and informed consent statement

The animal experiments involved in this study were approved by the Animal Ethics Committee of Binzhou Medical University (the animal ethics approval number: 2022-391) in accordance with ethical principles.

Patient consent for publication

Not applicable.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Xiaoyuan Ye: Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Conceptualization. **Yumin Wang:** Writing – original draft, Validation, Conceptualization. **Yanying Tian:** Validation, Methodology. **Ruonan Bi:** Formal analysis. **Mingyue Li:** Validation. **Chunyan Yang:** Writing – review & editing. **Li Zhang:** Writing – review & editing, Project administration. **Yuguang Gao:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

Acknowledgements

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27478.

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