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Resveratrol attenuates hydrogen peroxide-induced apoptosis in human umbilical vein endothelial cells

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Abstract. – BACKGROUND AND OBJECTIVES: Vascular endothelium injury caused by reactive oxygen species (ROS) plays an initial role in the pathogenesis of atherosclerosis. Resveratrol, a natural polyphenolic compound, is well known to possess a variety of cardioprotective activities. In this study we first investigated the effects of resveratrol against apoptosis of human umbilical vein endothelial cells (HUVECs) induced by hydrogen peroxide (H_2O_2) *in vitro* and the possible mechanisms.

MATERIALS AND METHODS: HUVECs were pre-incubated with resveratrol (0.5-10 microM) for 120 min, and then challenged with 100 microM H_2O_2 for 30 min. The cell viability was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. The superoxide dismutase (SOD) activities and the reduced glutathione (GSH) contents were measured spectrophotometrically by using commercially available kits. The apoptosis of HUVECs was detected by Hoechst 33258 and Annexin-V/PI staining by flow cytometry staining using Fluorescence microscope. We also measured the mitochondrial membrane potential with the fluorescent probe JC-1 through Fluorescence microscope.

RESULTS: The study showed that incubation with resveratrol caused significant increase of the viability of HUVECs and the SOD activities and GSH contents, and decrease of cell apoptosis induced by H_2O_2, which was accompanied with the restoration of the mitochondrial membrane potential.

CONCLUSIONS: Our data suggested that resveratrol protected HUVECs against apoptosis induced by H_2O_2 through enhancing the antioxidant defenses, and inhibiting the degression of the mitochondrial membrane potential.

Key Words:

HUVECs, Resveratrol, Hydrogen peroxide, Apoptosis, Mitochondrial membrane potential.

Introduction

Atherosclerosis is a slowly progressing and multifactorial disease, in which the abnormalities of endothelial cells (ECs) structure and function play an initial role in his development[1]. Many risk factors for atherosclerosis can lead to endothelial damage underlying the lumen of blood vessels, but now it is realized that exposure to local reactive oxygen species (ROS) is one of the main stimulator for ECs dysfunction/damage[2,3]. Generation of ROS can cause oxidative damage to lipids, proteins, enzymes in ECs, and then impair cellular function, resulting in the apoptosis of some severely damaged ECs[4,5]. As one of the most important ROS, hydrogen peroxide (H_2O_2) can easily cross the plasma membrane and damage the neighboring cells as well as H_2O_2-producing cells[6], so H_2O_2 has been extensively used to induce oxidative stress in *in vitro* models[7]. Numerous studies have reported that H_2O_2 could lead to the ECs dysfunction and apoptosis[4,8]. Therefore, the inhibition of the pro-apoptotic pathways in ECs induced by H_2O_2 has been considered as an attractive therapeutic strategy in atherosclerosis.

Resveratrol (*trans*-3, 4, 5-trihydroxystilbene), a natural polyphenolic compound, is mainly abundant in grapes skin, peanuts, mulberries, and in red wine. It has exerted a wide variety of pharmacological activities, including antioxidant activity, modulatory lipoprotein metabolism, antiplatelet aggregatory, anti-inflammatory, and antitumor[9-12]. Specially, the antioxidant activity of resveratrol has been considered to be essential in the cardiovascular protection, which was more likely attributed to the up-regulation of the natural antioxidant cell defenses rather than a direct free radical-scavenging effect[13].

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Although recent study has demonstrated that resveratrol might protect ECs from H_2O_2-induced oxidative stress[14], the mechanisms how resveratrol prevents H_2O_2-induced endothelial apoptosis remain incompletely understood. Considering the significance of oxidative stress-related vascular endothelial apoptosis and atherosclerosis, and to investigate the pharmaceutical functions of resveratrol in the treatment of atherosclerosis, H_2O_2 was used to mimic the effect of oxidative stress in HUVECs to explore how resveratrol protect the endothelium from oxidative stress and apoptosis.

Materials and Methods

Reagents

Resveratrol (purify >99%) was kindly provided by Changsha Organic Herb Inc. (Changsha, China), the concentration of saturated aqueous solution of resveratrol was determined to 440 μM by Freeze-drying method, and then the saturated resveratrol solution dissolved in M199 was diluted into desired concentrations for next experiments. H_2O_2 was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Dimethylsulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA), and streptomycin were obtained from Amresco (Solon, OH, USA). Penicillin, gelatin, glutamine, paraformaldehyde and Hoechst 33258 were purchased from Sigma (St. Louis, MO, USA), Type I collagenase, trypsinase and M199 were purchased from Gibco (Grand Island, NY, USA), Fetal bovine serum (FBS), Factor VIII were purchased from Hyclone (Logan, UT, USA), bECGF was from Roche Diagnostics (Mannheim, Germany), and heparin was purchased from Sinopharm Chemical Reagent Co. Ltd., (Shanghai, China). The superoxide dismutase (SOD) activity assay kit and the reduced glutathione (GSH) assay kit were purchased from Nanjing Institute of Jiancheng Bioengineering (Nanjing, China). Annexin V-FITC Apoptosis Detection Kit and Mitochondrial membrane potential assay kit with JC-1 were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). All the other reagents were of chemical analytical grade.

Isolation and Cultivation of HUVECs

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord of normal pregnancies as described previously[15-17] with some modifications. Briefly, the umbilical cord segments obtained under sterile conditions were stored at 4°C. When the experiment started, one end of the cord was cannulated with a blunt 12# gauge needle and secured with a 14 cm long hemostat, the vein lumen was perfused with phosphate buffered saline (PBS) (37°C) to wash out the blood. The other end of the umbilical vein was secured with a 14 cm long hemostat.10 mL of 0.1% type I collagenase was filled into the vein lumen and digested at 37°C for 15 min, then the digestive juice was collected and centrifuged (150×g for 10 min), then washed by M199 and centrifuged again (150×g for 10 min). Subsequently, resuspended cells were cultured in M199 supplemented with 20% FBS, 2 mg/mL glutamine, 50 U/mL heparin, 100 U/mL penicillin, 100 μg/mL streptomycin and 30 μg/mL bECGF, and cultured in a humidified atmosphere of 5% CO_2 at 37°C. HUVECs were identified by the characteristic "cobble stone" growth pattern and positive immunocytochemical staining with a monoclonal antibody against Factor VIII. Cells from 2th to 4th passages which grew well were used for experiments.

H_2O_2-induced Oxidant Stress

HUVECs were seeded in 96-well plates at a density of 1.0×10^5 cells/well and grew to 80% confluence. The cells were pretreated with resveratrol (0.5, 1, 10 μM) for 120 min, and then exposed to 100 μM H_2O_2 for another 30 min.

Cell Viability Assay

The MTT assay was used to estimate cell viability. Briefly, after different treatments as above, the culture supernatant was removed, then the cells were washed with PBS and incubated with MTT (5 mg/mL) in M199 medium at 37°C for another 4 h. Next, the culture medium with dye was removed; 200 μL DMSO was added to each well to dissolve the formazan crystals for 10 min. The optical density (O.D.) of each well were measured at 560 nm using a Microplate Reader (Bio-rad, Hercules, CA, USA). The viability of HUVECs each well was presented as percentage of control cells.

Measurement of Intracellular SOD Activities and GSH Contents

The medium in the 96-well plates was discarded and the HUVECs were washed with PBS twice, then the cells were lysed by Freeze Thaw method for three times. The activities of SOD

and the contents of GSH in cell lysate were determined by using commercial kits according to the manufacturer's instructions.

Hoechst 33258 Staining

HUVECs were collected, washed with PBS and fixed with 2% paraformaldehyde at room temperature for 15 min, and then the cells were washed with PBS again and stained with Hoechst 33258 staining solution (25 μg/mL) for 30 min at room temperature. The stained nuclei were observed using a fluorescence photomicroscope (Olympus, Shinjuku, Monolith, Tokyo, Japan).

Flow Cytometric Evaluation of Apoptosis

HUVECs were double-stained by using an Annexin V-FITC apoptosis detection kit according to the manufacturer's instructions. Samples stained with Annexin V and PI were quantitatively analyzed at an emission wavelength of 488 nm and an excitation wavelength of 570 nm by Flow Cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Determination of Mitochondrial Membrane Potential ($\Delta\Psi m$)

Mitochondrial membrane potential ($\Delta\Psi m$) of HUVECs was assessed by using Mitochondrial membrane potential assay kit with JC-1. Cells were washed with PBS and loaded with JC-1staining solution, and the $\Delta\Psi m$ was determined according to the manufacturer's instructions. The fluorescence in cells was quantitatively analyzed at an emission wavelength of 490 nm and an excitation wavelength of 530 nm with a fluorescence photomicroscope (Olympus, Japan).

Statistical Analysis

Results were expressed as mean±S.D. derived from at least three independent experiments. One way-ANOVA was performed to determine statistical significance test. Calculations were performed using the SPSS 18.0 for Windows software package (SPSS Inc., Chicago, IL, USA). Differences between groups were considered to be significant at $p < 0.05$.

Results

Resveratrol Inhibits H_2O_2-Induced Cytotoxicity in HUVECs

Endothelial cells were isolated from freshly obtained human umbilical cords and identified by morphologic and immunologic criteria in our previous works (data not shown). Initially we examined the cytotoxicity of resveratrol on HUVECs using the MTT assay. Resveratrol did not show obvious cytotoxic effects up to 55 μM (data not shown).

To evaluate whether resveratrol has protection against oxidative stress, HUVECs were pretreated with resveratrol for 120 min, then incubated by H_2O_2 for 30 min and cell viability was measured by MTT assay. The viability of cells was depressed significantly by 100 μM H_2O_2 for 30 min ($p <$ 0.001 versus untreated group), while resveratrol protected cells from H_2O_2-induced cytotoxicity in a concentration-dependent manner (0.5 μM, 56 ± 4.1%; 1 μM, 55.8 ± 5.0%; 10 μM, 71.2 ± 7.6%), as shown in Figure 1. Resveratrol could markedly enhance the cell viability at 10 μM ($p < 0.001$ versus H_2O_2 alone group). These results indicated that resveratrol could protect HUVECs from oxidative stress relative cellular injuries.

Resveratrol prevents H_2O_2-induced decrease of the SOD activities and GSH contents in HUVECs

To further investigate the protective effects of resveratrol, we measured the SOD activities and GSH contents in H_2O_2-treated cells, another indicators of cell toxicity. As shown in Figure 2, after treated with 100 μM H_2O_2 for 30 min, SOD activities declined from (28.3 ± 0.7) U/mg prot in normal cells to (14.6 ± 1.5) U/mg prot in H_2O_2-treated cells ($p < 0.001$ versus untreated group), and GSH contents decreased from (63.4 ± 8.1) mg/g

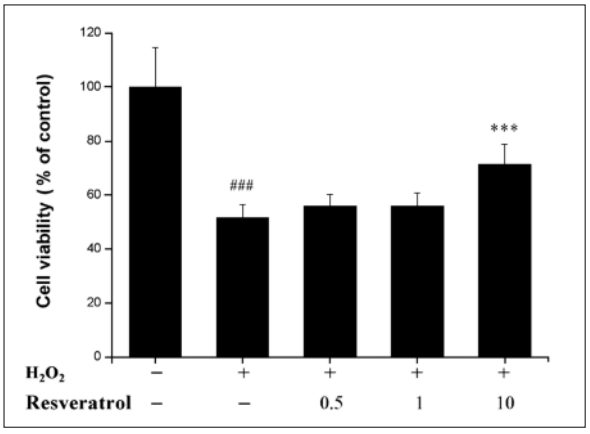

Figure 1. Resveratrol attenuated H_2O_2-induced cytotoxicity in HUVECs. The HUVECs pretreated with resveratrol (0.5, 1, 10 μM) for 120 min were exposed to 100 μM H_2O_2 for 30 min. $^{###}p < 0.001$ versus untreated group (control); $^{***}p < 0.001$ versus H_2O_2 alone group.

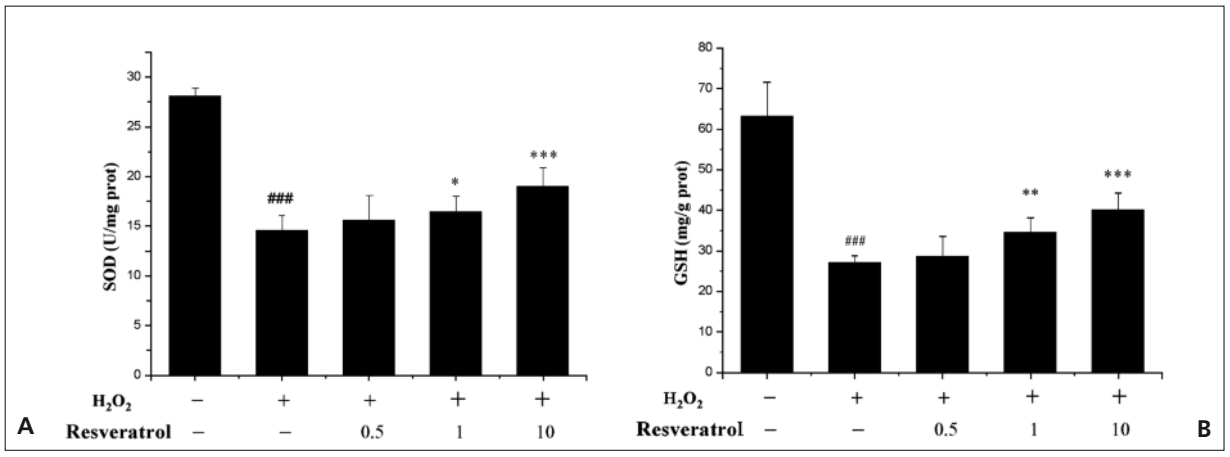

Figure 2. Resveratrol prevents H_2O_2-induced decrease of the SOD activities and GSH contents in HUVECs. The HUVECs pretreated with resveratrol (0.5, 1, 10 μM) for 120 min were exposed to 100 μM H_2O_2 for 30 min. ***A,*** The SOD activities. ***B,*** The GSH contents. $^{###}p < 0.001$ versus untreated group (control); $^{*}p < 0.05$ versus H_2O_2 alone group; $^{**}p < 0.005$ versus H_2O_2 alone group; $^{***}p < 0.001$ versus H_2O_2 alone group.

prot in normal cells to (27.3 ± 1.5) mg/g prot in H_2O_2-treated cells ($p < 0.001$ versus untreated group). On the contrary, 0.5, 1, 10 μM resveratrol increased SOD activities to (15.6 ± 2.5) U/mg prot, (16.4 ± 1.6) U/mg prot and (19.1 ± 1.9) U/mg prot, respectively, in which the effect of 10 μM resveratrol was more evident ($p < 0.01$ versus H_2O_2 alone group) (Figure 2A). Similarly, when pretreatment of HUVECs with different concentrations of resveratrol, GSH contents rose to (28.7 ± 4.8) mg/g prot, (34.7 ± 3.4) mg/g prot and (40.3 ± 3.9) mg/g prot, respectively. Also, 10 μM resveratrol increased the contents of GSH markedly ($p < 0.01$ versus H_2O_2 alone group) (Figure 2B).

Resveratrol Suppresses the Apoptosis of HUVECs Induced by H_2O_2

The uniform morphous of HUVECs nucleus and well-distributed deep blue fluorescence were revealed through Hoechst 33258 staining. After treating with 100 μM H_2O_2 for 30 min, HUVECs showed typical features of apoptosis, as showed in Figure 3. Resveratrol could improve the apoptosis morphous of HUVECs induced by H_2O_2.

To quantitatively gain insight into anti-apoptotic effects of resveratrol in H_2O_2-induced HUVECs, after treating with 100 μM H_2O_2 for 30 min, the apoptosis rate of HUVECs was measured by Annexin-V/PI staining. As shown in Figure 4, the apoptosis rate grew from 12.5 ± 0.4% to 24.3 ± 2.2% ($p < 0.01$ versus untreated group). By contrast, increased doses of resveratrol could evidently attenuate the apoptosis of HUVECs to 20.4 ± 2.7%, 19.1 ± 3.1% and 15.9 ± 4.6%, respectively. In which 10 μM resveratrol inhibited the apoptosis significantly ($p < 0.05$ versus H_2O_2 alone group).

Resveratrol Restores H_2O_2-induced Reduction of the Mitochondrial Membrane Potential (ΔΨm) in HUVECs

In order to test whether the inhibition of mitochondrial disruption may attribute to the anti-apoptotic effect of resveratrol, we tested its effects of on the mitochondrial permeability in the presence of H_2O_2. The normal HUVECs loaded by JC-1 revealed red fluorescence. After treating with 100 μM H_2O_2 for 30 min, the ΔΨm of mitochondrial was depolarized, as shown by reduction of the red fluorescence and the increase of green fluorescence. Resveratrol could dose-dependently restore the ΔΨm as indicated by increasing red fluorescence and repressing of the green fluorescence, and 10 μM resveratrol increased red fluorescence markedly as showed in Figure 5. The result was consistent with our previous results of intercellular SOD and GSH levels and apoptosis analysis.

Discussion

In the present study, we examined the effects of resveratrol on H_2O_2 induced HUVECs apoptosis *in vitro* and the possible mechanisms. Our data indicated that resveratrol could provide protection for HUVECs apoptosis induced by H_2O_2. It increased SOD activities and GSH contents. Furthermore, it markedly depressed the cell apopto-

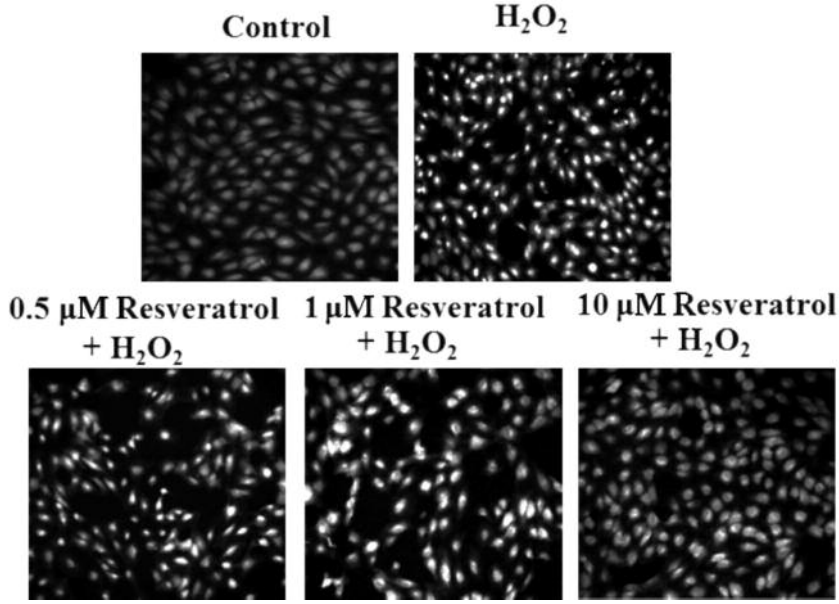

Figure 3. Morphological analysis of HUVECs treated with vehicle, resveratrol and/or H_2O_2. The HUVECs pretreated with resveratrol (0.5, 1, 10 μM) for 120 min were exposed to 100 μM H_2O_2 for 30 min, then cellular nucleus were fluorescence stained by Hoechst 33258 (fluorescence microscope, 20 ×).

sis. The underlying mechanisms of resveratrol against HUVECs apoptosis may be through increasing antioxidant defend systems and restoring the mitochondrial membrane potential.

It has been well established that the increased level of ROS from vessel tissue under pathological conditions is a main course leading to endothelial injury. After exposed to high level of H_2O_2, HUVECs will suffer remarkable cytotoxicity. Here, we demonstrated that H_2O_2, a precursor of other ROS, at 100 μM, can markedly decrease the HUVECs viability and increase the apoptosis of HUVECs. These findings were consistent with previous reports showing apoptosis in HUVECs treated by H_2O_2[18-19]. However, when resveratrol was added to the culture medium 2h before the treatment of H_2O_2, it increased cell viability and the resistance of endothelial cells against apoptosis. These findings suggested that resveratrol had a potential effect against H_2O_2-induced apoptosis.

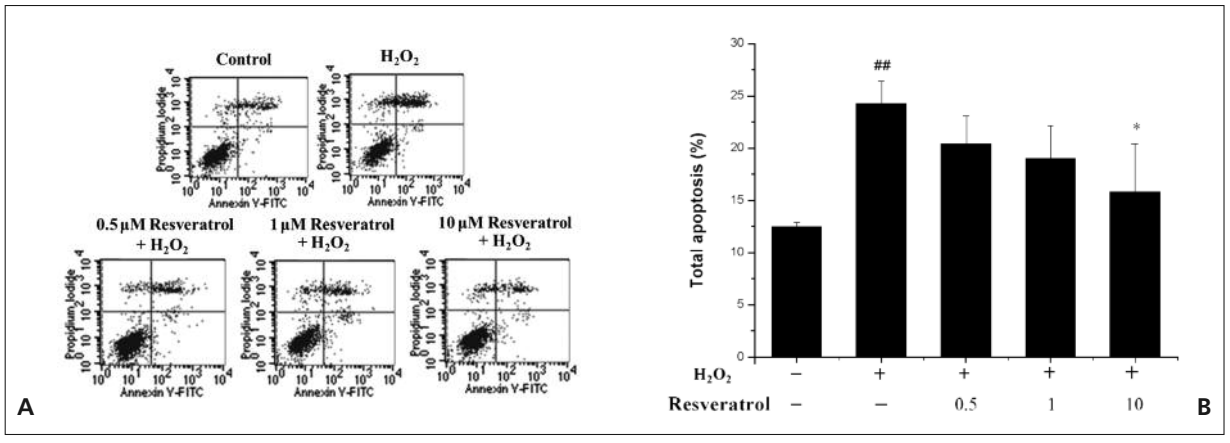

Figure 4. Resveratrol suppresses the apoptosis of HUVECs induced by H_2O_2 by flow cytometric analysis. The HUVECs pretreated with resveratrol for 120 min were exposed to 100 μM H_2O_2 for 30 min, and then the cells were labeled with Annexin-V/PI staining and analyzed by flow cytometer. ***A,*** Representative flow cytometric histograms of untreated group, H_2O_2 alone group and cells pretreated with various concentrations of resveratrol. ***B,*** Fluorescent intensities of HUVECs with untreated group, resveratrol and/or H_2O_2. $^{\#\#}p < 0.01$ versus untreated group (control); $^{*}p < 0.05$ versus H_2O_2 alone group.

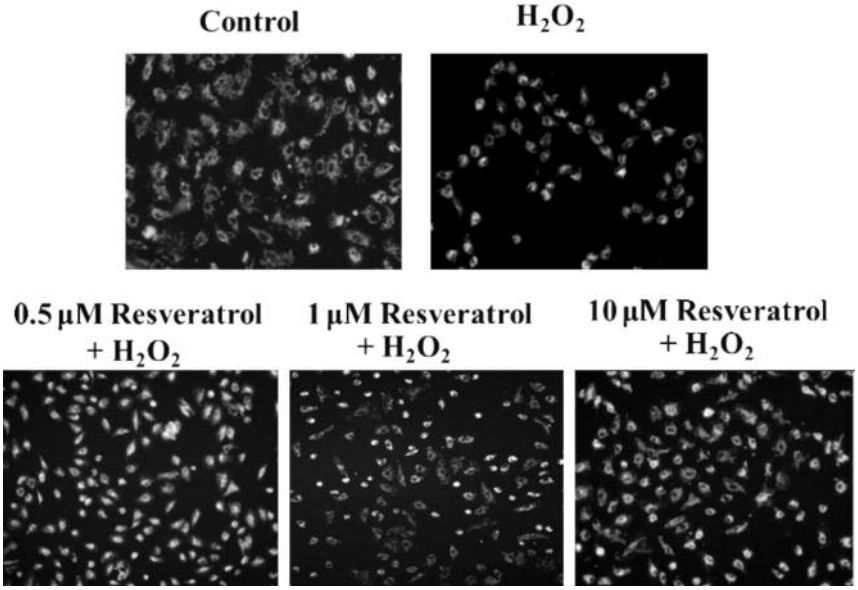

Figure 5. Resveratrol restores H_2O_2-induced reduction of the mitochondrial membrane potential in HUVECs (fluorescence microscope, 20×). The HUVECs pretreated with resveratrol for 120 min were exposed to 100 μM H_2O_2 for 30 min. The mitochondrial membrane potential in HUVECs was assessed by using mitochondrial membrane potential assay kit with JC-1.

However, the mechanisms by how resveratrol prevents endothelial cell apoptosis under oxidative stress are not well defined. Resveratrol is a natural polyphenolic compound and has been shown to be a free radical scavenger[20]. However, some researchers suggested that the direct antioxidant activities of resveratrol were rather low; the protective effects of resveratrol against oxidative stress were mainly attributed to the upregulation of cellular antioxidant defenses including SOD, catalase, and the content of GSH[13]. Thus, we hypothesized that the underlying mechanism of anti-apoptotic effect of resveratrol may be first due to its upregulation of endogenous cellular antioxidant systems to scavenging the ROS.

Antioxidant enzyme SOD is suggested to play an important role in antioxidant defenses in endothelial cells. SOD can dismutate superoxide radicals into hydrogen peroxide and subsequently detoxified into water by other antioxidant enzymes. As shown in Figure 2A, pretreatment with various concentrations of resveratrol markedly increased SOD activities. Previous studies also have demonstrated that treatment with resveratrol led to a significant increase in total cellular SOD activities[21,22]. These findings suggested that the protective effect of resveratrol may be related to the increased ability of scavenging H_2O_2.

Reduced glutathione (GSH) is a chief regulator of cellular redox balance which achieved by maintenance of the glutathione/glutathione disulfide (GSH/GSSG) status. It protects many cells with high intracellular GSH levels against oxidative damage. GSH may react with ROS nonenzymically and can be as a direct free radical scavenger[23]. GSH loss has been shown to be involved in apoptosis induced by various stimuli in endothelial cell[24-25]. This study reported that H_2O_2 decreased the GSH levels, while preincubation with resveratrol inhibited the decrease of GSH induced by H_2O_2 in a concentration-dependent manner, as shown in Figure 2A, suggesting that resveratrol modulated the GSH redox system.

It is well known that the mitochondria play a key role in cell death and cell survival, and the increase of mitochondrial membrane permeability constitutes a critical event in the apoptotic process[26]. Since resveratrol can decrease intracellular ROS level, thus we presumed that resveratrol was likely to go through the mitochondrial pathway to inhibit apoptosis, especially by restoring the $\Delta\Psi m$. In our present study, resveratrol can evidently reverse H_2O_2-induced $\Delta\Psi m$ decrease, which may inhibit pro-apoptotic factors released into the cytosol during apoptosis.

In conclusion, our present works showed that resveratrol had a protective effect against H_2O_2

induced cytotoxicity and apoptosis in HUVECs. This anti-apoptotic effect of resveratrol was possibly contributed to the combination of SOD and GSH upregulation and $\Delta\Psi m$ restoration. As oxidative stress induced endothelial cell injury plays an important role in the development of atherosclerosis, the findings of the present study may shed light on the pharmacological basis for the clinical application of resveratrol for treatment of atherosclerosis.

Acknowledgements

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