

Resveratrol attenuates hydrogen peroxide-induced aging through upregulation of autophagy in human umbilical vein endothelial cells

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Purpose: Resveratrol (RESV; trans-3,5,4'-trihydroxystilbene) has emerged as a potential new therapeutic for age-related atherosclerotic diseases. However, the effect of RESV on cellular aging and its underlying mechanisms remain unknown. Therefore, the aim of this study was to examine whether RESV can delay cellular aging through upregulation of autophagy.

Materials and methods: Human umbilical endothelial vein cells (HUVECs) were divided into four groups: the control group, and the hydrogen peroxide (H₂O₂) alone, H₂O₂ + RESV pretreatment, and H₂O₂ + 3-methyladenine (3-MA) + RESV pretreatment intervention groups. The cell viability was evaluated by a cell counting kit-8 assay. Superoxide dismutase (SOD) activity and intracellular reactive oxygen species (ROS) levels were tested using commercial kits. Senescence-related β-galactosidase activities were detected by immunohistochemical staining. The expression levels of aging-related and autophagy-related markers, including phosphorylated Rb (p-Rb), LC3, and p62, with or without RESV were measured by Western blotting.

Results: Pretreatment with 10 μM RESV increased the cell viability and SOD levels. The remarkably higher positive rate of senescence-associated β-galactosidase and increased intracellular ROS levels in the H₂O₂ treatment group were reversed by treatment with 10 μM RESV. As compared to the H₂O₂ treatment group, 10 μM RESV could upregulate autophagy through the regulation of p-Rb, LC3, and p62 levels. The anti-aging effect of RESV via an autophagy regulation mechanism was further confirmed by the suppression of these effects with 3-MA treatment.

Conclusion: RESV may reverse and delay the aging process of HUVECs via upregulation of autophagy and could be a candidate therapeutic for age-related atherosclerotic diseases.

Keywords: oxidative stress, senescence, LC3, p62, p-Rb

Introduction

The incidence of cardiovascular diseases, which is likely to be exacerbated by an aging population, is increasing worldwide. Epidemiology data have shown that aging is an independent risk factor of cardiovascular diseases, in addition to other traditional risk factors such as diabetes, hypertension, hyperlipidemia, and hyperuricemia.¹⁻⁴ Thus, controlling the risk factors of aging would help to reduce the incidence of cardiovascular disease. Accordingly, it is of great importance to understand the pathophysiological process of age-related cardiovascular disease for identifying a suitable therapeutic intervention to delay aging.

Oxidative stress participates in the pathological process of cardiac aging by producing excessive amounts of oxygen free radicals, which destroy mitochondrial DNA,

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alter mitochondrial function, trigger cell senescence, and activate apoptotic signals.⁵⁻⁸ Our previous study demonstrated that dehydroepiandrosterone reduced the expression level of endothelial nitric oxide synthase and weakened the function of oxidation resistance in rat arteries,⁹ indicating that drug intervention can reduce oxidative stress injury. More recently, we reported that estrogen can resist oxidative stress, protect endothelial function, and delay cellular aging.¹⁰ Several studies have also suggested the cardioprotective effects of estrogen, and its potential for the treatment of coronary heart disease.^{11,12} However, the long-term use of estrogen might pose serious health risks, including increasing the risk of cancer or thromboembolic disease,^{13,14} thereby limiting its clinical application.

Resveratrol (RESV) is a natural phytoestrogen that exhibits estrogen-like effects but with fewer adverse effects. In particular, RESV can promote cell proliferation through estrogen receptor and extracellular signal-regulated kinase activation.¹⁵ Moreover, RESV-mediated neuroprotective effects may be mediated by estrogen receptor.¹⁶ Our previous study showed that estrogen can delay the senescence of H₂O₂-induced endothelial cells, and more recent studies support that RESV has an estrogenic effect. Therefore, we hypothesized that RESV may inhibit oxidative stress, improve endothelial function, and delay aging.

Although the protective effects of RESV on the cardiovascular system have been revealed,^{17,18} the underlying mechanisms are not clear. Autophagy is an evolutionary conserved self-digesting process, which can degrade retired proteins and organelles to regenerate energy and nutrition.^{19,20} Moreover, RESV has been found to protect against neurologic diseases and tumors via regulating autophagy.²¹⁻²⁴ However, few studies have focused on the role of RESV-induced autophagy in age-related cardiovascular disease. As reported, RESV has been suggested to be an autophagy inducer. RESV was shown to induce basal autophagy and demonstrated a protective effect in atherosclerosis via an autophagic pathway. In 2010, Kao et al reported that RESV protects human endothelium from H₂O₂-induced oxidative stress and senescence via SirT1 activation.²⁵ Moreover, in 2013, Chen et al revealed the underlying signaling pathway through which RESV induces autophagy in human umbilical endothelial vein cells (HUVECs) under inflammation via the cAMP signaling pathway.²⁶ In our study, we established an H₂O₂-induced cellular aging model to examine whether RESV can delay the senescence of HUVECs via autophagy in a new degree. Rb is a crucial regulator of cell cycle progression, which can be

modulated by posttranslational modifications such as phosphorylation. In senescent cells, the level of p-Rb is reduced due to the inactivation of upstream kinases. Thus, the age-related Rb protein and its phosphorylated levels, along with the expression of key autophagy-related proteins LC3 and p62, were detected to examine the effect of RESV intervention on autophagy-related cellular aging. These results could provide a theoretical foundation for the potential of RESV as targeted therapy for aging-related atherosclerotic diseases.

Materials and methods

Cell culture and drug treatment

The HUVECs (ScienCell, San Diego, CA, USA) were cultured in endothelial cell culture medium (ECM; ScienCell) supplemented with 5% FBS (ScienCell), 1% endothelial cell growth supplement (ScienCell), and 1% penicillin/streptomycin solution (ScienCell), followed by incubation at 37°C in a humidified 5% CO₂ incubator. For all experiments, HUVECs were used between the third and fifth passages. The cells were cultured for 24 hours in the presence and absence of the potential autophagy agonist RESV (Sigma-Aldrich Co., St Louis, MO, USA) or 5 mM of the autophagy inhibitor 3-methyladenine (3-MA) (Sigma) prior to H₂O₂ intervention.

First, the HUVECs were incubated for 24 hours in ECM containing different concentrations of H₂O₂ (25–200 μM) and then the optimized concentration (100 μM) was selected to build a cellular aging model according to the references and our previous data. Second, the cells were incubated with 5, 10, or 50 μM of RESV in the presence of 100 μM H₂O₂ for 24 hours to determine the effect of RESV on H₂O₂-induced cellular senescence. Finally, to examine the effects of RESV on cellular aging, HUVECs were divided into the following four groups: 1) control group: the cells were cultured in ECM only; 2) H₂O₂ intervention group: the cells were exposed to 100 μM H₂O₂ in ECM for 24 hours; 3) RESV intervention group (H₂O₂ + RESV): cells were treated with 10 μM RESV 1 hour before stimulation with H₂O₂; and 4) 3-MA intervention group (H₂O₂ + RESV + 3-MA): cells were treated with 5 mM 3-MA 1 hour before the 10 μM RESV intervention and subsequent H₂O₂ stimulation. Five parallel controls were set up for each group.

Cell viability assays

HUVECs with density of 1×10⁴ cells per well were seeded in 96-well plates and exposed to different concentrations of H₂O₂ in the absence and presence of RESV. The cells were then incubated with 5% CO₂ at 37°C for 24 hours. Subsequently, 10 μL of cell counting kit-8 reagent

(Beyotime, Jiangsu, China) and 100 μL of fresh ECM were added to each well after changing the medium, followed by incubation at 37°C, 5% CO_2 for 2 hours. The cell viability was measured at 450 nm on a microplate reader.

Measurement of SOD levels

HUVECs were seeded in 24-well plates at a density of 1×10^5 cells per well in ECM and incubated for 24 hours. The measurement of superoxide dismutase (SOD) levels was performed using a kit from Nanjing Jianchen (Jiangsu, China) according to the manufacturer's protocol.

Measurement of intracellular ROS levels

Intracellular reactive oxygen species (ROS) accumulation was measured using an ROS assay kit (Nanjing Jianchen) according to the manufacturer's protocol. Cultured cells were incubated with 100 μM H_2O_2 for 24 hours in the absence and presence of 10 μM RESV. Treated cells were then washed three times with appropriate ECM to remove extracellular fluorescent substances. The cells were then incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) at 37°C for 20 minutes; the DCF fluorescence of treated cells was detected by a fluorescence microscope directly.

SA- β -galactosidase (SA- β -gal) staining

The cells were fixed by incubating in 4% paraformaldehyde and 0.2% glutaraldehyde at 4°C for 5 minutes and were then stained overnight with freshly prepared senescence-associated SA- β -gal (1 mg/mL X-gal, 40 mM citric acid/sodium phosphate [pH 6.0], 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl_2) at 37°C. The next day, the stained cells were quantified under an optical microscope.

Western blot analysis

The cells were lysed in RIPA lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mM ethylenediaminetetraacetic acid. Total protein content in the supernatant was measured using a bicinchoninic acid protein assay kit (Beyotime). Samples of cell lysate supernatant (50 μg protein) were resolved by SDS-PAGE and electrotransferred onto a polyvinylidene fluoride membrane (Millipore, Burlington, MA, USA) and then blocked with 5% skim milk for 1 hour, followed by overnight incubation with specific antibodies against LC3 (catalog no. 3868), p62 (catalog no. 8025), p-Rb (catalog no. 8516), Rb (catalog no. 9313) and

β -actin (catalog no. 4970) (1:1,000 dilution; all from Cell Signaling Technology, Danvers, MA, USA) at 4°C. The following day, the membranes were washed three times for 30 minutes each and then incubated with the secondary antibody horseradish peroxidase-conjugated anti-IgG for 1 hour. After washing with buffer, the membranes were incubated with enhanced chemiluminescence reagents (Applygen, Beijing, China) to visualize the protein signals. Quantification of band intensity was carried out using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The band intensity of β -actin was used as a control.

Statistical analyses

Data for three independent experiments are expressed as the mean \pm SD. Multiple comparisons between groups were performed using one-way ANOVA followed by the least-significant difference test. Differences between the treatment groups were analyzed by a two-tailed Student's *t*-test. *P*-values of <0.05 were considered statistically significant.

Results

RESV improved cell viability

Figure 1A shows the morphology of HUVECs incubated in ECM with 5% CO_2 at 37°C. H_2O_2 markedly inhibited cell viability (Figure 1B) in a dose-dependent manner, with a significant difference in the degree of reduction in viability between the 100 μM H_2O_2 -treated group and 200 μM H_2O_2 -treated group compared to the control group. This suggested that a high concentration (200 μM) of H_2O_2 may induce cell growth arrest, leading to cell apoptosis or necrosis; therefore, treatment of 100 μM of H_2O_2 for 24 hours was selected for subsequent experiments.

Moreover, pretreatment with 10 μM of RESV significantly increased cell viability compared to that in the H_2O_2 group (Figure 1C). However, pretreatment with 50 μM of RESV did not significantly increase cell viability compared to that in the H_2O_2 group (Figure 1C). Therefore, 10 μM was selected as the concentration of RESV treatment for subsequent experiments.

RESV increased SOD levels

As shown in Figure 1D, H_2O_2 significantly decreased the SOD concentration compared to that of the control group, and the addition of 10 μM RESV reversed this decrease in H_2O_2 -induced SOD levels. However, there were no statistically

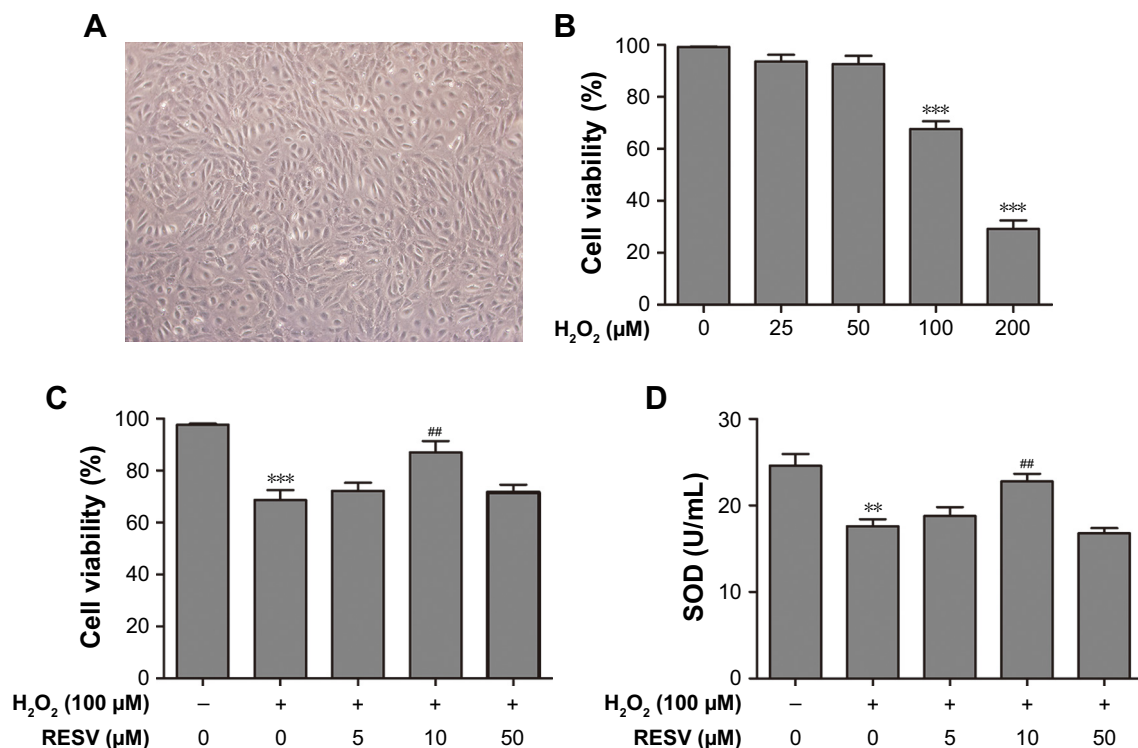


Figure 1 Effects of RESV on cell viability and SOD levels in HUVECs treated with H₂O₂. **Notes:** (A) Morphology of HUVECs under normal culture condition (100×). (B) Effect of different concentrations of H₂O₂ on HUVECs viability. (C) Effects of different concentrations of RESV on HUVECs viability in the presence of 100 μM H₂O₂. (D) SOD level of HUVECs treated with different concentrations of RESV and in the presence of 100 μM H₂O₂. N=5. ****P*<0.01, *****P*<0.001 vs the control group; ***P*<0.01 vs the H₂O₂ group. **Abbreviations:** HUVECs, human umbilical endothelial vein cells; RESV, resveratrol; SOD, superoxide dismutase.

significant differences between the 5 and 50 μM RESV groups as compared with the H₂O₂ group.

RESV suppressed the accumulation of H₂O₂-induced SA-β-gal

The SA-β-gal staining assay was used to detect the level senescence in HUVECs induced by H₂O₂. As illustrated in Figure 2, there was a significant difference in the positive staining rates of cells among the four groups (*P*<0.001). Compared to the control group, the positive rate of cells in the H₂O₂ group increased significantly (*P*<0.001), and this increase was reversed by pretreatment with RESV (*P*<0.001). However, pretreatment with the autophagy antagonist 3-MA partially blocked this anti-aging effect of RESV (*P*<0.001).

RESV suppressed H₂O₂-induced ROS generation

The ROS levels significantly differed among the four groups (*P*<0.001, Figure 3A and B). The intracellular ROS levels in the cells treated with H₂O₂ were increased significantly as compared to those of the control group (*P*<0.001),

and decreased levels of ROS were detected in the RESV intervention group compared to those of the H₂O₂ group (*P*<0.001). However, pretreatment with 3-MA significantly increased the ROS levels compared to those of the RESV intervention group (*P*<0.001).

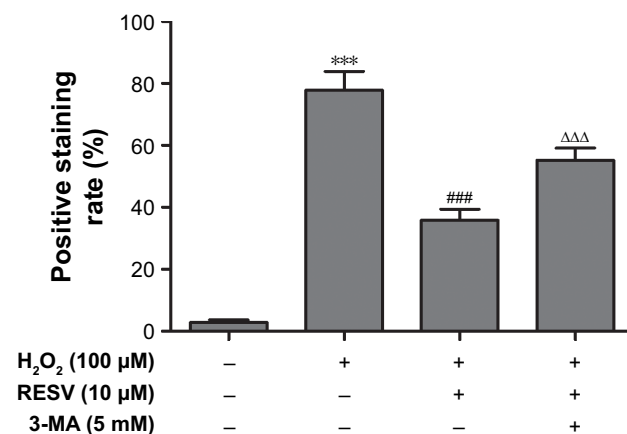


Figure 2 Percentage of SA-β galactosidase-positive HUVECs cells treated with H₂O₂, RESV, and 3-MA. N=5. **Notes:** ****P*<0.001 vs control group; ###*P*<0.001 vs H₂O₂ group; ΔΔΔ*P*<0.001 vs RESV group. **Abbreviations:** HUVECs, human umbilical endothelial vein cells; 3-MA, 3-methyladenine; RESV, resveratrol.

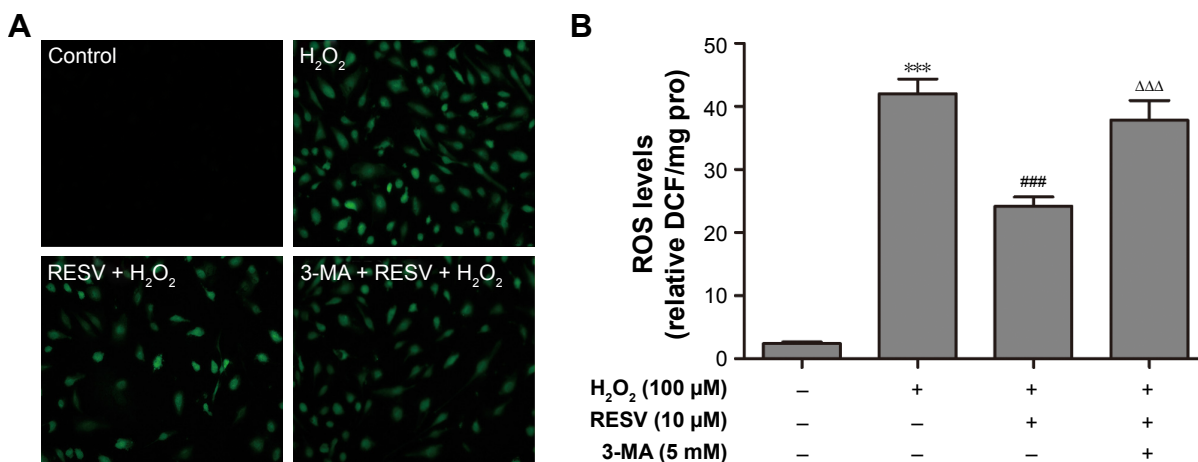


Figure 3 Detections of ROS in various groups.

Notes: (A) Detection of ROS in various groups by DCFH-DA probes and representative images taken under a fluorescence microscope (100×). The green fluorescence intensity reflects the presence of ROS. (B) Comparison of ROS levels across different treatment groups. N=5. *** $P < 0.001$ vs control group; ### $P < 0.001$ vs H₂O₂ group; ΔΔΔ $P < 0.001$ vs RESV group.

Abbreviations: DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; 3-MA, 3-methyladenine; RESV, resveratrol; ROS, reactive oxygen species.

The protective effect of RESV was associated with autophagy regulation

As shown in Figure 4, the levels of p-Rb decreased as the H₂O₂ concentrations increased from 25 to 200 μM ($P < 0.05$) compared to those of the control group.

The autophagy indexes of the LC3 II/LC3 I ratio and p62 expression were measured by Western blotting, demonstrating that the protective effect of RESV was largely dependent on the autophagy progress: the LC3 II/LC3 I protein ratio increased significantly, while the expression level of p62 decreased significantly in the 10 μM RESV treatment group compared to the H₂O₂ group ($P < 0.05$, Figure 5A and B). However, there were no significant differences in p62 expression levels between the 5 and 50 μM RESV groups and the H₂O₂ group (Figure 5A and B). These results suggested that the progress of autophagic degradation of the substrates was blocked, and the ability of autophagy was limited in the presence of 5 and 50 μM RESV (Figure 5A and B).

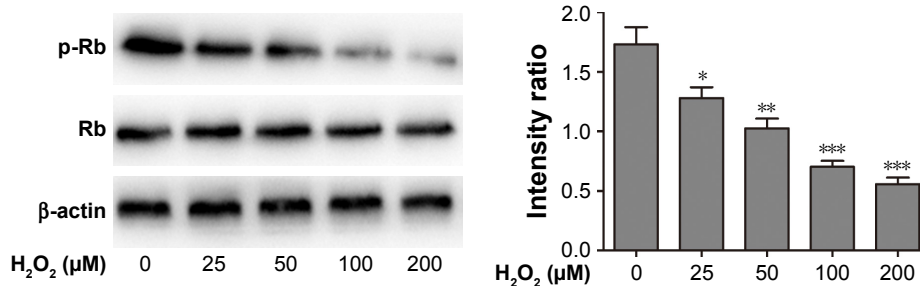


Figure 4 Expression levels of p-Rb in response to different concentrations of H₂O₂.

Notes: A total of 50 μg lysates collected from cells treated with or without H₂O₂ were separated on 6% SDS-PAGE. Quantification of band intensity was carried out using ImageJ software. According to the results, treatment of 100 μM of H₂O₂ for 24 hours was selected for subsequent experiments. N=3. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs the control group.

Moreover, the concentrations of p-Rb and the LC3 II/LC3 I ratio decreased ($P < 0.05$, Figure 6A and B), while the expression of p62 increased in the H₂O₂ group compared to those of the control group ($P < 0.05$, Figure 6A and B). These results indicated that HUVECs senescence was associated with a decrease in autophagy to accelerate aging. In contrast, after combined H₂O₂ and RESV treatment, there was a significant increase in the LC3 II/LC3 I protein ratio and p-Rb levels and a reduction in p62 expression levels ($P < 0.05$) as compared to those in the H₂O₂ treatment-only group. These results indicated that RESV (10 μM) could upregulate autophagy and delay aging in H₂O₂-induced HUVECs.

3-MA suppressed the anti-aging effect of RESV by inhibiting autophagy

To further confirm the relationship between the anti-aging effect of RESV and autophagy, the cells were treated with the autophagy inhibitor 3-MA along with RESV.

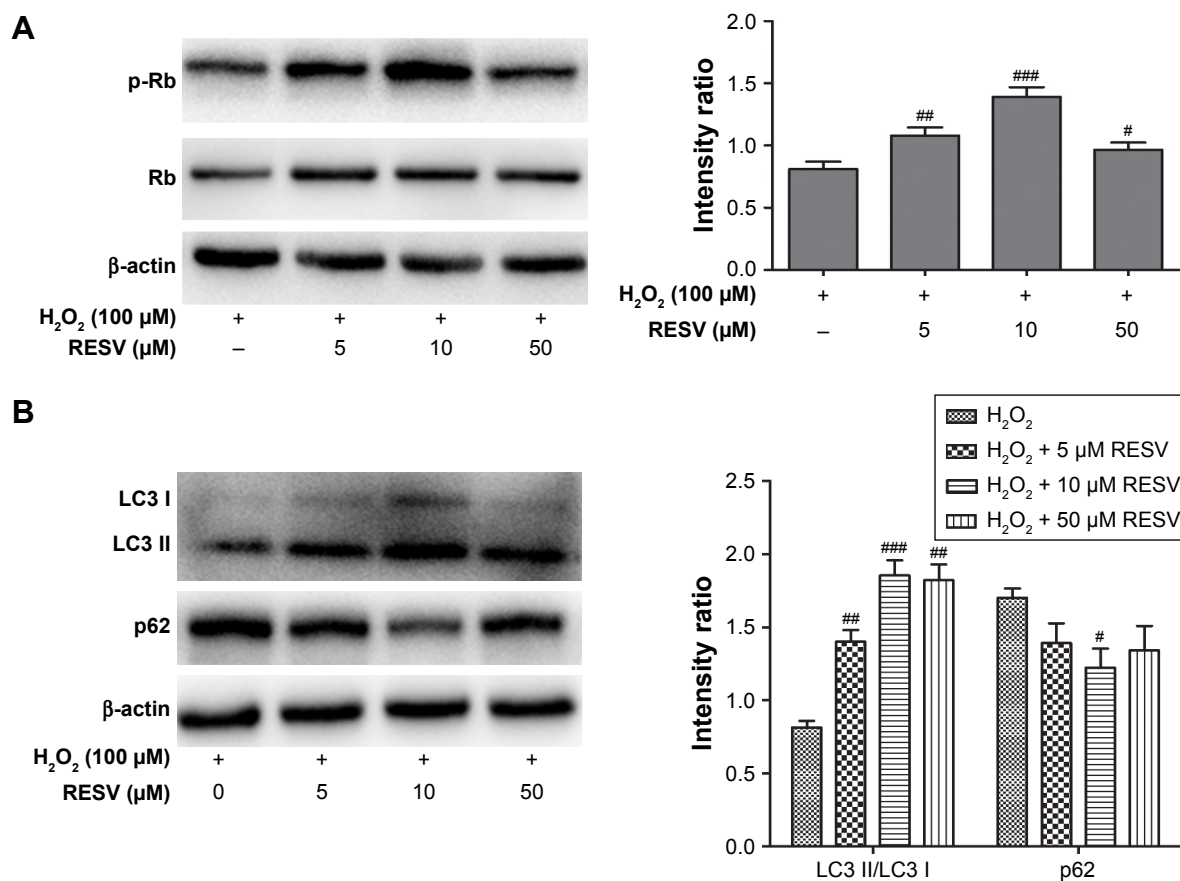


Figure 5 Anti-aging effect and autophagy-related markers of H₂O₂-induced HUVECs treated with different concentrations of RESV.

Notes: (A) Expression levels of p-Rb and Rb in HUVECs treated with different concentrations of RESV and in the presence of 100 μM H₂O₂. **P*<0.05, ****P*<0.001 vs H₂O₂ group (N=3). (B) LC3 II/LC3 I ratios and expression levels of p62 in HUVECs treated with different concentrations of RESV and in the presence of 100 μM H₂O₂. **P*<0.05, ***P*<0.01, ****P*<0.001 vs H₂O₂ group (N=3).

Abbreviations: HUVECs, human umbilical endothelial vein cells; LC3, microtubule light chain 3; RESV, resveratrol.

After pretreatment with 3-MA, the levels of p-Rb and LC3 proteins decreased (Figure 6A and B), while the expression level of p62 protein increased as compared to those of the RESV treatment group (Figure 6B). These results further suggested that RESV may delay the senescence of HUVECs via the autophagy pathway.

Discussion

Oxidative damage to cells is considered to be one of the major causes of aging.^{27,28} Oxidative stress can be caused by endogenous ROS overproduction and impairment of mitochondrial homeostasis. Such excessive accumulation of abnormal substances may cause lipid peroxidation and destruction of the cell membrane, endoplasmic reticulum, and mitochondrial membrane,^{29,30} resulting in cell cycle stagnation if the cell repair mechanism is impaired. Persistent and severe oxidative stress can also initiate apoptotic signaling by releasing pro-apoptotic proteins, thereby activating the downstream caspase family proteins through a series of cascade

amplification reactions, and eventually leading to cell death.³¹ Our previous study showed that endothelial cells can induce cellular senescence after stimulation with H₂O₂.¹⁰ The present study further demonstrated that the intensity of oxidative stress is associated with the positive rate of aging, and H₂O₂-induced oxidative stress affected HUVECs senescence in a concentration-dependent manner. In addition, we found that induction of autophagy was closely related to the concentrations of RESV. When cells were in the aging state, ROS levels increased (Figure 3A and B), the positive rate of senescent cells increased (Figure 2), and the level of the aging-related protein p-Rb decreased gradually (Figure 4), which suggested that excessive oxidative stress can trigger cellular senescence and accelerate cell death. Moreover, the level of the antioxidant protein SOD decreased during oxidative stress (Figure 1D), suggesting an imbalance between oxidation and anti-oxidation during the aging process. Our study also revealed that 50 μM of RESV did not significantly improve cell viability and SOD levels after H₂O₂ treatment.

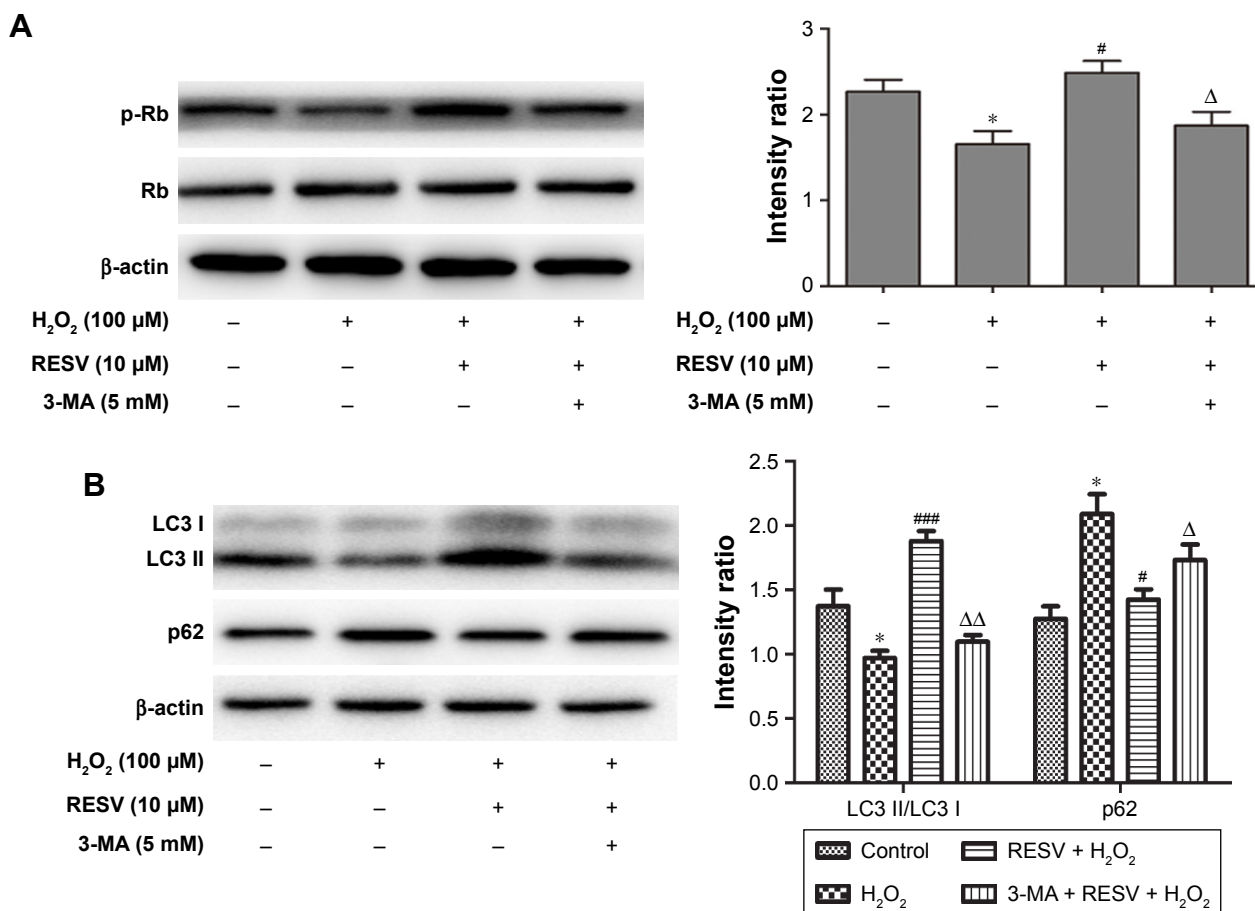


Figure 6 Effect of 3-MA on the anti-aging effect of RESV by inhibiting autophagy.

Notes: (A) Expression levels of p-Rb and Rb in different treatment groups. (B) LC3 II/LC3 I ratios and expression levels of p62 proteins in different treatment groups. N=3. * $P < 0.05$ vs control group; # $P < 0.05$, ### $P < 0.001$ vs H₂O₂ group; ^Δ $P < 0.05$, ^{ΔΔ} $P < 0.01$ vs RESV group.

Abbreviations: LC3, microtubule light chain 3; 3-MA, 3-methyladenine; RESV, resveratrol.

We speculate that the antioxidant capacity of RESV may be related to cell concentration and that excessive RESV may inhibit cell growth. In a healthy physiological state, the production and elimination of oxygen free radicals in the human body are dynamically regulated to achieve a balance. Antioxidant enzymes can catalyze the decomposition of free radicals and thus maintain a constant internal environment in the body.³² However, when the production of ROS exceeds the capacity of the antioxidant defense systems, the aging process can be accelerated. We here demonstrated that cell senescence decreased the autophagy ability, while RESV treatment could delay senescence by regulating the autophagy pathway. Thus, RESV shows potential as an effective therapeutic option to prevent oxidative injury to aging cells and maintain endothelial cell survival.

Our previous study confirmed that estrogen protects endothelial cells and delays cellular senescence, and subsequent studies confirmed the role of estrogen in protecting cardiac function.^{33,34} However, the long-term usage of estrogen can

lead to serious side effects such as increasing the risk of stroke, breast cancer, ovarian cancer, and others.^{13,14,35} Therefore, the clinical application of estrogen is limited, pointing to the potential for RESV as a new phytoestrogen with estrogenic effects but without the side effects and risks.^{36,37}

Although the underlying mechanisms of RESV in delaying the senescence of HUVECs are currently not clear, our results suggest that RESV can upregulate autophagy, reduce oxidative stress, and delay the aging of cells. Autophagy can remove misfolded or aggregated proteins, eliminate intracellular pathogens, and degrade damaged organelles, including the mitochondria, endoplasmic reticulum, and peroxisomes. Some studies have also suggested that RESV can protect neurological functions and inhibit tumor development through induction of autophagy.^{38,39} To further confirm the anti-aging effect of RESV, we observed the changes in autophagy-related markers and cellular senescence between the RESV and 3-MA intervention groups. The autophagy effect of RESV was blocked by 3-MA treatment,

which further established that RESV may play an important role in anti-aging treatment through the autophagy pathway. The mechanism may be that oxidative stress causes damage to cells by inducing endothelial cell barrier dysfunction, thereby blocking the autophagic pathway so that abnormal generation of ROS exceeds the body's ability to metabolize them to ultimately accelerate the aging process. By reducing the ROS levels, RESV could decrease DNA damage accumulation by removal of damaged mitochondria and toxic free radicals.^{40–42} Therefore, RESV can inhibit oxidative stress, promote autophagy, improve vascular endothelial function, maintain cellular homeostasis of the internal and external environment, and eventually delay the aging process.

The aging of endothelial cells induces vascular senescence and changes the molecular biology of cells. Vascular aging is one of the risk factors for atherosclerosis, which can increase arterial stiffness and weaken elasticity, leading to endothelial dysfunction and the occurrence of atherosclerotic disease.^{43,44} RESV, through promoting the autophagy of aging cells, can degrade harmful substances such as damaged proteins and organelles, delay the pathological progress of aging, and prevent the occurrence of aging cardiovascular diseases. These findings provide new insights into the prevention and treatment of aging-related diseases, indicating that RESV has good application prospect and suggesting that it will be a novel lead compound for the treatment of aging-related atherosclerotic diseases.

The current understanding of RESV-mediated autophagy is still in its preliminary stage, and there are some limitations of our study. First, we only examined the effect of RESV on aged HUVECs at the protein level. Second, further studies on the specific autophagic pathways triggered by RESV are needed. More experimental data are required to support the use of RESV in the treatment of aging-related atherosclerotic diseases.

Conclusion

Oxidative stress plays an important role in the cellular aging progress, whereas RESV could delay aging through upregulating autophagic pathways in HUVECs. These findings support the potential of RESV for the prevention and treatment of age-related arteriosclerotic diseases.

Acknowledgments

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Disclosure

The authors report no conflict of interest in this work.

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