Original Research

Resveratrol alleviates alveolar epithelial cell injury induced by hyperoxia by reducing apoptosis and mitochondrial dysfunction

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Impact statement

With the progression of medical treatment in premature infants, the incidence of BPD has not decreased; thus, it is important to find new treatment and prevention strategies for BPD. This study aimed to provide experimental evidence for the potential application of resveratrol to protect the lungs during hyperoxia. Our study revealed that resveratrol may alleviate hyperoxia-induced mitochondrial dysfunction and apoptosis in alveolar epithelial cells through the SIRT1/PGC-1a signaling pathway. Resveratrol-induced SIRT1 upregulation is involved in lung protection. This study revealed the possible mechanism of resveratrol's lung protection in hyperoxia and provides a theory for the future application of resveratrol in the clinic.

Abstract

Bronchopulmonary dysplasia is a severe and long-term pulmonary disease in premature infants. Hyperoxia-induced acute lung injury plays a critical role in bronchopulmonary dysplasia. Resveratrol is a polyphenolic phytoalexin and a natural agonist of Sirtuin 1. Many studies have shown that resveratrol has a protective effect on hyperoxia-induced lung damage, but its specific protective mechanism is still not clear. Further exploration of the possible protective mechanism of resveratrol was the main goal of this study. In this study, human alveolar epithelial cells were used to establish a hyperoxia-induced acute lung injury cell model, and resveratrol (Res or R), the Sirtuin 1 activator SRT1720 (S) and the Sirtuin 1 inhibitor EX-527 (E) were administered to alveolar epithelial cells, which were then exposed to hyperoxia to investigate the role of Res in mitochondrial function and apoptosis. We divided human alveolar epithelial cells into the following groups: (1) the control group, (2) hyperoxia group, (3) hyperoxia+Res20 group, (4) hyperoxia+Res20+E5 group, (5) hyperoxia+Res20+E10 group, (6) hyperoxia+S2 group, (7) hyperoxia+S2+E5 group, and (8) hyperoxia+S2+E10 group. Hyperoxia-induced cell apoptosis and mitochondrial dysfunction were alleviated by Res and SRT1720. Res and SRT1720 upregulated Sirtuin 1, PGC-1α, NRF1, and TFAM but decreased the expression of acetyl-p53 in human alveolar epithelial cells that were exposed to hyperoxia. These findings revealed that Res may alleviated hyperoxia-induced mitochondrial dysfunction and apoptosis in alveolar epithelial cells through the SIRT1/PGC-1a signaling pathway. Thus, Sirtuin 1 upregulation plays an important role in lung protection.

Keywords: Bronchopulmonary dysplasia, resveratrol, SRT1720, EX-527, apoptosis, mitochondrial dysfunction

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Introduction

Oxygen therapy is widely used for hypoxic conditions for various reasons, especially in neonates. Hyperoxia can cause a variety of side effects,[1–4] and the lung is usually the first organ to be damaged. Bronchopulmonary dysplasia (BPD) is a severe and long-term pulmonary disease in premature infants that affects as many as 25% of extreme preterm newborn infants.[5] Long-term and high-concentration oxygen exposure is recognized as the main pathogenic factor in the occurrence of BPD.[6] However, with the advancement of medical treatment in preterm infants, the survival rate of preterm infants has greatly improved, and the prevalence of BPD has not decreased; therefore, it is important to find new treatment and prevention strategies for BPD.

Sirtuin 1 (SIRT1), a NAD^+-dependent protein deacetylase, modulates many essential metabolic processes, including apoptosis, inflammation, oxidative stress, aging, and mitochondrial regulation, via deacetylation of substrate proteins, such as p53, nuclear factor-κB (NF-κB), STAT3, and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α).[7–11] Deacetylation of

SIRT1 may affect the transactivation function of p53, thereby reducing cell cycle arrest and apoptosis.[12] The expression of acetyl-p53 (Ac-p53) increases in hyperoxia.[13] SIRT1 also regulates mitochondrial function and biosynthesis by interacting with PGC1α.[14,15] Resveratrol (3,5,4′-trihydroxy-trans-stilbene, Res, or R) is a polyphenolic compound and is reported to have a wide range of biological activities, including anti-inflammation, antioxidation, anti-apoptosis, and anti-fibrosis.[16,17] Many studies have reported that in respiratory diseases, Res exerts its protective effect by regulating SIRT1, reactive oxygen species (ROS) production and apoptosis.[13,18] SRT1720 (S) is a selective SIRT1 activating agent.[19,20] EX-527 (SEN0014196, E) is an effective, selective SIRT1 inhibitor that effectively inhibits SIRT1 deacetylase activity and thus restores global acetylation levels.[21] Res and SRT1720 can stimulate PGC-1α to remodel the metabolism, especially in skeletal muscle.[22–24]

Mitochondria provide energy for cell survival and function, which are very important in all cell types. In addition to participating in energy metabolism, increasing evidence shows that mitochondria are also involved in nonenergy metabolism, including lipid synthesis, calcium signaling, cell death, and metabolism, by changing mitochondrial protein expression, structure, and function.[25–28] Mitochondria also regulate cell apoptosis by promoting the release of proapoptotic mediators.[29–33] Studies have shown that mitochondrial dysfunction is related to respiratory diseases, including airway inflammation, pulmonary fibrosis, and BPD.[34,35] Under oxidative stress conditions, the mitochondrial genome is a cellular target, such as with acute lung injury (ALI) or pneumonia.[36] Mitochondrial DNA (mtDNA) is more vulnerable to oxidative stress than nuclear DNA.[37,38] Mitochondrial transcription factor A (TFAM) is considered essential for the stability and transcription of mtDNA.[39,40] Studies have shown that TFAM can regulate mitochondrial function by interacting with PGC-1α and nuclear respiratory factor 1 (NRF1).[41]

However, it is still unclear how Res exerts its protective effect during hyperoxia-induced lung injury. We hypothesized that Res plays a protective role in hyperoxic damage by alleviating mitochondrial dysfunction and that the SIRT1/PGC-1α signaling pathway may be involved. Hence, we developed a hyperoxia model to study the possible mechanism by which Res protects alveolar epithelial cells.

Materials and methods

Cell culture and hyperoxia model

Human alveolar epithelial cells (HPAEpiCs) were used in this experiment (Shanghai Institute of Cell Biology, Chinese Academy Sciences). HPAEpiCs were cultured in Dulbecco's modified Eagle medium (DMEM, high glucose, Invitrogen, Shanghai, China) at 37°C in a 5% CO_2 atmosphere.[42] The medium included fetal bovine serum (10%, Beyotime, Shanghai, China), penicillin (100 U/mL), and streptomycin (100 μg/mL). Hyperoxia model: 100% CO_2 and 100% O_2 were mixed in a 5:95 ratio using an air mixing apparatus. Cells were exposed to hyperoxia (inlet mixture gas contained O_2 (950 mL/L) and CO_2 (50 mL/L) at a speed of 3 L/minutes for 10 minutes) and then cultured in a humidified incubator (5% CO_2, 37°C) for 24 h. The oxygen concentration was dynamically measured using a ML-IICB digital intelligent oxygen.

Cell counting kit-8 assay

After the cells reached 50–60% confluence, the cells were incubated with several different concentrations of Res (5 μM, 10 μM, 20 μM, 50 μM, 100 μM, 150 μM, 200 μM, 250 μM), SRT1720 (0.5 μM, 1 μM, 2 μM, 4 μM, 6 μM, 8 μM), and EX-527 (1 μM, 2.5 μM, 5 μM, 10 μM, 20 μM, 50 μM, 100 μM), and three wells were used for each concentration. Cell proliferation was measured after 24 h by WST-8 assay (Cell Counting Kit-8, Dojindo, Kumamoto, Japan). The absorbance was measured with a microplate reader (450 nm).

Research groups

Based on the results of CCK8 and ROS detection and reference related research, we finally determined the drug concentration of Res (20 μM), SRT1720 (2 μM), and EX-527 (5 μM and 10 μM). The cells were divided into the following groups after they reached 50 to 60% confluence: (1) a control group in which the cells were exposed to normoxia (21% O_2), (2) hyperoxia group in which the cells were exposed to hyperoxia, (3) hyperoxia+Res20 group in which 20 μM Res (DESITE, Chengdu, China) was added and the cells were exposed to hyperoxia, (4) hyperoxia+Res20+E5 group in which 20 μM Res and 5 μM EX-527 (MCE, New Jersey, US) were added and the cells were exposed to hyperoxia, (5) hyperoxia+Res20+E10 group in which 20 μM Res and 10 μM EX-527 were added and the cells were exposed to hyperoxia, (6) hyperoxia+S2 group in which 2 μM SRT1720 (Selleck, Houston, TX, USA) was added, and the cells were exposed to hyperoxia (7) hyperoxia+S2+E5 group in which 2 μM SRT1720 and 5 μM EX-527 were added and the cells were exposed to hyperoxia, and (8) the hyperoxia+S2+E10 group in which 2 μM SRT1720 and 10 μM EX-527 were added and the cells were exposed to hyperoxia. All cell cultures were cultured in a humidified incubator (5% CO_2, 37°C) for 24 h and then harvested for the next steps.

Measurement of intracellular total ROS

The cells were incubated in 2′,7′-dichlorofluorescein diacetate (DCHF-DA) (Beyotime, Shanghai, China) medium for 20 min. After cell incubation, the cells were washed, and serum-free high glucose DMEM was used. Fluorescence was measured with a fluorescence enzyme labeling instrument.

Measurement of mitochondrial ROS by MitoSOX staining

First, 1 μM of MitoSOX™ (Invitrogen, Shanghai, China) working solution and 10 mg/l of Hoechst 33342 (Solarbio, Beijing, China) working solution were used. The cells were washed twice with Hank's solution and then incubated

with the configured Mito SOX^{TM} working solution for 20 min (37°C). This process was carried out in a dark room. After incubation, the cells were washed again with Hank's solution. The cells were then incubated in the prepared Hoechst 33342 working solution for 10 min (37°C) in the dark. Finally, a fluorescence microscope was used for observation and image collection.

Measurement of mitochondrial membrane potential

The JC-1 Kit (Solarbio, Beijing, China) was used to evaluate the mitochondrial membrane potential in the cells. The JC-1 dying working solution was prepared according to the manufacturer's instructions. The cells were incubated with the JC-1 staining working solution in the dark for 20 min (37°C). Finally, a fluorescence microscope was used for observation and image collection. The average fluorescence intensity of red or green fluorescence was evaluated by ImageJ software. The membrane potential is represented by the ratio of red/green fluorescence intensity, and a decrease in the ratio means that the membrane potential is relatively low.

Flow cytometry analysis of apoptosis

FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, Franklin Lakes, NJ) was used to measure cell apoptosis, and apoptotic cells were quantitated using a flow cytometer (Thermo Fisher Scientific).

Western blot

After treatment for 24 h as described before, the cells were lysed with lysis buffer (4°C), followed by protein concentration detection using a BCA Protein Assay kit (Beyotime, Shanghai, China). The proteins were separated by SDS-PAGE with the same amount of proteins for each lane (30 μg/lane). The separated proteins were transferred to PVDF membranes. The membranes were blocked with 5% (w/v) dry nonfat milk and incubated with the corresponding primary antibodies against SIRT1 (1:1000, Abcam, UK, ab110304), acetyl-p53 (1:1000, Abcam, UK, ab183544), PGC-1α (1:1000, Cell Signaling, USA, #2178), NRF1 (1:1000, Cell Signaling, USA, #46743), TFAM (1:1000, Cell Signaling, USA, #8076), and GAPDH (1:15000, Proteintech, USA, Cat no: 10494–1-AP) at 4°C with gentle shaking overnight. Goat anti-rabbit IgG-HRP (Beyotime, Shanghai, China) and goat anti-mouse IgG-HRP (Beyotime, Shanghai, China) were used as secondary antibodies, and these secondary antibodies were added to the membranes. GAPDH (Proteintech, Chicago, USA) was used as an internal control. Finally, the electrochemiluminescence (ECL) system was adopted for band detection.

Statistical analysis

SPSS 19.0 statistical software was used to perform statistical analysis. The data are expressed as the mean ± standard deviation (SD) of four independent experiments and statistically analyzed using one-way analysis of variance

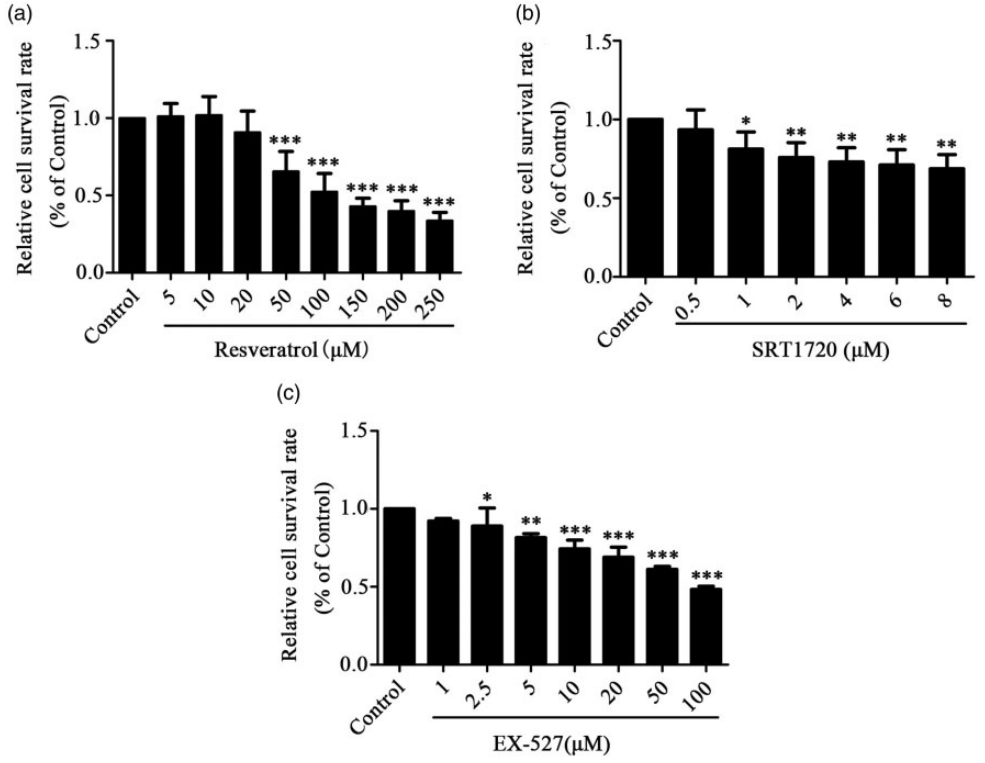

Figure 1. Effects of Res, SRT1720 and EX-527 on the viability of HPAEpiC cells. (a) Res inhibits the growth of HPAEpiC cells in a dose-dependent manner. (b) SRT1720 inhibits the growth of HPAEpiC cells in a dose-dependent manner. (c) EX-527 inhibits the growth of HPAEpiC cells in a dose-dependent manner. Data from four independent experiments. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$.

(ANOVA) or a nonparametric test (Kruskal–Wallis). Statistical significance was set at $P < 0.05$.

Results

Cytotoxicity of res, SRT1720, and EX-527 on HPAEpiCs in vitro

We added Res, SRT1720 and EX-527 drugs in different concentrations to HPAEpiCs, and then CCK8 was used for drug cytotoxicity detection. All three drugs showed dose-dependent cytotoxicity, as shown in Figure 1. Next, different concentrations of Res (IC_{50}, 119 μM), SRT1720 (IC_{50}, 32 μM), and EX-527 (IC_{50}, 93 μM) were added to HPAEpiCs before hyperoxia exposure, and the subsequent drug concentration of the experiment was explored by detecting ROS. We found that Res 20 μM and SRT1720 2 μM could not only reduce the production of ROS, but also had little effect on the inhibition of cell growth, as shown in Figure 2(a) and (c). Based on previous studies,[43–45] we chose Res 20 μM and SRT1720 2 μM as the concentrations used in this study. EX-527 can promote the production of ROS at 5 μM and 10 μM, and has little effect on the growth inhibition of cells, as shown in Figure 2(b). At the same time, we also refer to previous related studies.[45,46] Therefore, we chose 5 μM and 10 μM as the concentrations used in this study.

Res reduced the total ROS of alveolar epithelial cells exposed to hyperoxia

As shown in Figure 2(a), the total ROS levels were significantly increased in the hyperoxia group compared with the control group, indicating that total ROS

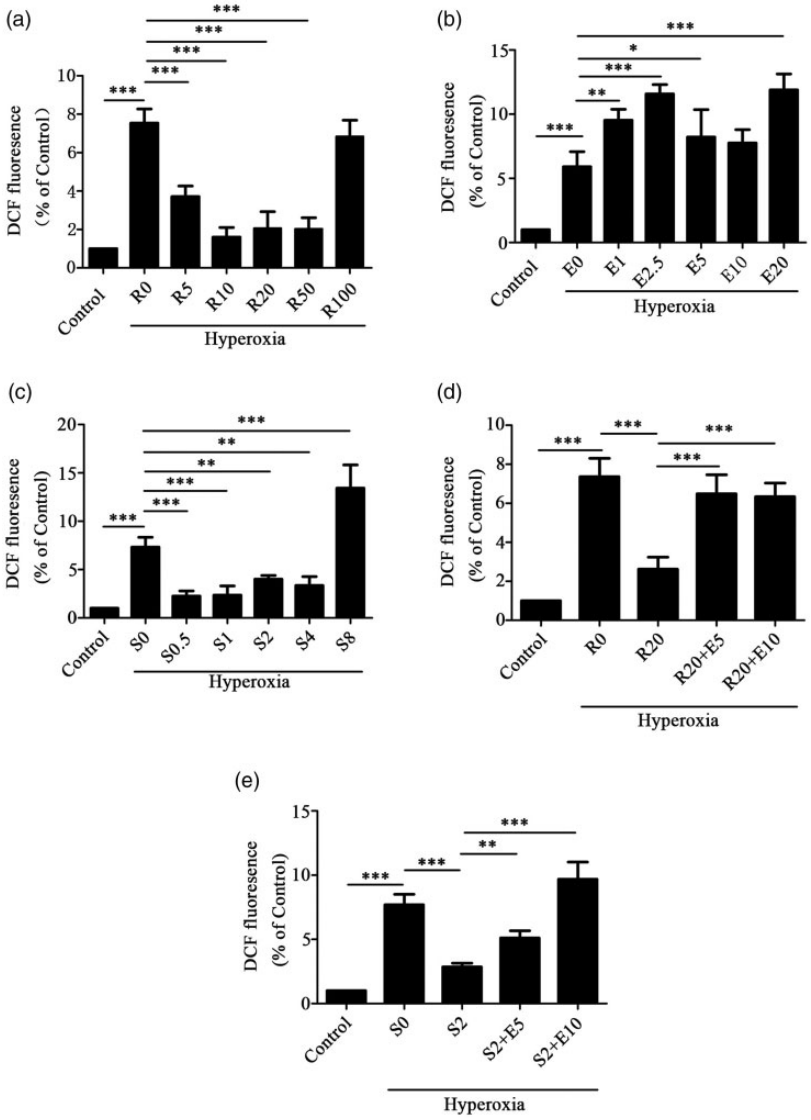

Figure 2. The effects of Res, SRT1720, and EX-527 on the total ROS of HPAEpiC cells. (a) Effects of Res at different concentrations on the total ROS of HPAEpiC cells induced by hyperoxia. (b) Effects of EX-527 at different concentrations on the total ROS of HPAEpiC cells induced by hyperoxia. (c) Effects of SRT1720 at different concentrations on the total ROS of HPAEpiC cells induced by hyperoxia. (d) Effects of Res and EX-527 on the total ROS of HPAEpiC cells induced by hyperoxia. (e) Effects of SRT1720 and EX-527 on the total ROS of HPAEpiC cells induced by hyperoxia. Data from four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

production was increased under hyperoxic conditions. Res significantly decreased the hyperoxia-induced total ROS increase in the HPAEpiC cells. Figure 2(b) shows that compared with the hyperoxia group, EX-527 significantly increased the total ROS in the HPAEpiC cells. As shown in Figure 2(c), compared with the hyperoxia group, SRT1720 significantly reduced the increase in total ROS that was induced by hyperoxia. As shown in Figure 2(d), the ROS level of the hyperoxia+Res20 group was significantly lower than that of the hyperoxia group. In the hyperoxia+Res20+E5 and hyperoxia+Res20+E10 groups, in which the cells were pretreated with EX-527 and Res and then exposed to hyperoxia, the levels of total ROS were significantly increased compared with those of the hyperoxia + Res20 group. However, there was no significant difference in ROS levels under different EX-527 concentrations. SRT1720 (Figure 2(e)) demonstrated similar results to Res. The ROS level in the hyperoxia+S2+E10 group was higher than that in the hyperoxia+S2+E5 group.

Res reduced mtROS production in alveolar epithelial cells that were exposed to hyperoxia

We also used MitoSOX^{TM} to detect the production of mtROS. As shown in Figure 3, the levels of mtROS were significantly increased in the hyperoxia group compared to those in the control group, indicating that exposure to hyperoxia increased the production of mtROS. In the hyperoxia+Res20 group, the level of mtROS was lower than that in the hyperoxia group with no Res treatment. In the hyperoxia+Res20+E5 and hyperoxia+Res20+E10 groups, the levels of mtROS were significantly increased with no statistically significant difference between the two groups. As shown in Figure S1, SRT1720 and Res had similar results.

Res alleviated the decrease in the mitochondrial membrane potential of alveolar epithelial cells when exposed to hyperoxia

The decreased mitochondrial membrane potential is important in the early stage of apoptosis. The JC-1 Kit was used to

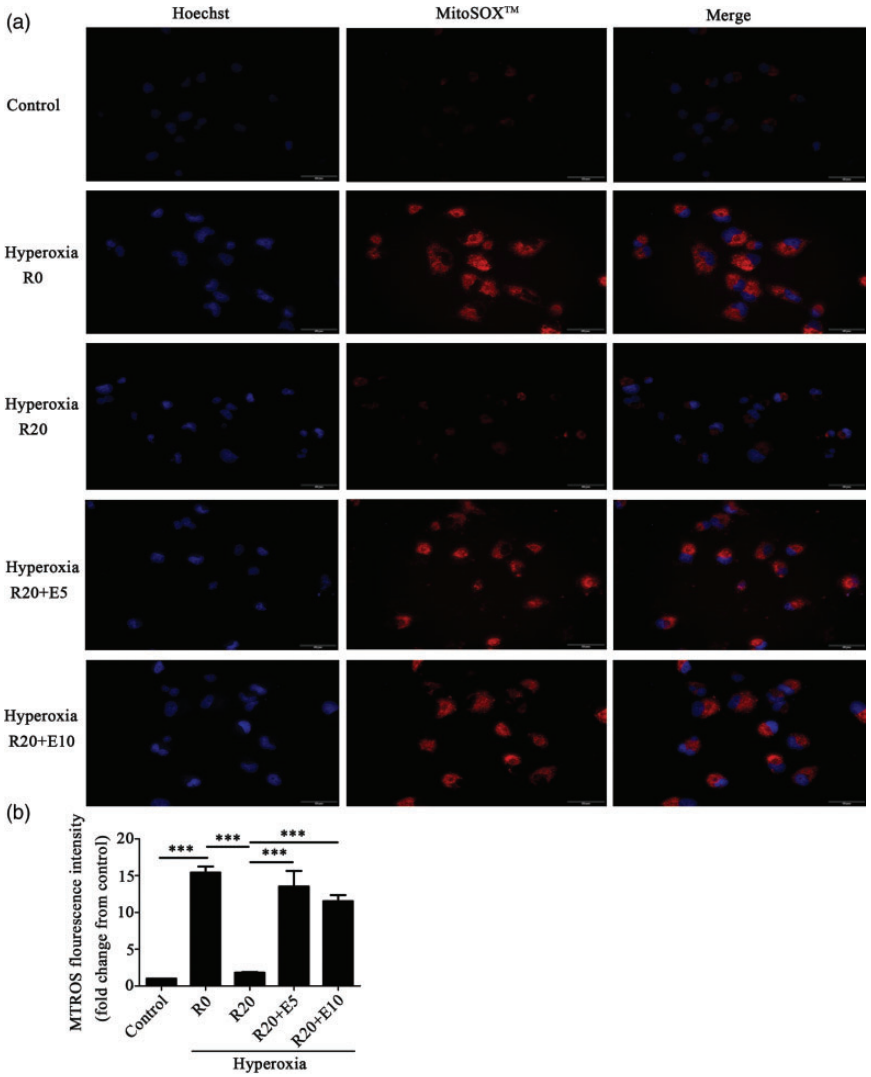

Figure 3. Res reduced mtROS production in HPAEpiC cells that were exposed to hyperoxia. (a) The effects of Res on mtROS induced by hyperoxia. The blue fluorescence represents the nucleus, while the red fluorescence represents mtROS (400× magnification, Scale bar: 50 μm). (b) The relative level of mtROS compared to the control group. Data from four independent experiments. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$.(A color version of this figure is available in the online journal.)

measure the mitochondrial membrane potential. As shown in Figure 4, the mitochondrial membrane potential was significantly decreased in the hyperoxia group compared with the control group. In the hyperoxia+Res20 group, the level of mitochondrial membrane potential was higher than that in the hyperoxia group with no Res. In the hyperoxia+Res20+E5 and hyperoxia+Res20+E10 groups, the levels of mitochondrial membrane potential were decreased with no statistically significant difference between the two groups. Additionally, SRT1720 and Res showed similar results, as shown in Figure S2.

Res reduced the apoptosis of alveolar epithelial cells that were exposed to hyperoxia

As shown in Figure 5, the apoptotic rate in the hyperoxia group was significantly increased compared with that of the control group. The apoptotic rate of the hyperoxia+Res20 group was significantly decreased compared with that of the hyperoxia group. Compared with the hyperoxia+Res20 group, the apoptotic rates of the hyperoxia+Res20+E5 and hyperoxia+Res20+E10 groups were significantly increased with no statistically significant difference between the two groups. As shown in Figure S3, SRT1720 and Res demonstrated similar results.

Res upregulated the expression of SIRT1, PGC-1α, NRF1, and TFAM in alveolar epithelial cells and downregulated Ac-p53 when exposed to hyperoxia

As shown in Figure 6(a) and (b), the expression levels of SIRT1, PGC-1α, NRF1, and TFAM were decreased, and the expression of Ac-p53 was increased in the hyperoxia group compared with that of the control group. In the hyperoxia+Res20 group, the expression levels of SIRT1, PGC-1α, NRF1 and TFAM were higher, while the expression of Ac-p53 was lower than that in the hyperoxia group with no Res. In the

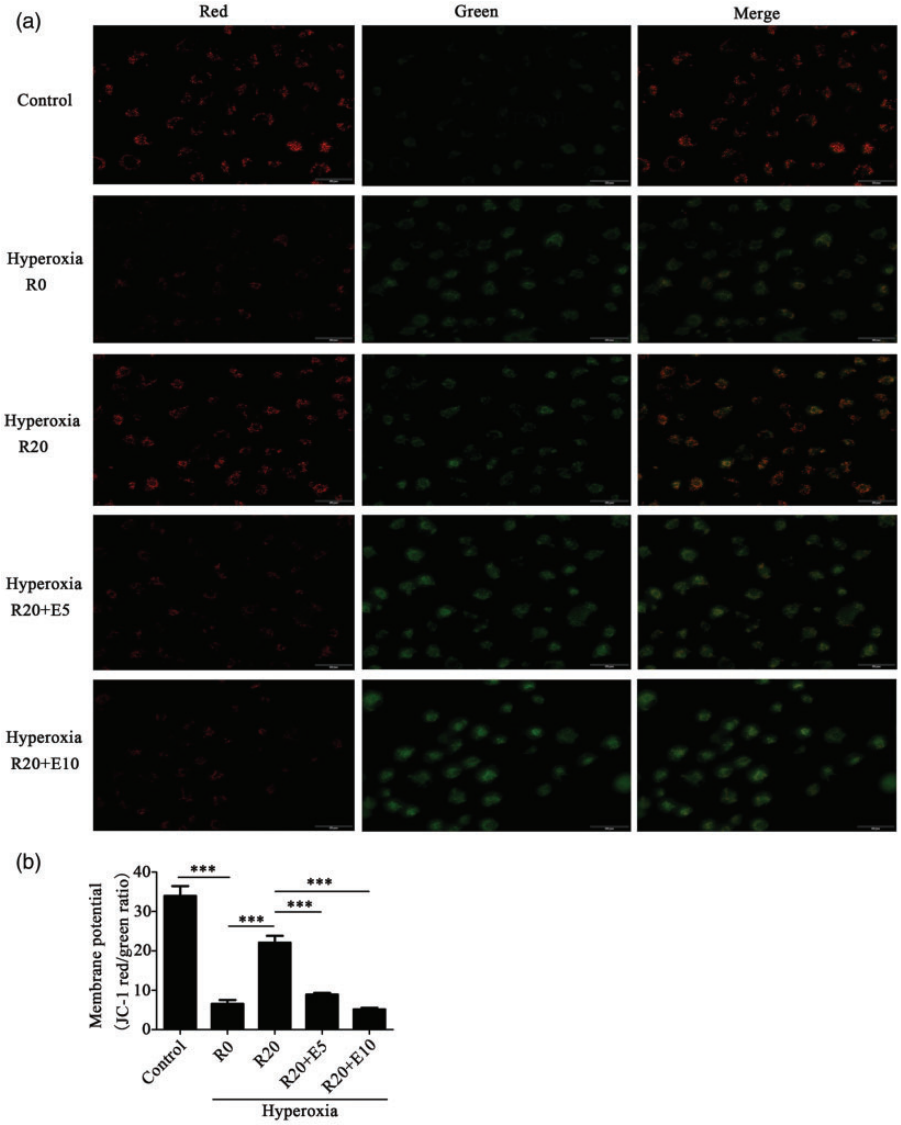

Figure 4. Res alleviated the decrease in mitochondrial membrane potential of alveolar epithelial cells when exposed to hyperoxia. (a) The effects of Res on the decrease in mitochondrial membrane potential in HPAEpiC cells induced by hyperoxia (400× magnification, Scale bar: 50 μm). (b) The red/green ratio of each group. Data from four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

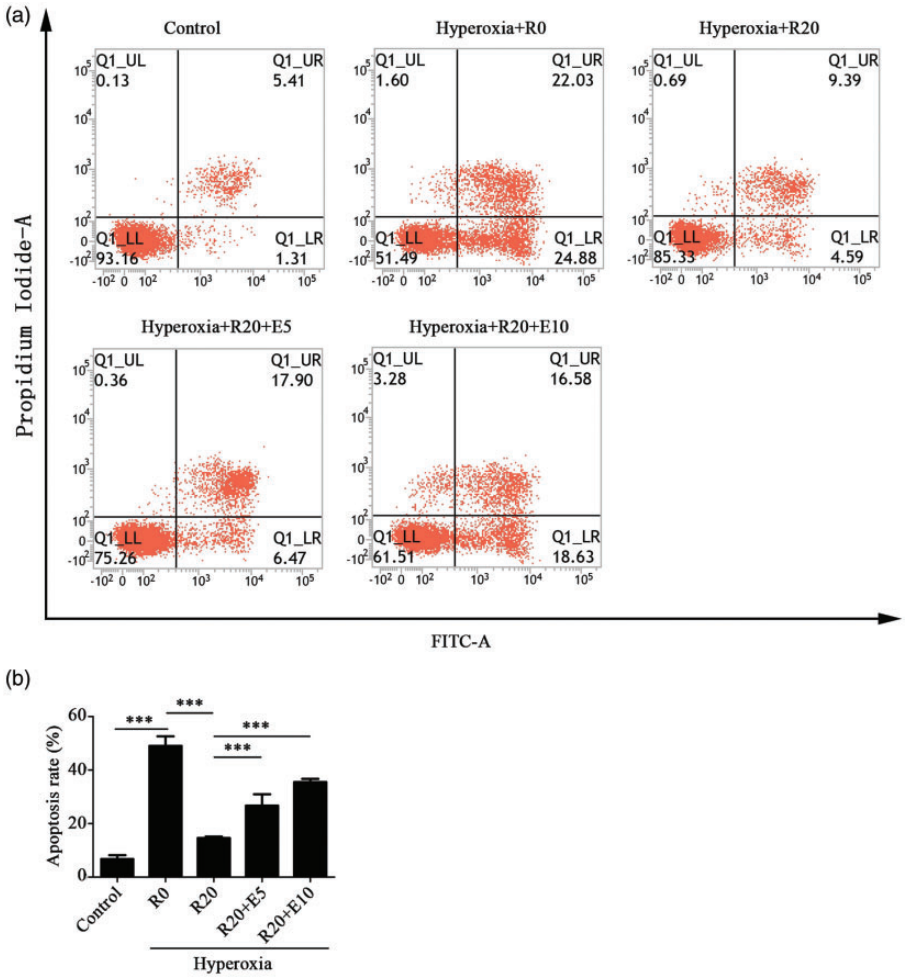

Figure 5. Res reduced the apoptosis of alveolar epithelial cells that were exposed to hyperoxia. (a) The effects of Res and EX-527 on apoptosis of HPAEpiC cells induced by hyperoxia. (b) The apoptotic rates of each group. Data from four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

hyperoxia+Res20+E5 and hyperoxia+Res20+E10 groups, the expressions of SIRT1, PGC-1α, NRF1, and TFAM were decreased, and the expression of Ac-p53 was increased. The expression of PGC-1α and NRF1 in the hyperoxia+Res20+E10 group decreased more significantly than that in the hyperoxia+Res20+E5 group. As shown in Figure 6 (c) and (d), SRT1720 and Res demonstrated similar results. The changes in SIRT1, PGC-1α, and TFAM expression were more obvious in the hyperoxia+Res20+E10 group.

Overall, the results of this study suggested that hyperoxia exposure can increase total ROS and mtROS production, decrease mitochondrial membrane potential, downregulate SIRT1, PGC-1α, NRF1, and TFAM, upregulate Ac-p53, and induce apoptosis and mitochondrial dysfunction in human alveolar epithelial cells. Pretreatment with Res or SRT1720 decreased the total ROS and mtROS production, increased the mitochondrial membrane potential, upregulated SIRT1, PGC-1α, NRF1, and TFAM, and downregulated Ac-p53 to alleviate hyperoxia-induced apoptosis and mitochondrial dysfunction. The results were in contrast to those of the Res or SRT1720 pretreatment groups when the cells were pretreated with EX-527 before Res or SRT1720 treatment.

Discussion

BPD has a serious impact on the growth of children, and can affect their respiratory systems, nervous systems, movement, and hearing. The impact of the respiratory system is most obvious, as it is manifested in childhood and even after reaching adulthood. Incidence of respiratory diseases, respiratory medicine usage, hospital re-admission rate, incidence of pulmonary hypertension, and abnormal lung function are significantly increased. Due to the severe adverse effects of BPD, it is very important to conduct early intervention for premature infants with high-risk factors of BPD. In the past, many studies have explored the prevention and treatment of BPD, including vitamin A, glucocorticoids and caffeine, which are administered shortly after the birth of premature infants. Therefore, in this study, resveratrol was added before hyperoxia exposure to explore the preventive effect of resveratrol on BPD.

Increasing evidence has indicated that hyperoxia can cause a variety of side effects.[1–4] Excessive ROS and inflammation induced by hyperoxia lead to lung injury during the development of BPD.[6,47] Excessive ROS, which are produced under hyperoxic conditions, are considered to be the main cause of lung injury. Hyperoxia causes excessive

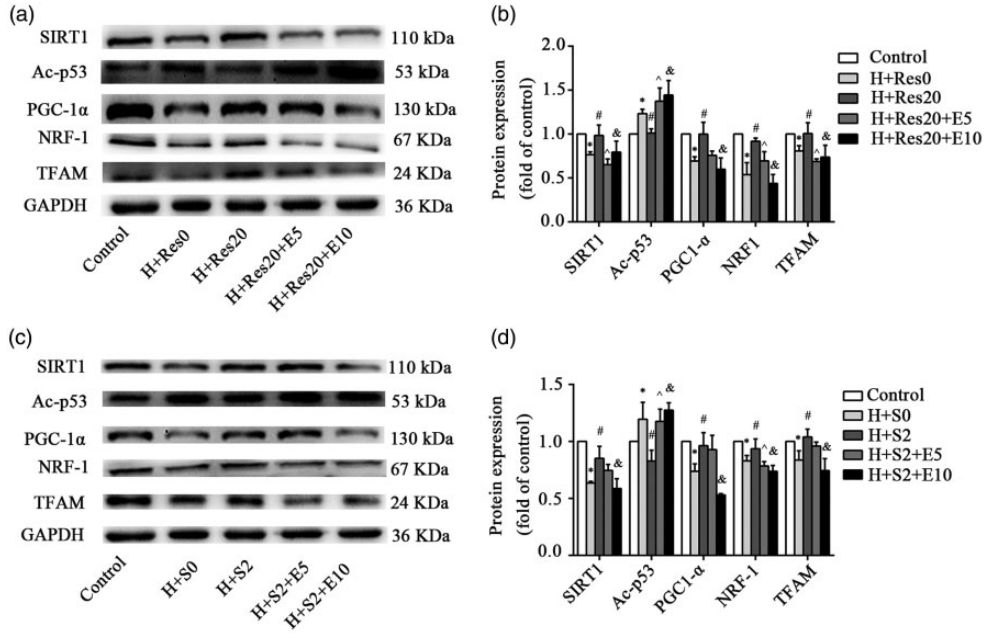

Figure 6. Res and SRT1720 upregulated the expression of SIRT1, PGC-1α, NRF1, and TFAM in alveolar epithelial cells and downregulated Ac-p53 when exposed to hyperoxia. (a, b) The effects of Res on the protein expression of SIRT1, PGC-1α, NRF1, TFAM, and Ac-p53 in HPAEpiC cells during hyperoxia. Data from four independent experiments. *: Control group versus hyperoxia (H+R0) group, $P < 0.05$; #: Hyperoxia (H+R0) group versus hyperoxia+Res20 (H+R20) group, $P < 0.05$; ^: Hyperoxia+Res (H+R20) group versus hyperoxia+Res20 + 5 μM EX-527 (H+R20+E5) group, $P < 0.05$; &: Hyperoxia+Res20 (H+R20) group versus hyperoxia+Res20 + 5 μM EX-527 (H+R20+E5) group, $P < 0.05$. (c, d) The effects of SRT1720 on the protein expression of SIRT1, PGC-1α, NRF1, TFAM and Ac-p53 in HPAEpiC cells during hyperoxia. Data from four independent experiments. *: Control group versus hyperoxia (H+S0) group, $P < 0.05$; #: Hyperoxia (H+S0) group versus hyperoxia+SRT1720 (H+S2) group, $P < 0.05$; ^: Hyperoxia+SRT1720 (H+S2) group versus hyperoxia+SRT1720 + 5 μM EX-527 (H+S2+E5) group, $P < 0.05$; &: Hyperoxia+SRT1720 (H+S2) group versus hyperoxia+SRT1720 + 10 μM EX-527 (H+S2+E10) group, $P < 0.05$.

production of ROS, resulting in lung endothelial and epithelial cell damage, destruction of the alveolar-capillary barrier, increased lung permeability, and activation of the release of pro-inflammatory factors.[48]

Res has many pharmacological activities, including anti-inflammation, antioxidation, anti-apoptosis, and anti-fibrosis.[16] Previous studies have shown that Res alleviated apoptosis induced by hyperoxia via ROS reduction both *in vitro* and *in vivo*.[17,18] A recent study showed that hyperoxia promoted the production of ROS and NO, enlarged the Bax/Bcl-2 ratio, activated the mitochondria-dependent apoptotic pathway, and thus induced the apoptosis of pulmonary epithelial cells. This indicates that the mitochondrial-dependent apoptotic pathway is involved in hyperoxia injury.[49] In the study of hyperoxia-induced lung injury, SRT1720 may play a similar role as Res.[19,20] EX-527 can effectively inhibit SIRT1 deacetylase activity and thus restore global acetylation levels.[21] In this study, we further showed that after Res pretreatment, the total ROS and mtROS decreased significantly, and SRT1720 produced similar results as Res. However, after pretreatment with EX-527 and Res (or SRT1720), the total ROS and mtROS were significantly increased. These data indicate that Res and SRT1720 can inhibit the increase in intracellular ROS induced by hyperoxia, while EX-527 can eliminate the inhibition of Res and SRT1720 on the increase in intracellular ROS induced by hyperoxia in which mitochondria play an important role.

Alveolar epithelial cell apoptosis is regarded as a significant feature in hyperoxia-induced acute lung injury.[50,51] One of the protective mechanisms of Res in respiratory diseases is anti-apoptosis.[16] Previous studies have shown that Res alleviated apoptosis induced by hyperoxia both *in vitro* and *in vivo*.[17,18] The decrease in mitochondrial membrane potential is an early feature of apoptosis. The expression of SIRT1 changes with changes in oxygen content. SIRT1 is a deacetylase that regulates many important metabolic processes by interacting with substrate proteins, including p53, NF-κB, STAT3, and PGC1-α. In our previous *in vitro* research, it was shown that hyperoxia induces a decrease in SIRT1 expression, and the use of resveratrol can increase SIRT1 expression.[18,52] Our animal experiments showed that resveratrol may reduce apoptosis by stimulating SIRT1.[13] Therefore, we used resveratrol, a SIRT1 agonist to further study the role of SIRT1 in hyperoxia. In this study, the mitochondrial membrane potential was shown to be significantly decreased in the hyperoxia group compared with the control group, while pretreatment with Res, such as SRT1720, mitigated hyperoxia-induced mitochondrial membrane potential declination. Flow cytometry was used to evaluate the apoptotic rates of each group. The results showed that the apoptotic rate of the hyperoxia group was significantly increased compared with that of the control group, while pretreatment with Res or SRT1720 decreased the apoptotic rate. However, when pretreated with EX-527 before Res or SRT1720 treatment, the apoptotic rate was increased. The SRT1720 and Res groups demonstrated similar results. In terms of protein expression, the use of Res and SRT1720 can offset the downregulation of SIRT1 expression and upregulation of acetyl-p53

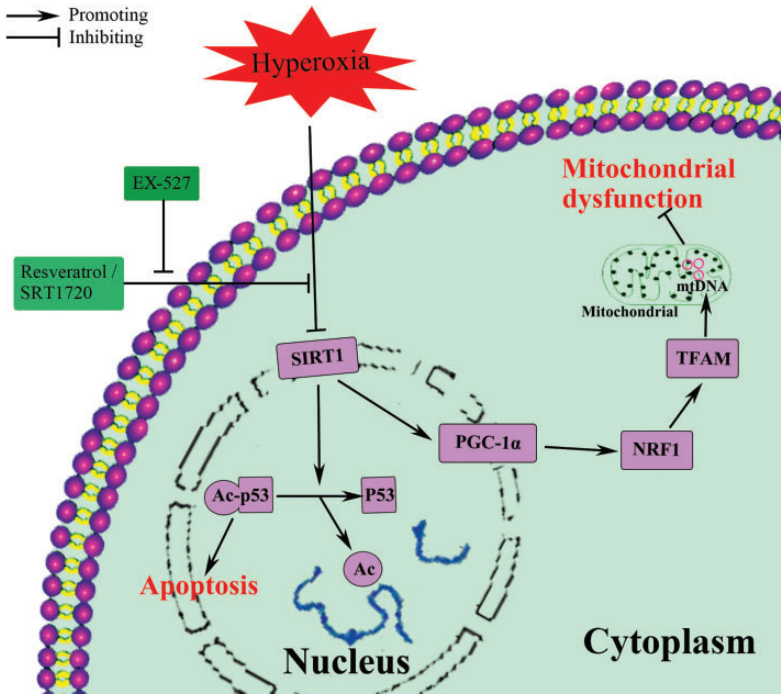

Figure 7. Protective mechanism of resveratrol against hyperoxia-induced cell damage. (A color version of this figure is available in the online journal.)

expression induced by hyperoxia, while the effect of Res and SRT1720 can be alleviated by EX-527. We can conclude that hyperoxia can induce cell apoptosis, and the activation of SIRT1 can alleviate this change, which is consistent with our previous experiments.

Mitochondria play an important role in cell function, including lipid synthesis, calcium signaling, cell death, and metabolism, by changing the mitochondrial protein expression, structure, and function.[25–28] Therefore, the maintenance of normal mitochondrial function remains crucial to cell life. Under oxidative stress conditions, the mitochondrial genome has always been considered a cellular target.[15] PGC-1α and its downstream transcription factors NRF1 and TFAM are involved in the regulation of mitochondrial function, and TFAM is essential for mtDNA stabilization and transcription.[39,40] SIRT1 can interact with PGC-1α to regulate mitochondrial function and biosynthesis.[13,14] In our study, mtROS and mitochondrial membrane potential were decreased when exposed to hyperoxia, and the expression levels of PGC-1α, NRF1, and TFAM were decreased, suggesting that hyperoxia caused damage to human alveolar epithelial cells via mitochondrial dysfunction. When pretreated with Res or SRT1720, the expressions of PGC-1α, NRF1, and TFAM increased, while the expressions of these proteins decreased when they were pretreated with EX-527 before Res or SRT1720 treatment, accompanied by changes in mtROS and the mitochondrial membrane potential. Resveratrol can improve hyperoxia-induced mitochondrial dysfunction, and the SIRT1/PGC-1a signaling pathway may be involved in this protective effect.

In conclusion, this study suggests that hyperoxia-induced apoptosis and mitochondrial dysfunction in alveolar epithelial cells can be alleviated by pretreatment with Res and may occur through the SIRT1/PGC-1a signaling pathway. Res-induced SIRT1 upregulation was involved in lung protection (Figure 7). However, our research was limited to the cell level, and we did not further study the relationship between SIRT1 and PGC-1a. Therefore, in future experiments, we will further study the SIRT1/PGC-1a signaling pathway and conduct animal experiments, including gene knockout, to further explore the protective effect of resveratrol. On the other hand, although resveratrol has many benefits, due to its low bioavailability and instability, it is currently mainly used in cell and animal research, with only a few clinical studies. Therefore, improving the bioavailability of resveratrol and turning to clinical research will also be a focus for future research.

AUTHORS' CONTRIBUTIONS

XZ mainly designed the research, analyzed the data and prepared the figures; FW mainly performed the experiments and drafted the manuscript. XZ, FW, XL, and WD approved the final version of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SUPPLEMENTAL MATERIAL

Supplementary material for this article is available online.

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