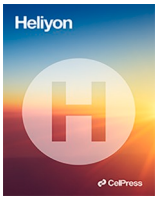
Heliyon 10 (2024) e24586

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Ginsenoside Rb1 prevents age-related endothelial senescence by modulating SIRT1/caveolin-1/enos signaling pathway

Bin Zhou [1], Guangyao Sh [1], Dongmei Xie [1], Xiaoli Zhao [1], Baoshun Hao, Dinhui Liu, Min Wang, Lin Wu [***], Liangying Lin [**], Xiaoxian Qian [*]

The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China

ARTICLE INFO

Keywords:
Ginsenoside Rb1
Senescence
Caveolin-1
Deacetylation
Human umbilical vein endothelial cells

ABSTRACT

Background: Advancing age is one of the independent risk factors for cardiovascular disorders. The *Compendium of Materia Medica*, a classic book on traditional Chinese medicine, states that ginseng "harmonizes the five internal organs, calming the spirit and prolonging the years of life." Considered one of the primary bioactive compounds derived from Panax ginseng, ginsenoside Rb1 (g-Rb1) has been scientifically suggested to possess anti-senescence efficacy. More research is needed to explore the vascular pharmacological activity and potential clinical application value of g-Rb1.

Aims of the study: Our previous study demonstrated that g-Rb1 could mitigate cellular senescence via the SIRT1/eNOS pathway. This study was performed to explore the exact mechanisms by which g-Rb1 modulates the SIRT1/eNOS pathway.

Materials and methods: We used human primary umbilical vein endothelial cells (HUVECs) to establish a replicative ageing model. Real-time (RT−PCR), western blotting, small interfering RNA (siRNA), and immunoprecipitation were conducted to detect the effect of g-Rb1 on the SIRT1/caveolin-1/eNOS axis.

Results: G-Rb1 increased NO production and alleviated replicative senescence of HUVECs. The application of g-Rb1 elevated the mRNA and protein abundance of both SIRT1 and eNOS while concomitantly suppressing the expression of caveolin-1. Inhibition of SIRT1 and eNOS by siRNAs suppressed the anti-senescence function of g-Rb1, while caveolin-1 siRNA could enhance it. G-Rb1 decreased the acetylation level of caveolin-1 and increased NO production, which was suppressed by SIRT1 siRNA. Both g-Rb1 and caveolin-1 siRNA could reduce the acetylation level of eNOS and increase NO production.

Conclusion: G-Rb1 prevents age-related endothelial senescence by modulating the SIRT1/caveolin-1/eNOS signaling pathway.

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: wulin23@mail.sysu.edu.cn (L. Wu), linly35@mail.sysu.edu.cn (L. Lin), qianxx@mail.sysu.edu.cn (X. Qian).
[1] These authors contributed equally to this study.

https://doi.org/10.1016/j.heliyon.2024.e24586
Received 15 August 2023; Received in revised form 9 January 2024; Accepted 10 January 2024
Available online 17 January 2024

1. Introduction

With the worsening of the ageing problem, the incidence of atherosclerosis has been increasing yearly. Individual ageing inevitably accompanies cellular senescence [1], and endothelial cell senescence has been determined to be a critical factor in the occurrence and development of atherosclerosis [2]. Previous studies have shown that alleviating endothelial cell senescence can significantly improve atherosclerosis [3]. Therefore, an in-depth exploration of the mechanism of endothelial cell senescence and the search for related anti-ageing drugs would contribute to the development of new therapies for cardiovascular disease and reduce the burden of cardiovascular disease.

Endothelial nitric oxide synthase (eNOS) is the rate-limiting enzyme for synthesizing nitric oxide (NO), an important neurotransmitter and signaling molecule in the cardiovascular system. Many studies have confirmed that the activation of eNOS and subsequent NO production can slow endothelial cell senescence and prevent atherosclerosis [4,5]. Binding with caveolin-1 inhibits eNOS activity, but the specific mechanism has yet to be fully elucidated [6].

Caveolin-1 is the main component of caveolae. Caveolin-1 indirectly associates with cytoskeletal proteins, maintaining the invagination of caveolae and inhibiting eNOS activity by binding to it [6]. Previous studies have shown that in aged endothelial cells, the expression of caveolin-1 increases while eNOS activity decreases [7]. The delivery of caveolin-1 and its ability to bind to eNOS may significantly affect eNOS-mediated NO production. The expression level of caveolin-1 and the deacetylation level of eNOS are regulated by SIRT1. SIRT1 is a deacetylase closely related to the delay of ageing and lifespan extension. Studies have shown that in aged organs, the expression of SIRT1 significantly decreases, while overexpression of SIRT1 can delay ageing [8,9]. However, whether SIRT1 regulates eNOS function by affecting caveolin-1 remains unclear.

Panax ginseng is a traditional Chinese herb with anti-ageing effects. According to the *Compendium of Materia Medica,* long-term consumption of ginseng can prolong life. Modern pharmacological research has confirmed that the main active ingredients of ginseng are various ginsenosides, including Rb, Rc, Rd, Rg, and Rf. Ginsenoside Rb1 (g-Rb1) is one of the main components of ginseng

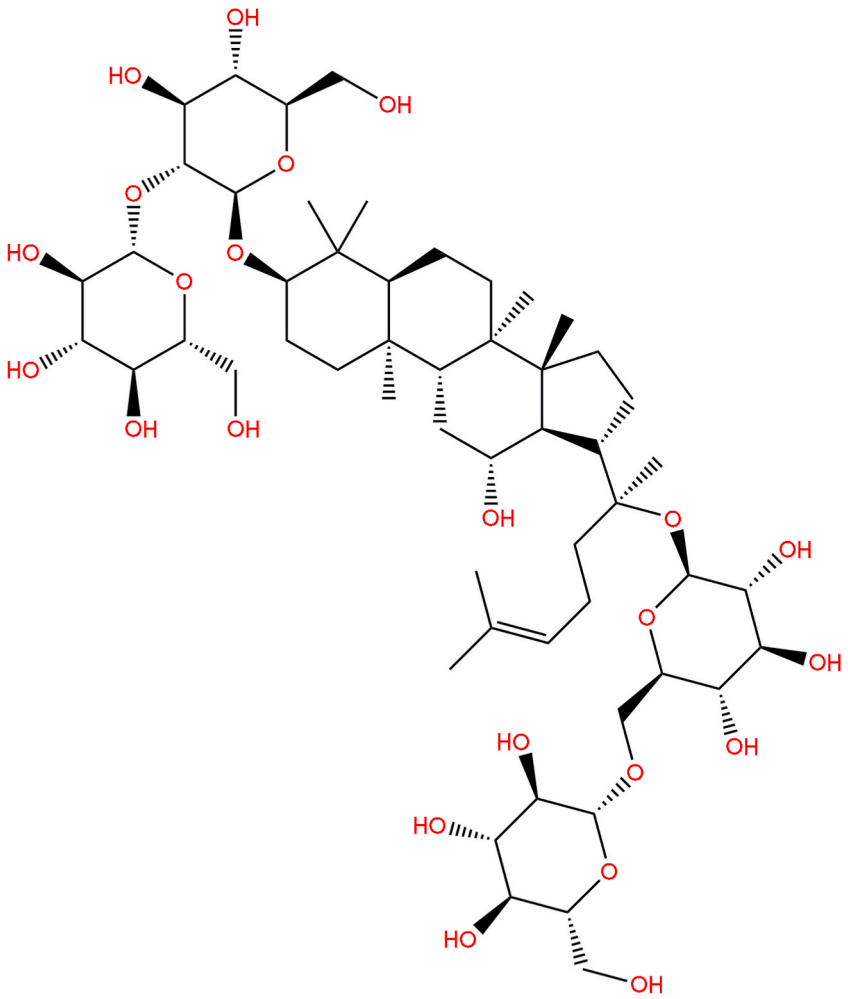


Fig. 1. Structure diagram of g-Rb1.

and has a significant protective effect on vascular endothelial cells. Previous studies by our research group have shown that g-Rb1 can enhance eNOS activity and NO secretion, reduce oxidative stress, and delay endothelial cell senescence, possibly through the involvement of SIRT1 [8,10]. However, the specific mechanisms are still unclear.

We established a replicative senescence model of human umbilical vein endothelial cells to simulate the natural ageing of human vascular endothelium and further explored whether g-Rb1 affects the caveolin-1/eNOS axis through SIRT1 and its potential mechanisms. We measured the acetylation levels of caveolin-1 and eNOS. We established the significant regulatory role of SIRT1 on the caveolin/eNOS axis and clarified, for the first time, the impact of caveolin acetylation level on eNOS activity, providing a theoretical basis for exploring new targets for anti-ageing therapy to combat atherosclerosis.

2. Materials and methods

2.1. Isolation of primary human umbilical vein endothelial cells (HUVECs)

HUVECs from donors (The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, China) were isolated according to a protocol [11]. Culturing of the cells was carried out in Medium 199 (HyClone, Brussels, Belgium), with the addition of 20 % foetal bovine serum (FBS), 1 % glutamine (Sigma Aldrich, USA), and 5 % CO_2 at 37 °C. The isolation of HUVECs from donors was approved by the Research Committee of the Third Affiliated Hospital of Sun Yat-sen University (approval no. [2022]02-317-01). The donors all provided written consent for the donation of umbilical cords.

2.2. Establishment of a replicative senescent model and agents

We passaged the primary cells when their density was more than 80 %. Cell detachment was achieved by treating the cells with 0.05 % trypsin and EDTA (Gibco, Thermo, USA). The senescence markers of the 2nd, 16th, and 30th passage cells were detected. G-Rb1 (CAS No. 41753-43-9, n = 1109, molecular structure is shown in Fig. 1) was obtained from Pufei De Biotech Co., Ltd, Chengdu, China. G-Rb1 was added to the medium for 48 h at concentrations of 0 μmol/L, 20 μmol/L, 40 μmol/L, 80 μmol/L, 100 μmol/L and 120 μmol/L.

If siRNA transfection was needed, the target RNA was detected 24 h after transfection, and then g-Rb1 intervention was carried out. After the intervention, the culture medium, cell protein, and total RNA were collected for follow-up experiments.

2.3. Flow cytometry assay

Influx flow cytometry was conducted to identify and indicate the stability of isolated HUVECs (B.D., USA). Data were analysed by FlowJo 7.5 software (Tree star, Ashland, USA). The anti-mouse antibody CD31-PE (1:200, B.D.) was used.

2.4. SA-β-gal staining

SA-β-gal staining was executed using a commercially available kit sourced from Beyotime, Shanghai, China. Briefly, PBS was used to wash the HUVECs, followed by fixation at room temperature for a period of 10 min. Subsequently, the cells were exposed to a stain solution containing senescence-associated β-galactosidase (SA-β-gal) and incubated at 37 °C for 12–16 h. We detected lysosomal β-gal by microscopy. The quantification of senescent cells (SA-β-gal-positive) was accomplished by manually counting a range of 150–200 cells.

2.5. MTT assay

The MTT assay was performed according to the protocol described in the instructions (Beyotime, China). Briefly, the division of the HUVECs into six groups was performed based on distinct concentrations of g-Rb1 (0 μmol/L, 20 μmol/L, 40 μmol/L, 80 μmol/L, 100 μmol/L, 120 μmol/L) and incubated in 96-well plates (4 × 103/well) for 72 h. Then, the cells were immersed for 4 h in 5 mg/ml MTT. The cells were washed with PBS, DMSO (150 μl, Sigma Aldrich, USA) was added, and the plates were gently shaken for 15 min. The recorded absorbance at 490 nm was obtained using a microplate spectrophotometer (BioTek EON, BioTek Corporation, Vermont, USA).

2.6. Total RNA isolation and qPCR

TRIzol reagent (Thermo Fisher Scientific, MA, USA) was employed to isolate total RNA from the cells. and quantified at 260 nm. Following the guidelines provided in the Prime Script® 1st strand cDNA Synthesis Kit manual, the conversion of 1 μg total RNA into cDNAs was facilitated through the use of reverse transcriptase and oligo deoxythymidine primers (Takara, USA). Following reverse transcription, the amplified samples were prepared using a total volume of 25 μl solution containing SYBR Green master mix (BioLine, London, United Kingdom) as described in the SYBR® Premix Ex Taq™ II instructions. β-actin was used for normalizing gene expression. The gene sequences are as follows: SIRT1 (GenBank No. NM_012238.4) forward 5′-TGTGGTAGAGCTTGCATTG, ATCTT-3′, backwards 5′-GGCCTGTTGCTCTCCTCATT-3’; caveolin-1 (GenBank NC_000007.14) forward 5′-CCTCCTCACAGTTTTCATCC-3′, backward 5′-CAATCACATCTTCAAAGTCAATC-3’; eNOS (GenBank No. NM_001160110.1) forward 5′-

TGGTACATGAGCACTGAGATCG-3′, backwards 5′-CCACGTTGATTTCCACTGCTG-3’; PAI-1 (GenBank No. NM_001165413) forward 5′-TGCTGGTGAATGCCCTCTACT-3′, backwards 5′- CGG TCATTC CCA GGT TCT CTA-3’; β-actin (GenBank No. NM_001101.3) forward 5′-AGCGGGAAATCGTGC GTGAC-3′, backwards 5′-TCCATGCCCAGGAAGGAAGG-3’.

2.7. Western blot

RIPA lysis buffer supplemented with proteinase inhibitors, phosphatase inhibitors, and deacetylase inhibitors was utilized for the extraction of total protein. The isolated proteins were subjected to Western blot analysis as the next step. Subsequent analysis of the protein bands was conducted using Quantity One Software provided by Bio-Rad (USA). The following primary antibodies were used: SIRT1 (1:2000, Proteintech Group, USA), caveolin-1 (1:3000, Cell Signaling TechnologyTechnology, USA), PAI-1 (1:10,000, Proteintech Group, USA), eNOS (1:2000, Abcam, USA), and Ac-lysine (1:500, Cell Signaling Technology, USA).

2.8. Transfection of siRNA

SiRNA-control and siRNAs specific to SIRT1 and caveolin-1 were purchased from Biomics (Biomics Biotechnologies, China). Their sequences were as follows: SIRT1: forward, 5′-GGUCAAGGGAUGGUAUUUATT-3′, backwards 5′-UAAAUACCAUCCCUUGACCTT-3’; caveolin-1: forward, 5′- CUUCACUGUGACGAAAUATT -3′, backwards, 5′- UAUUUCGUCACAGUGAAGGTT-3’. When cells grew at a density of 30%–50 %, mixtures of Lipofectamine, Opti-MEMI, and siRNA were added to the medium for 6 h. Then, the cells were reincubated in fresh medium with or without g-Rb1 for 48 h. Then, the cells and their supernatant were collected for further experiments.

2.9. Nitrate reductase assay

The nitric oxide (NO) assay kit quantitatively measures total NO (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). To measure nitrate levels, this assay kit employs nitrate reductase to enzymatically convert nitrate into nitrite, followed by the utilization of the Griess reagent for analysis. The level of NO in the samples of cell supernatants was determined following the manufacturer’s instructions and normalized by μmol/L.

2.10. Superoxide dismutase (SOD) and malondialdehyde (MDA) assays

Following a centrifugation step at 3000 rpm/min for 15 min, the supernatant from the cellular supernatant samples was retrieved to measure SOD and MDA concentrations. The SOD assay kit and MDA assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were selected for the measurements. The studies adhered to the manufacturer’s guidelines, and the results were normalized to nmol/ml units.

2.11. Immunoprecipitation

After treatments, the cells were subjected to ice-cold RIPA buffer (Keygentech, Nanjing, China) for a duration of 30 min. The cell lysate containing the protein was transferred to a precooled E.P. tube and then centrifuged at 14,000 rpm for 30 min at 4 °C, after which the supernatant was carefully transferred to a newly precooled E.P. tube. The corresponding antibodies (eNOS, caveolin-1 antibodies) were added to the supernatant, and a small amount of RIPA lysate was added simultaneously. The solution was incubated overnight at 4 °C by slowly shaking. After rinsing with RIPA lysis buffer three times, the protein A agarose beads (10 μL) were centrifuged at 3000 rpm for 3 min per wash to separate the beads from the liquid. Once the agarose beads were ready, they were introduced to the cell lysate in the presence of the antibody. The mixture was then gently incubated at 4 °C for 4 h. The agarose beads were subjected to centrifugation at 3000 rpm at 4 °C for 3 min. The excess liquid was cautiously removed, and the agarose beads were subjected to triple washes using 1 ml of RIPA lysis buffer, ensuring thorough cleansing. The resulting precipitate was reconstituted by adding 60 μL of 2 × SDS loading buffer. Afterwards, the sample was exposed to boiling water for 5 min prior to its application onto the SDS−PAGE gel. The subsequent steps in the procedure followed the Western blot protocol.

2.12. Statistical analysis

The experimental trials were replicated three times, and the outcomes were reported as the average values ± standard deviation (S.D.). Statistical significance was assessed using unpaired Student’s *t*-test or one-way ANOVA. A significance threshold was set at a p value less than 0.05 to determine the statistical importance of the findings. IBM SPSS Statistics v22.0 was employed for performing the statistical analysis.

3. Results

3.1. Primary HUVECs were extracted and passaged to establish a replicative senescence model

After the primary HUVECs were extracted, they were identified by morphology (paving stone) and CD31 expression level (high

expression of CD31) (Fig. 2A and B). From the 16th generation, proliferation slowed, the morphology became irregular (Fig. 2C), and the percentage of cells exhibiting positive β-galactosidase staining increased (Fig. 2D). At the same time, the expression of PAI-1 (plasminogen activator inhibitor-1), one of the markers of endothelial cell ageing, rose from the 16th generation (Fig. 2E). The above results suggest that the 16th generation of primary human umbilical vein endothelial cells stably show senescence characteristics. Therefore, the 16th-generation cells were recognized as senescent cells, while the 2nd-generation cells were recognized as young cells in the following experiments.

3.2. G-Rb1 attenuated the senescence of endothelial cells

According to previous research [9], we designed a g-Rb1 concentration gradient for ageing human umbilical vein endothelial cells, which was 0 μmol/L, 20 μmol/L, 40 μmol/L, 80 μmol/L, 100 μmol/L, and 120 μmol/L. First, the effects of different concentrations of g-Rb1 on cell proliferation were observed. It was observed from the results that a dose of 120 μmol/L g-Rb1 exhibited a potentially

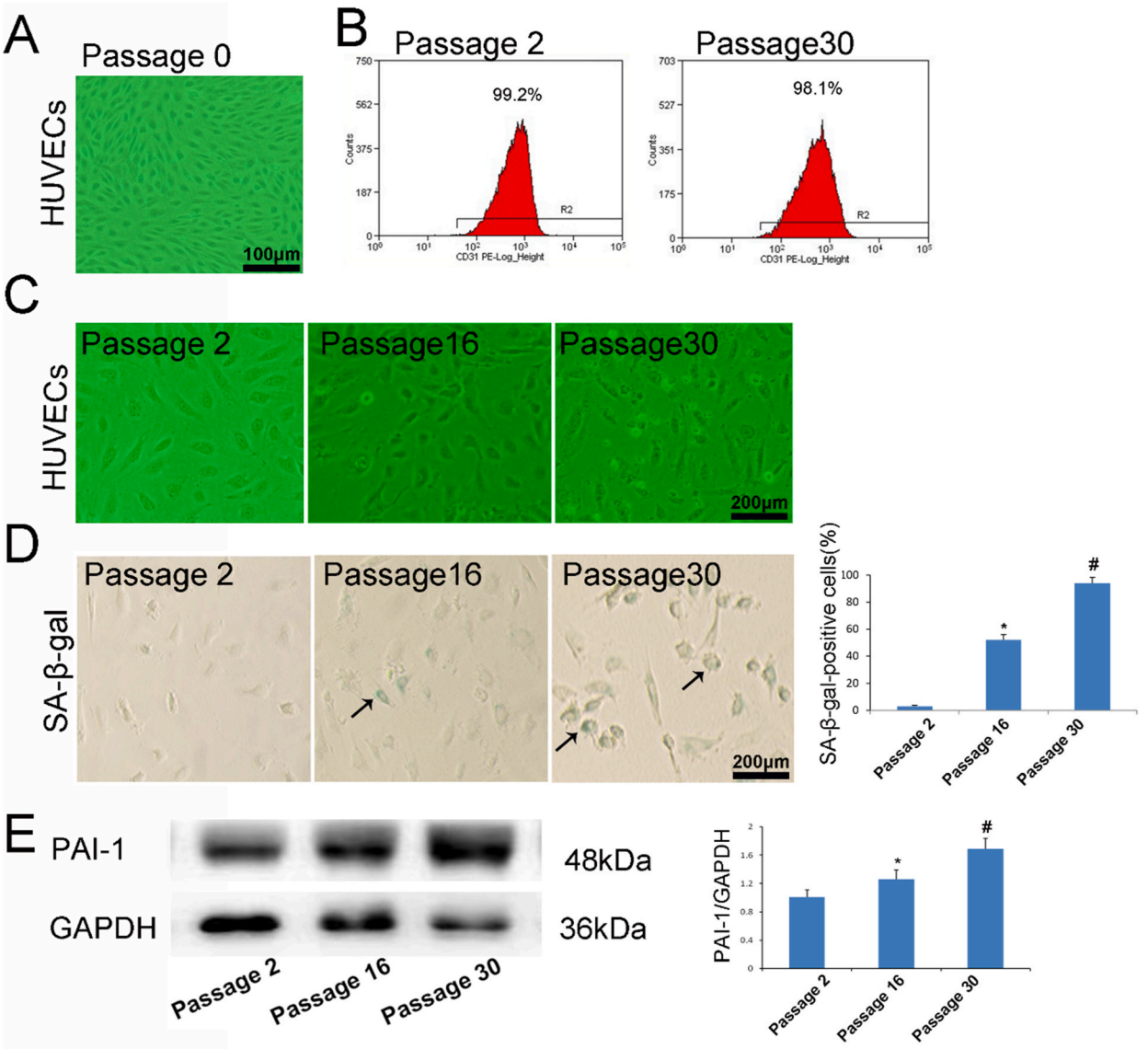


Fig. 2. Identification of senescent HUVECs.
A. Freshly isolated HUVECs exhibited adherent growth with rich cytoplasm. Scale bar = 100 μm. **B.** Different passages of primary HUVECs significantly and stably expressed CD31. **C.** Replicative senescent HUVECs exhibited increased size and apparent nuclear changes. Scale bar = 200 μm **D.** SA-*β*-gal stained. Scale bar = 200 μm. Data are shown as the mean ± SD, $^{*}p < 0.05$ vs. Passage 2, $\#p < 0.05$ vs. Passage 16, n = 3. **E.** The expression of PAI-1 increased during replicative senescence progression. Data are shown as the mean ± SD, $^{*}p < 0.05$ vs. Passage 2, $\#p < 0.05$ vs. Passage 16, n = 3.

deleterious influence on the cells (Fig. 3A). Second, measuring the expression level of PAI-1 and the proportion of β-galactosidase--positive cells confirmed that g-Rb1 at 80 μmol/L had a noticeable alleviating effect on replicative senescence (Fig. 3B and C). Here, 80 μmol/L will be used as the concentration of g-Rb1 in the following experiments.

3.3. G-Rb1 modulated SIRT1, caveolin-1, and eNOS

Aligning with precedented studies, our outcomes suggest a reduction in the mRNA and protein levels of SIRT1 and eNOS in senescent cells relative to young cells, while caveolin-1 presents an inverted relationship [8,10,12]. However, g-Rb1 alleviated these changes (Fig. 4A and B). At the same time, g-Rb1 can also lessen the decrease in NO levels in senescent cells (Fig. 4C). By regulating the expression of SIRT1 and caveolin-1 through siRNA, we found that the reduction in SIRT1 expression and the increase in caveolin-1 expression aggravated cell senescence (Fig. 4D and E), suggesting that SIRT1 and caveolin-1 play an essential role in cell senescence.

3.4. G-Rb1 prevents age-related endothelial senescence by modulating the SIRT1/caveolin-1/eNOS pathway

To further confirm the role of SIRT1/caveolin-1/eNOS in the anti-ageing properties of g-Rb1 and explore its potential mechanism, we successfully inhibited the expression of SIRT1 and caveolin-1 in HUVECs by siRNA interference, and then HUVECs were treated with g-Rb1. The mRNA and protein expression of SIRT1, caveolin-1, eNOS, and PAI-1 in different experimental groups was measured. The results showed that inhibition of SIRT1 expression could completely counteract the upregulation of eNOS but only partially counteract the anti-ageing effect of g-Rb1 (Fig. 5A and B). Inhibition of caveolin-1 expression had no significant impact on the upregulation of eNOS by g-Rb1 but could further enhance the anti-ageing effect of g-Rb1 (Fig. 5A and B). The observed outcomes provide compelling evidence that the modulation of SIRT1/caveolin-1/eNOS is crucial for the anti-senescence efficacy of g-Rb1. Further experiments showed that inhibiting the expression of SIRT1 could not completely counteract the effect of g-Rb1 on the expression of NO, and inhibiting the expression of caveolin-1 could further increase the expression of NO, so g-Rb1 may affect the activity of eNOS through mechanisms other than the expression level of SIRT1/caveolin-1/eNOS (Fig. 5C).

Furthermore, we found that g-Rb1 significantly decreased the acetylation of caveolin-1 and eNOS, but this effect could be reversed by SIRT1 silencing (Fig. 5D). Acetylated caveolin-1 and eNOS decreased when caveolin-1 was silenced, and g-Rb1 further enhanced this effect (Fig. 5D and E). All our results suggested that g-Rb1 achieved anti-ageing effects by regulating the expression level and acetylation level of the SIRT1/caveolin-1/eNOS pathway.

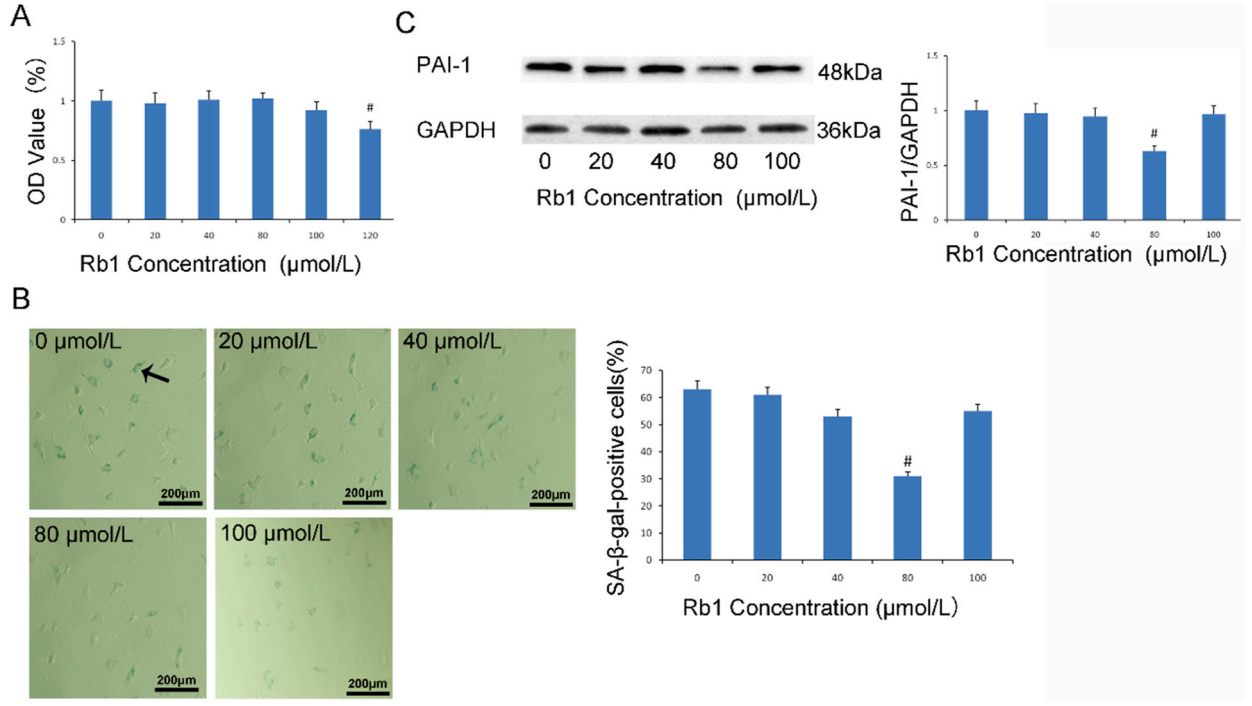


Fig. 3. Rb-1 ginsenosides in anti-senescence of endothelial cells.
A. MTT assay showing the effect of ginsenoside Rb-1 on HUVECs. HUVECs without ginsenoside Rb-1 induction were normalized to 1. Data are shown as the mean ± SD, $\#p < 0.05$ vs. 0 μmol/L, n = 3, OD: optic density. **B.** SSA-*β*-gal staining. Bar = 200 μm. Data are shown as the mean ± SD, $\#p < 0.05$ vs. 0 μmol/L, n = 3. **C.** The expression of PAI-1 was decreased by 80 μmol/L g-Rb1. Data are shown as the mean ± SD, $\#p < 0.05$ vs. 0 μmol/L, n = 3.

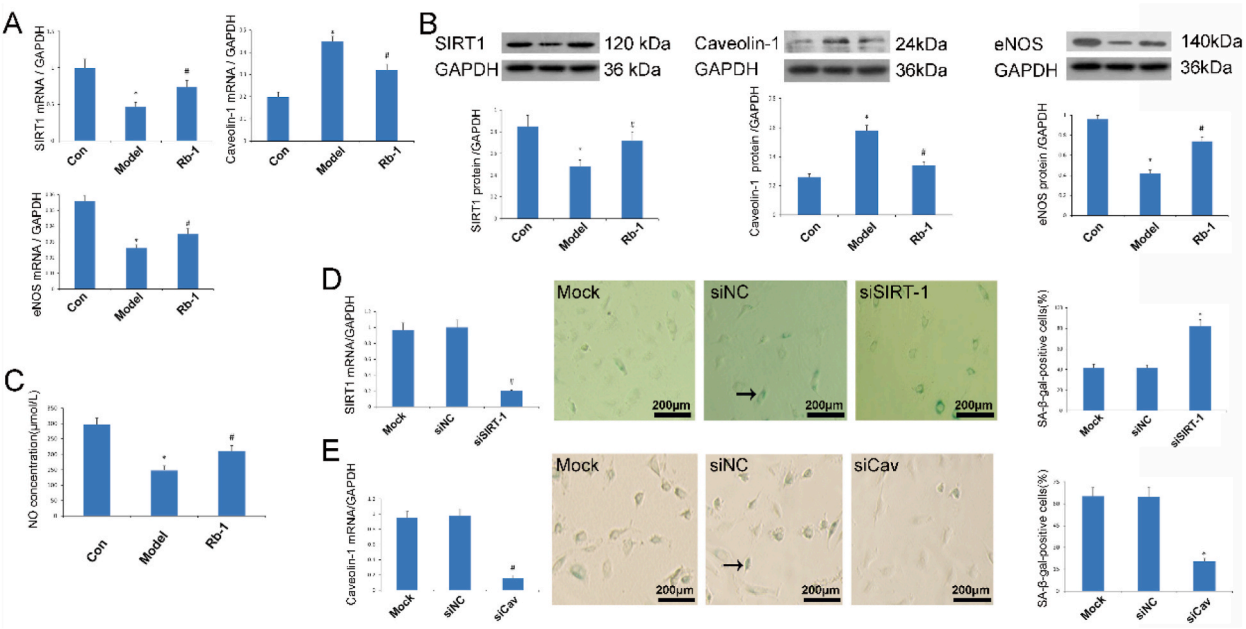


Fig. 4. Rb-1 modulates SIRT1, caveolin-1, and eNOS in the senescent endothelial cell model.
A. mRNA expression of sirtuin 1 (SIRT1), caveolin-1, and eNOS. **B.** Protein expression of SIRT1, caveolin-1, and eNOS. **C.** Nitric oxide (NO) concentration of the cultured medium. Data (A–C) are shown as the mean ± SD, $*p < 0.01$ vs. Control group, $\#p < 0.05$ vs. Model group, n = 3. **D-E.** Downregulation of SIRT1 and caveolin-1 gene expression by siRNA in the senescent endothelial model, and SA-β-gal staining of each group. Scale bar = 200 μm, arrows indicate positively stained cells. Data are shown as the mean ± SD, $\#p < 0.05$ vs. siNC, $*p < 0.05$ vs. siNC, n = 3. siNC, nonsilencing control. Mock, transfection reagents alone.

4. Discussion

Our research provides evidence for the significant effect of g-Rb1 in delaying senescence in HUVECs. In vitro experiments using SA-β-GAL, PAI-1, and NO as markers of cellular senescence demonstrated that g-Rb1 improves the function of senescent cells. Notably, the results suggest that the target of g-Rb1 is associated with the SIRT1/caveolin-1/eNOS axis. The main findings of this study are as follows: (1) SIRT1 delays the replicative senescence of vascular endothelial cells by regulating the expression and acetylation levels of caveolin-1 and eNOS; (2) g-Rb1 alleviates replicative senescence of vascular endothelial cells through the SIRT1/caveolin-1/eNOS axis (Fig. 6).

Ageing endothelial cells have been found in human atherosclerotic plaques [13]. Anti-ageing may represent an innovative therapeutic approach for atherosclerosis [14]. Atherosclerosis is an age-related disease, and replicative senescence is the best cellular model of ageing [15]. Therefore, we chose a primary endothelial cell model of replicative senescence for our experiments. In China, some drugs, such as Compound Danshen Dripping Pills and total Panax notoginsenosides, which contain ginsenosides as one of their main components, have been used to treat coronary atherosclerosis. Previous studies have indicated the protective effects of these drugs in humans [16,17]. Our study confirms that g-Rb1 exerts anti-ageing effects in vitro via the SIRT/caveolin-1/eNOS axis, providing a new theoretical basis for the clinical application of g-Rb1 in preventing and treating atherosclerosis.

SIRT1 is closely associated with ageing and atherosclerosis [18]. Known as a "longevity gene," SIRT1 can delay ageing by alleviating oxidative stress, inhibiting inflammation, and activating autophagy [19,20]. Our previous studies and the current study have confirmed that increased SIRT1 expression protects endothelial cells and delays endothelial senescence. Furthermore, SIRT1 can counteract atherosclerosis. Studies on apoE−/− mice have demonstrated that activating SIRT1 reduces plaque formation in atherosclerosis [3]. Recently, a study on human serum found that SIRT1 is related to the formation and stability of carotid plaques [21]. Therefore, SIRT1 may be a new target for the clinical prevention and treatment of atherosclerosis [14]. Our previous study indicated that g-Rb1's modulation of SIRT1 may be related to AMPK [22]. However, the exact mechanisms require further exploration.

Caveolin-1 is involved in various cellular functions. Our study demonstrates that g-Rb1 significantly downregulates Caveolin-1 expression, confirming the observations reported by Zhang et al. [23]. Our research also found that g-Rb1 can reduce caveolin-1 acetylation levels, thereby delaying endothelial cell senescence, which may be related to the upregulation of SIRT1 expression by g-Rb1. Caveolin-1 is involved in the development of atherosclerosis. It promotes the early occurrence and progression of atherosclerosis by increasing adhesion molecule expression in HUVECs and facilitating LDL transport into cells [24]. Similar to our study, Zhao et al. found that the deacetylation of Caveolin-1 induces autophagy and has anti-atherosclerotic effects [25]. Therefore, inhibiting Caveolin-1 expression may protect endothelial cells and suppress atherosclerosis through various pathways.

eNOS plays a crucial role in maintaining normal vascular function [26,27]. In senescent cells, eNOS activity decreases, resulting in

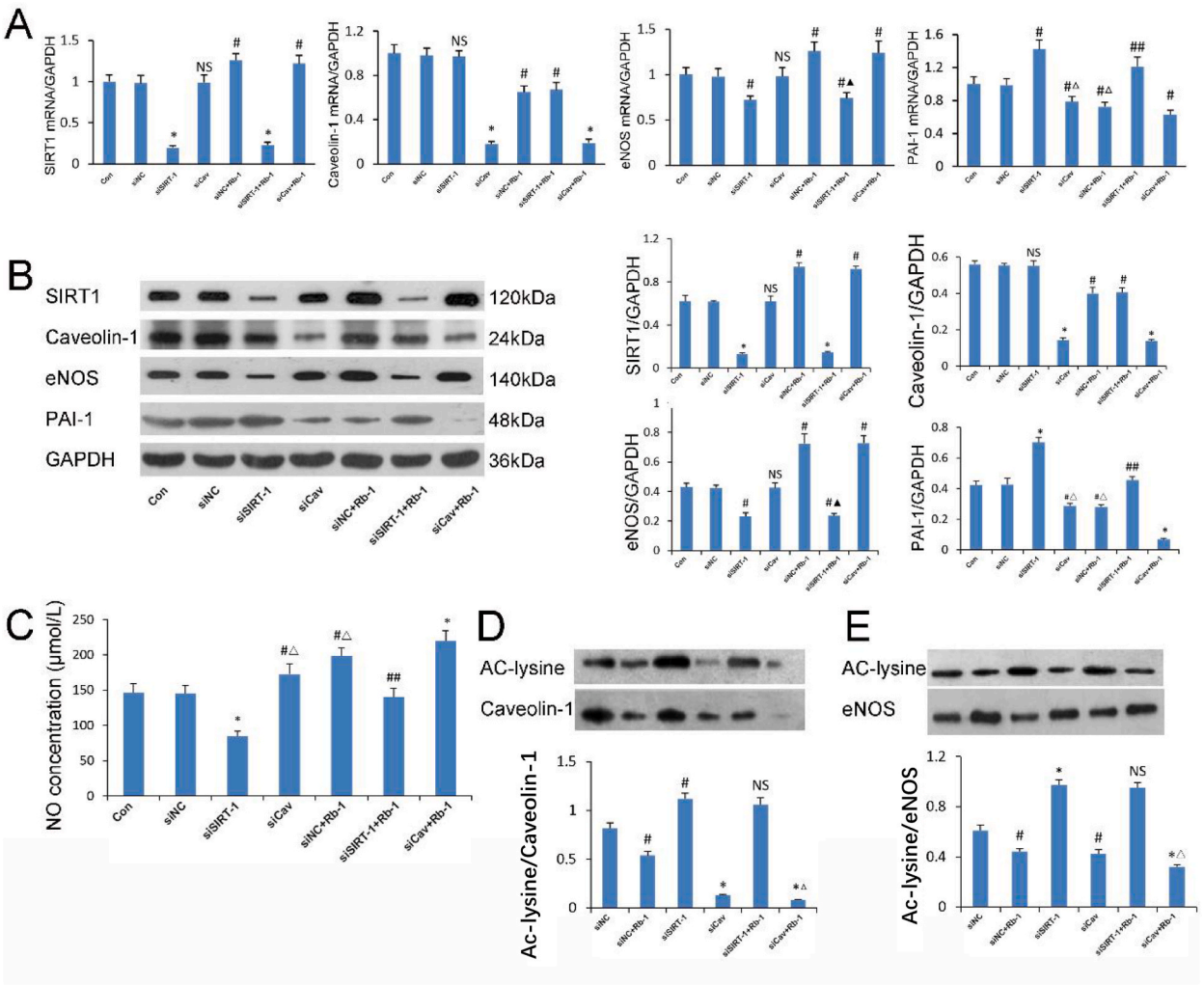


Fig. 5. G-Rb1 regulated eNOS activity through SIRT1/caveolin-1.
A. mRNA levels of SIRT1, caveolin-1, eNOS, and PAI-1 in HUVECs. **B.** Protein levels of SIRT1, caveolin-1, eNos, and PAI-1 in HUVECs. **C.** Nitric oxide (NO) concentration of the cellular supernatant. A-C, Data are shown as the mean ± SD, $*p < 0.01$ vs. control; $\#p < 0.05$ vs. control; $\triangle p <$ 0.05 vs. sicaveolin-1 +g-Rb1; $\#\#p < 0.05$ vs. siSIRT1, n = 3. **D-E.** Acetylation of caveolin-1 and eNOS. $*p < 0.01$ vs. control; $\#p < 0.05$ vs. control; $\triangle p < 0.05$ vs. sicaveolin-1; NS $p > 0.05$ vs. siSIRT1, n = 3. Con, control group; siNC, nonsilencing control.

reduced NO production [28]. Our study also demonstrates that g-Rb1 inhibits replicative senescence of HUVECs by modulating SIRT1/eNOS expression, consistent with our previous research findings [10]. Previous studies have shown that upregulation of eNOS can reduce the secretion of inflammatory factors by vascular endothelial cells and inhibit inflammation and atherosclerosis [27,29]. Activation of eNOS increases NO expression, leading to arterial dilation and reduced vascular damage caused by shear stress [30]. The corresponding counterpart to eNOS is iNOS, which is inducible nitric oxide synthase. iNOS is not commonly expressed, but it can be induced under conditions of cellular damage or inflammation [31]. It is generally believed that increased expression of iNOS leads to excessive synthesis of nitric oxide (NO) and increased oxidative stress, contributing to damage. Additionally, iNOS may be associated with COX-2, which promotes inflammation in atherosclerotic lesions [32]. Further exploration is required to investigate whether gRb1 can inhibit iNOS expression.

In addition, there are several other limitations in our study. First, the direct mechanisms by which g-Rb1 upregulates SIRT1 and eNOS and downregulates Caveolin-1 expression are still unclear. Second, the acetylation sites of caveolin-1 remain unknown. Last, the lack of animal experiments to validate the in vivo effects of g-Rb1 is a limitation. These shortcomings need to be further investigated and addressed.

5. Conclusion

In conclusion, our study demonstrates that g-Rb1 increases NO expression by upregulating SIRT1/caveolin-1/eNOS and reducing

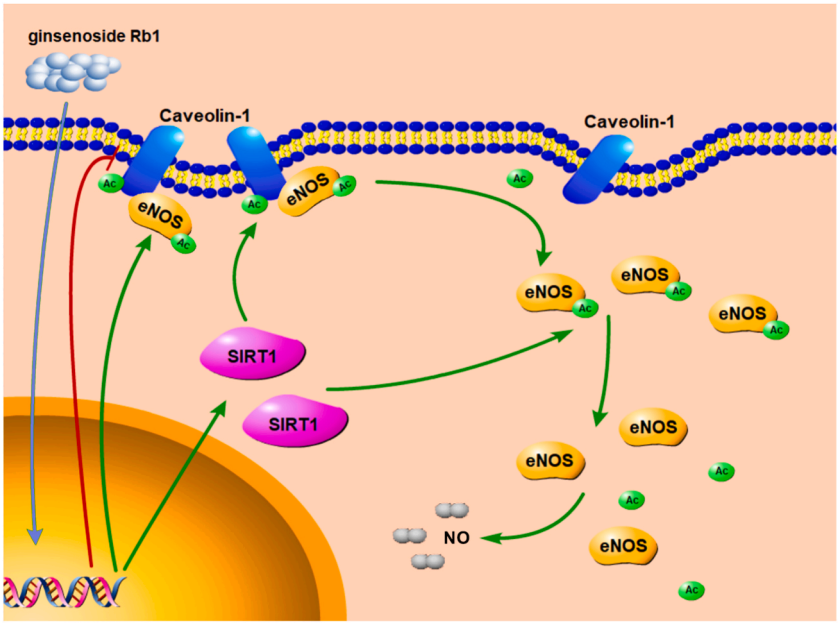


Fig. 6. Regulation of the SIRT1/caveolin-1/eNOS axis by g-Rb1.

caveolin-1 and eNOS acetylation levels, thereby alleviating replicative senescence of HUVECs. For the first time, we have identified that g-Rb1 regulates eNOS activity through modulation of Caveolin-1 expression and acetylation, which may be associated with SIRT1 upregulation. These findings provide a new theoretical basis for the use of g-Rb1 in delaying endothelial cell ageing. However, further experimental research is needed to explore the clinical application of g-Rb1.

6. Ethics declaration

Isolation of HUVECs from donors was approved by the Research Committee of the Third Affiliated Hospital of Sun Yat-sen University (approval no. [2022]02-317-01). The donors have all supplied written consent for the donation of the umbilical cords.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. The data are not publicly available due to privacy or ethical restrictions.

CRediT authorship contribution statement

Bin Zhou: Validation, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Guangyao Sh:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis. **Dongmei Xie:** Writing – review & editing, Writing – original draft, Software, Project administration, Methodology, Formal analysis. **Xiaoli Zhao:** Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Formal analysis. **Baoshun Hao:** Software, Methodology, Formal analysis. **Dinhui Liu:** Resources, Project administration, Methodology. **Min Wang:** Software, Methodology, Formal analysis. **Lin Wu:** Writing – review & editing, Validation, Supervision, Software, Resources, Methodology, Data curation, Conceptualization. **Liangying Lin:** Validation, Supervision, Investigation, Data curation, Conceptualization. **Xiaoxian Qian:** Validation, Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors reported no potential conflict of interest.

Acknowledgments

This work was supported by grants from the Natural Science Foundation of Guangdong Province of China (Grant Number: 2021A1515010599 and 2022A1515012262) and the Scientific Research Fund of Guangdong Provincial Bureau of Traditional Chinese Medicine of China.(Grant Number: 20221084).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24586.

References

[1] L. Zhang, L.E. Pitcher, M.J. Yousefzadeh, L.J. Niedernhofer, P.D. Robbins, Y. Zhu, Cellular senescence: a key therapeutic target in aging and diseases, J. Clin. Invest. 132 (2022).
[2] G. Jia, A.R. Aroor, C. Jia, J.R. Sowers, Endothelial cell senescence in aging-related vascular dysfunction, Biochim. Biophys. Acta, Mol. Basis Dis. 1865 (2019) 1802–1809.
[3] S. Karnewar, S. Pulipaka, S. Katta, D. Panuganti, P.K. Neeli, R. Thennati, M.K. Jerald, S. Kotamraju, Mitochondria-targeted esculetin mitigates atherosclerosis in the setting of aging via the modulation of SIRT1-mediated vascular cell senescence and mitochondrial function in Apoe(-/-) mice, Atherosclerosis 356 (2022) 28–40.
[4] X.X. Liang, R.Y. Wang, Y.Z. Guo, Z. Cheng, D.Y. Lv, M.H. Luo, A. He, S.X. Luo, Y. Xia, Phosphorylation of Akt at Thr308 regulates p-eNOS Ser 1177 during physiological conditions, FEBS Open Bio 11 (2021) 1953–1964.
[5] H. Niu, J. Li, H. Liang, G. Wu, M. Chen, Exogenous hydrogen sulfide activates PI3K/Akt/eNOS pathway to improve replicative senescence in human umbilical vein endothelial cells, Cardiol. Res. Pract. 2023 (2023) 7296874.
[6] S.D.S. Oliveira, R.D. Minshall, Caveolin and endothelial NO signaling, Curr. Top. Membr. 82 (2018) 257–279.
[7] E.E. Powter, P.R. Coleman, M.H. Tran, A.J. Lay, P. Bertolino, R.G. Parton, M.A. Vadas, J.R. Gamble, Caveolae control the anti-inflammatory phenotype of senescent endothelial cells, Aging Cell 14 (2015) 102–111.
[8] Z. Song, Y. Liu, B. Hao, S. Yu, H. Zhang, D. Liu, B. Zhou, L. Wu, M. Wang, Z. Xiong, et al., Ginsenoside Rb1 prevents H2O2-induced HUVEC senescence by stimulating sirtuin-1 pathway, PLoS One 9 (2014) e112699.
[9] G. Shi, D. Liu, B. Zhou, Y. Liu, B. Hao, S. Yu, L. Wu, M. Wang, Z. Song, C. Wu, et al., Ginsenoside Rb1 alleviates oxidative low-density lipoprotein-induced vascular endothelium senescence via the SIRT1/beclin-1/autophagy Axis, J. Cardiovasc. Pharmacol. 75 (2020) 155–167.
[10] D.H. Liu, Y.M. Chen, Y. Liu, B.S. Hao, B. Zhou, L. Wu, M. Wang, L. Chen, W.K. Wu, X.X. Qian, Ginsenoside Rb1 reverses H2O2-induced senescence in human umbilical endothelial cells: involvement of eNOS pathway, J. Cardiovasc. Pharmacol. 59 (2012) 222–230.
[11] A.L. Cheung, Isolation and culture of human umbilical vein endothelial cells (HUVEC), Curr Protoc Microbiol (2007). *Appendix* 4, Appendix 4B.
[12] S. Oh, D.Y. Rhee, S. Batsukh, K.H. Son, K. Byun, High-Intensity Focused Ultrasound Increases Collagen and Elastin Fiber Synthesis by Modulating Caveolin-1 in Aging Skin, 2023. Cells *12*.
[13] T. Minamino, H. Miyauchi, T. Yoshida, Y. Ishida, H. Yoshida, I. Komuro, Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction, Circulation 105 (2002) 1541–1544.
[14] Z. Cui, X. Zhao, F.K. Amevor, X. Du, Y. Wang, D. Li, G. Shu, Y. Tian, X. Zhao, Therapeutic application of quercetin in aging-related diseases: SIRT1 as a potential mechanism, Front. Immunol. 13 (2022) 943321.
[15] K.E. Foreman, J. Tang, Molecular mechanisms of replicative senescence in endothelial cells, Exp. Gerontol. 38 (2003) 1251–1257.
[16] W. Shao-Mei, Y. Li-Fang, W. Li-Hong, Traditional Chinese medicine enhances myocardial metabolism during heart failure, Biomed. Pharmacother. 146 (2022) 112538.
[17] B. He, D. Chen, X. Zhang, R. Yang, Y. Yang, P. Chen, Z. Shen, Oxidative stress and ginsenosides: an update on the molecular mechanisms, Oxid. Med. Cell. Longev. 2022 (2022) 9299574.
[18] M. Kitada, Y. Ogura, D. Koya, The protective role of Sirt1 in vascular tissue: its relationship to vascular aging and atherosclerosis, Aging (Albany NY) 8 (2016) 2290–2307.
[19] S. Ministrini, Y.M. Puspitasari, G. Beer, L. Liberale, F. Montecucco, G.G. Camici, Sirtuin 1 in endothelial dysfunction and cardiovascular aging, Front. Physiol. 12 (2021) 733696.
[20] Y.P. Liu, R. Wen, C.F. Liu, T.N. Zhang, N. Yang, Cellular and molecular biology of sirtuins in cardiovascular disease, Biomed. Pharmacother. 164 (2023) 114931.
[21] J. Chen, J. Li, Y. Wang, Z. Zhang, H. Yang, Z. Li, T. Wang, T. Peng, X. Mo, F. Xu, Serum ANGPTL4 and SIRT1 factor levels and the Carotid Atherosclerotic plaque stability relationship analysis, Cell. Mol. Biol. 69 (2023) 62–66.
[22] Z. Zheng, M. Wang, C. Cheng, D. Liu, L. Wu, J. Zhu, X. Qian, Ginsenoside Rb1 reduces H2O2-induced HUVEC dysfunction by stimulating the sirtuin-1/AMP-activated protein kinase pathway, Mol. Med. Rep. 22 (2020) 247–256.
[23] Y. Zhang, K. Sun, Y.Y. Liu, Y.P. Zhang, B.H. Hu, X. Chang, L. Yan, C.S. Pan, Q. Li, J.Y. Fan, et al., Ginsenoside Rb1 ameliorates lipopolysaccharide-induced albumin leakage from rat mesenteric venules by intervening in both trans- and paracellular pathway, Am. J. Physiol. Gastrointest. Liver Physiol. 306 (2014) G289–G300.
[24] S. Pavlides, J.L. Gutierrez-Pajares, J. Iturrieta, M.P. Lisanti, P.G. Frank, Endothelial caveolin-1 plays a major role in the development of atherosclerosis, Cell Tissue Res. 356 (2014) 147–157.
[25] Y. Zhao, X. Jia, X. Yang, X. Bai, Y. Lu, L. Zhu, W. Cheng, M. Shu, Y. Zhu, X. Du, et al., Deacetylation of Caveolin-1 by Sirt6 induces autophagy and retards high glucose-stimulated LDL transcytosis and atherosclerosis formation, Metabolism 131 (2022) 155162.
[26] H. Li, U. Förstermann, Uncoupling of endothelial NO synthase in atherosclerosis and vascular disease, Curr. Opin. Pharmacol. 13 (2013) 161–167.
[27] F.F. Hong, X.Y. Liang, W. Liu, S. Lv, S.J. He, H.B. Kuang, S.L. Yang, Roles of eNOS in atherosclerosis treatment, Inflamm. Res. 68 (2019) 429–441.
[28] A.M. Pourbagher-Shahri, T. Farkhondeh, M. Talebi, D.M. Kopustinskiene, S. Samarghandian, J. Bernatoniene, An overview of NO signaling pathways in aging, Molecules 26 (2021).
[29] M.X. Duan, H. Zhou, Q.Q. Wu, C. Liu, Y. Xiao, W. Deng, Q.Z. Tang, Andrographolide protects against HG-induced inflammation, apoptosis, migration, and impairment of angiogenesis via PI3K/AKT-eNOS signalling in HUVECs, Mediat. Inflamm. 2019 (2019) 6168340.
[30] M. Siragusa, A.F. Oliveira Justo, P.F. Malacarne, A. Strano, A. Buch, B. Withers, K.G. Peters, I. Fleming, VE-PTP inhibition elicits eNOS phosphorylation to blunt endothelial dysfunction and hypertension in diabetes, Cardiovasc. Res. 117 (2021) 1546–1556.
[31] S. Anavi, O. Tirosh, iNOS as a metabolic enzyme under stress conditions, Free Radic. Biol. Med. 146 (2020) 16–35.
[32] M. Lind, A. Hayes, M. Caprnda, D. Petrovic, L. Rodrigo, P. Kruzliak, A. Zulli, Inducible nitric oxide synthase: good or bad? Biomed. Pharmacother. 93 (2017) 370–375.