Protective effects of hyperoside against H_2O_2-induced apoptosis in human umbilical vein endothelial cells

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Abstract. The vascular endothelium is important in the physiological homeostasis of blood vessels. Increasing evidence demonstrates that oxidative stress-induced endothelial damage is involved in the pathogenesis of several cardiovascular diseases, including atherosclerosis. Hyperoside, one of major active components from *Apocynum venetum* L. (Luo-Bu-Ma), which is a traditional Chinese herbal medicine commonly used for the prevention of cardiovascular diseases, exhibits diverse bioactivities, including anti-inflammatory and antioxidant effects. In the present study, the protective effects of hyperoside against hydrogen peroxide (H_2O_2)-induced apoptosis of human umbilical vein endothelial cells (HUVECs) were investigated. The results demonstrated that hyperoside significantly prevented the loss of cell viability, the increase of endothelial Ca^{2+} content and apoptosis in H_2O_2-induced HUVECs. Additionally, reverse transcription-polymerase chain reaction and western blot analysis revealed that hyperoside significantly decreased the mRNA expression levels of B-cell lymphoma (Bcl)-2 associated X protein (Bax), cleaved caspase-3 and phosphorylated-p38, while increasing the mRNA expression of Bcl-2 in H_2O_2-induced HUVECs. The present findings suggested that hyperoside has protective effects against H_2O_2-induced apoptosis in HUVECs and serves a key role in the prevention of cardiovascular diseases.

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Introduction

The vascular endothelium is a physiological barrier separating the circulating blood from the underlying tissue, and serves an important role in maintaining physiological homeostasis of blood vessels (1). Endothelial cell injury induced by oxidative stress, which is initiated by excessive generation of reactive oxygen species (ROS) in response to diverse extracellular detrimental stimuli, is involved in the pathogenesis of several diseases, including coronary heart disease, neurodegenerative disorders, diabetes, arthritis, inflammation and cancer (2-5). Therefore, the protection of endothelial cells against ROS-induced damage must be considered as an important strategy for intervention in cardiovascular diseases.

Apocynum venetum L. (Luo-Bu-Ma) is a traditional Chinese herbal medicine exhibiting diverse activities, including inhibition of platelet aggregation and myocardial ischemia/reperfusion injury, hypotension and an antioxidative effect, and is widely used for the prevention of cardiovascular diseases (6-10). Hyperoside (Fig. 1), a flavonoid, is the predominant active component abundant in *A. venetum*. Increasing evidence has demonstrated that hyperoside exhibits anti-inflammatory, antioxidative and cellular protective effects. Ku *et al* (11) reported that hyperoside exhibited anti-inflammatory effects via the regulation of the high-mobility group box (HMGB)1-mediated signaling pathway and was beneficial for the inhibition of vascular inflammation diseases (11). It was also reported that hyperoside has a powerful capacity to modulate oxidative stress-induced melanogenesis through the inhibition of the formation of peroxynitrite, O_2 and NO in B16F10 melanoma cells (12). Numerous previous studies also indicated that hyperoside exerted protective effects on oxidative stress-induced injury of several types of cell (13-17). The present study demonstrated the protective effects of hyperoside against H_2O_2-induced apoptosis of HUVECs through the detection of endothelial Ca^{2+} content, expression levels of B-cell lymphoma (Bcl)-2, Bcl-2 associated X protein (Bax) and cleaved caspase-3, which was performed to investigate whether hyperoside was involved in the prevention of cardiovascular diseases.

Materials and methods

Materials. Hyperoside was purchased from the Chinese Food and Drug Inspection Institute (Beijing, China). Dimethyl

sulfoxide (DMSO), 4% paraformaldehyde, H_2O_2 and 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin and streptomycin, and TRIzol reagent were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The bicinchoninic acid (BCA) assay kit and cridine orange/ethidium bromide (AO/EB) were purchased from Beyotime Institute of Biotechnology, Inc. (Nanjing, China). Antibodies against phosphorylated (p-)p38 (#9211), p38 (#9212), Bcl-2 (#2876), Bax (#2772) and cleaved-caspase-3 (#9661) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (#CW0103) and β-actin (#CW0097) were purchased from CoWin Biotech Co., Ltd. (Beijing, China). H_2O_2 was prepared freshly for each experiment from a 33% (v/v) stock solution. All other chemicals and reagents were commercially available and of standard biochemical quality.

Cell culture and treatments. The HUVEC line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 0.1% penicillin/streptomycin at 37˚C in 5% CO_2. For all experiments, HUVECs were grown to 70-80% confluence and were subsequently treated as designed for different experiments.

Cell viability analysis. MTT assays were used to evaluate cell viability. HUVECs were seeded into 96-well plates at a density of $1x10^4$ cells/well and cultured at 37˚C for 24 h. The culture medium was then removed and fresh medium for different treatments was added to each well. Following treatment, 10 μl of 5 mg/ml MTT solution was added to each well and the cells were incubated for another 4 h. The culture medium was subsequently replaced with 100 μl DMSO. The optical density in each well was determined using a Bio-Rad Microplate Reader (Model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 570 nm.

Assessment of apoptosis using AO/EB fluorescent staining. The cells were seeded at $5x10^5$ cells/well in 6-well plates and cultured for 24 h. The cells were subsequently treated with hyperoside for 24 h prior to exposure to H_2O_2 for 4 h. The cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min and were washed with ice-cold PBS three times. The cells were stained with AO/EB solution (5 μl) for 30 sec and changes in cell morphology were observed using a fluorescence microscope (Olympus IX-71; Olympus, Tokyo, Japan).

Assessment of intercellular Ca^{2+} levels. The cells were seeded at a density of $5x10^5$ cells/well into 6-well plates and were cultured for 24 h. The cells were subsequently treated with hyperoside for 24 h prior to exposure to H_2O_2 for 4 h. The Fluo-3/AM fluorescent probe was added to the HUVEC suspension at a final concentration of 10 μM and incubated at 37˚C for 40 min. The intracellular Ca^{2+} content in the HUVECs was subsequently measured with laser-scanning confocal microscopy (Olympus FV1000, Olympus) with an excitation wavelength of 488 nm and a measured emission at 530 nm.

Reverse transcription-polymerase chain reaction (RT-PCR). The total RNA was extracted from the HUVECs using TRIzol reagent, according to the manufacturer's protocol. The concentration of purified RNA samples were measured spectrophotometrically using a Picodrop (Picodrop Ltd., Walden, UK). The RT reaction was performed using a SuperScript III First-Strand Synthesis system (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Amplification of the RT product by PCR was performed using Promega Taq DNA Polymerase (Promega Co., Madison, WI, USA). All reactions were performed in a thermal cycler (Model 2400; Perkin-Elmer, Norwalk, CT, USA) with primers (Sangon Biotech Co., Ltd., Shanghai, China) specific for Bcl-2, forward: 5'-CTTCGCCGAGATGTCCAGCCA-3' and reverse: 5'-CGCTCTCCACACACATGACCC-3'; Bax, forward: 5'-TGC TTCAGGGTTTCATCCAGGA-3' and reverse: 5'-ACGGCG GCAATCATCATCCTCTG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward: 5'-TCTCTGCTCCTC CTGTTCGAC-3' and reverse: 5'-TTAAAAGCAGCCCTG GTGAC-3'. Thermal cycling conditions involved an initial denaturation step at 94˚C for 10 min, followed by 30 cycles of denaturation at 94˚C for 10 sec, and primer annealing at 60˚C for 1 min, which was followed by agarose gel electrophoresis.

Quantitation of protein samples. Following treatment with various concentrations of hyperoside prior to H_2O_2 exposure, the HUVECs were washed with ice-cold PBS and harvested by trypsinization. The cells were centrifuged (1,000 rpm for 5 min) and washed with ice-cold PBS three times. The cells were then suspended in 100 μl ice-cold radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology, Inc.), sonicated 10 times for 5 sec with 10 sec pauses in an ice-water bath, and the samples were centrifuged (13,000 rpm for 5 min at 4˚C). The supernatants were stored at -80˚C. Quantification of the protein was performed using a BCA assay.

Western blot analysis. Equal quantities of protein extracts (40 μg) were separated on 12% sodium dodecyl sulfate-polyacrylamide gels. The proteins were subsequently transferred onto nitrocellulose membranes (Pall Gelman Laboratory Corporation, Ann Arbor, MI, USA). The membranes were blocked with 5% (w/v) non-fat milk powder in Tris-buffered saline/0.1% Tween-20 (TBST) for 1.5 h at room temperature. Following blocking, the membranes were incubated overnight at 4˚C with the primary antibody (dilution, 1:1,000). After three washes with TBST, the membranes were incubated for 1 h with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G as the secondary antibody at room temperature (dilution, 1:2,000). After three washes, the proteins were detected using an enhanced chemiluminescence detection kit (CoWin Biotech Co., Ltd., Beijing, China).

Statistical analysis. The data are presented as the mean ± standard deviation. Statistical comparisons were performed using a Student's t-test, and the differences between multiple groups were assessed by one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

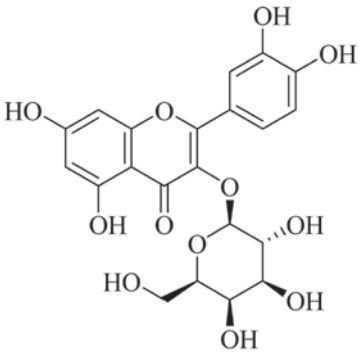

Figure 1. Chemical structure of hyperoside.

Results

Effects of hyperoside on the viability of HUVECs. To evaluate the apoptotic induction of H_2O_2 on HUVECs, HUVECs were incubated with 200 μM H_2O_2 for different durations. As shown in Fig. 2A, the viability of HUVECs decreased ~50% following exposure to 200 μM H_2O_2 for 4 h. Therefore, the exposure of HUVECs to 200 μM H_2O_2 for 4 h was selected to induce apoptosis of HUVECs for the subsequent experiments.

To ensure the suitable concentrations of hyperoside, the cytotoxicity of hyperoside was evaluated by MTT assay. HUVECs were cultured with various concentrations of hyperoside for 24 h and the cell viability was assessed. The result revealed that hyperoside at concentrations of <20 μM caused no affect the viability of HUVECs, as shown in Fig. 2B. Therefore, the concentrations of hyperoside were confirmed as 10, 15 and 20 μM in the subsequent experiments.

As shown in Fig. 2C, the H_2O_2-induced decrease of cell viability was significantly attenuated by hyperoside treatment in a dose-dependent manner (P<0.01), which suggested that hyperoside exhibited protective effect on H_2O_2-induced HUVECs injury.

Effects of hyperoside on H_2O_2-induced apoptosis in HUVECs. As shown in Fig. 3, AO/EB staining demonstrated that exposure of HUVECs to 200 μM H_2O_2 for 4 h induced HUVEC apoptosis. However, the treatment of HUVECs with 10, 15 and 20 μM hyperoside significantly attenuated the apoptosis of HUVECs induced by H_2O_2, which demonstrated that hyperoside exhibited anti-apoptosis effects in H_2O_2-induced HUVECs.

Effects of hyperoside on intercellular Ca^{2+} levels in HUVECs. As shown in Fig. 4, compared with the control group, intercellular Ca^{2+} content, presented as the fluorescence intensity, was significantly overloaded in the H_2O_2-induced HUVECs (P<0.01). However, the treatment of HUVECs with different concentrations of hyperoside (10, 15 and 20 μM) significantly inhibited the increase of fluorescence intensity induced by H_2O_2 in a dose-dependent manner (P<0.01). This indicated that hyperoside exhibited protective effects on the oxidative stress-induced increase of intercellular Ca^{2+} content.

Effects of hyperoside on the mRNA expression levels of Bcl-2 and Bax. As shown in Fig. 5, the mRNA expression of Bax

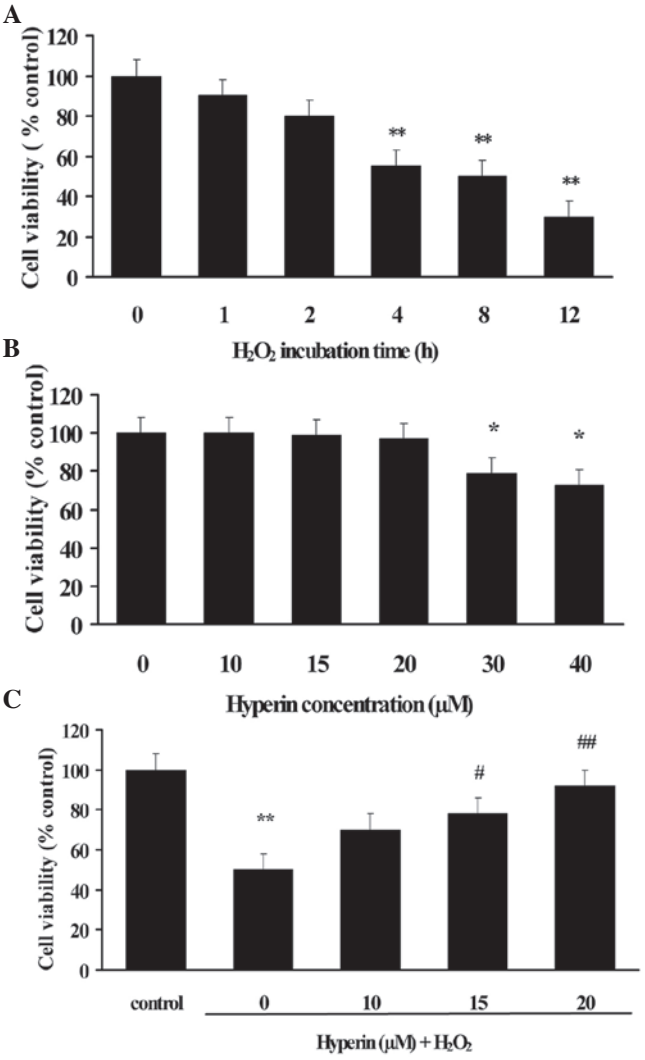

Figure 2. Cytotoxic activity of (A) H_2O_2 and (B) hyperoside was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide assay. (C) The protective effect of hyperoside on H_2O_2-induced cytotoxicity was determined in human umbilical vein endothelial cells. The data are presented as the mean ± standard deviation (*P<0.05 and **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. H_2O_2 treatment). H_2O_2, hydrogen peroxide.

was significantly increased in the H_2O_2-induced group. By contrast, the mRNA expression of Bcl-2 was significantly decreased compared with the control group. However, different concentrations of hyperoside (10, 15 and 20 μM) exhibited significant inhibition on the H_2O_2-induced increase of Bax and decrease of Bcl-2 mRNA in a dose-dependent manner (P<0.01).

Effects of hyperoside on the expression of apoptotic-associated proteins. As shown in Figs. 6 and 7, compared with the control group, the expression of cleaved caspase-3, Bax and p-p38 was significantly increased, while Bcl-2 was significantly decreased, in the H_2O_2-induced group. However, the treatment with different concentrations of hyperoside (10, 15 and 20 μM) revealed a significant inhibition on the H_2O_2-induced increase of cleaved caspase-3, Bax and p-p38, and the decrease of Bcl-2 in a dose-dependent manner (P<0.01). This indicated that hyperoside exhibited antiapoptotic effects on H_2O_2-induced HUVECs.

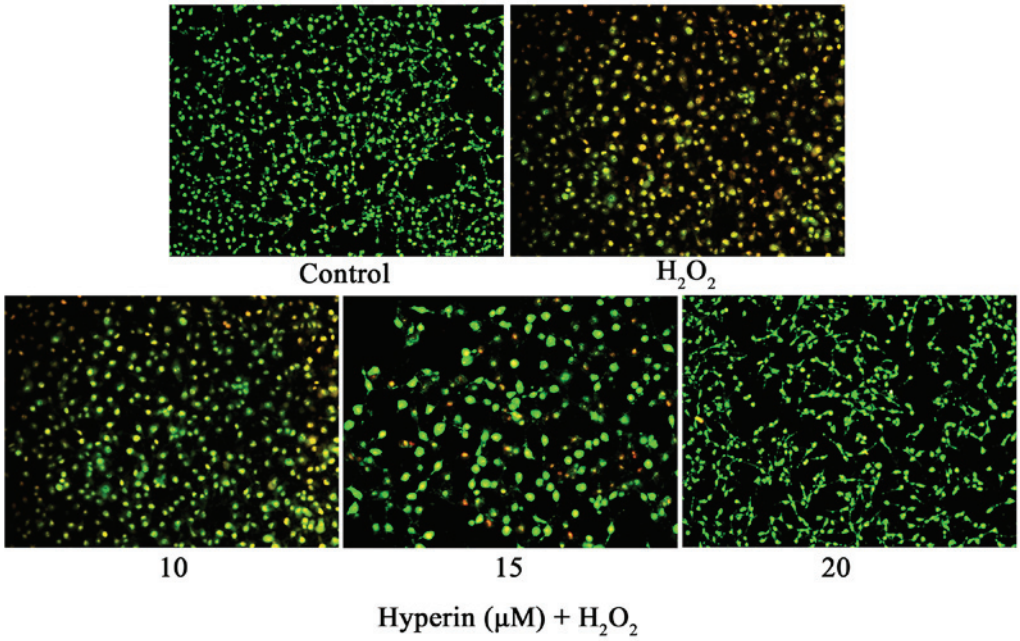

Figure 3. Apoptosis of human umbilical vein endothelial cells were evaluated by acridine orange/ethidium bromide. Magnification, x100. Green = normal, intact cells; yellow + orange = apoptotic cells. H_2O_2, hydrogen peroxide.

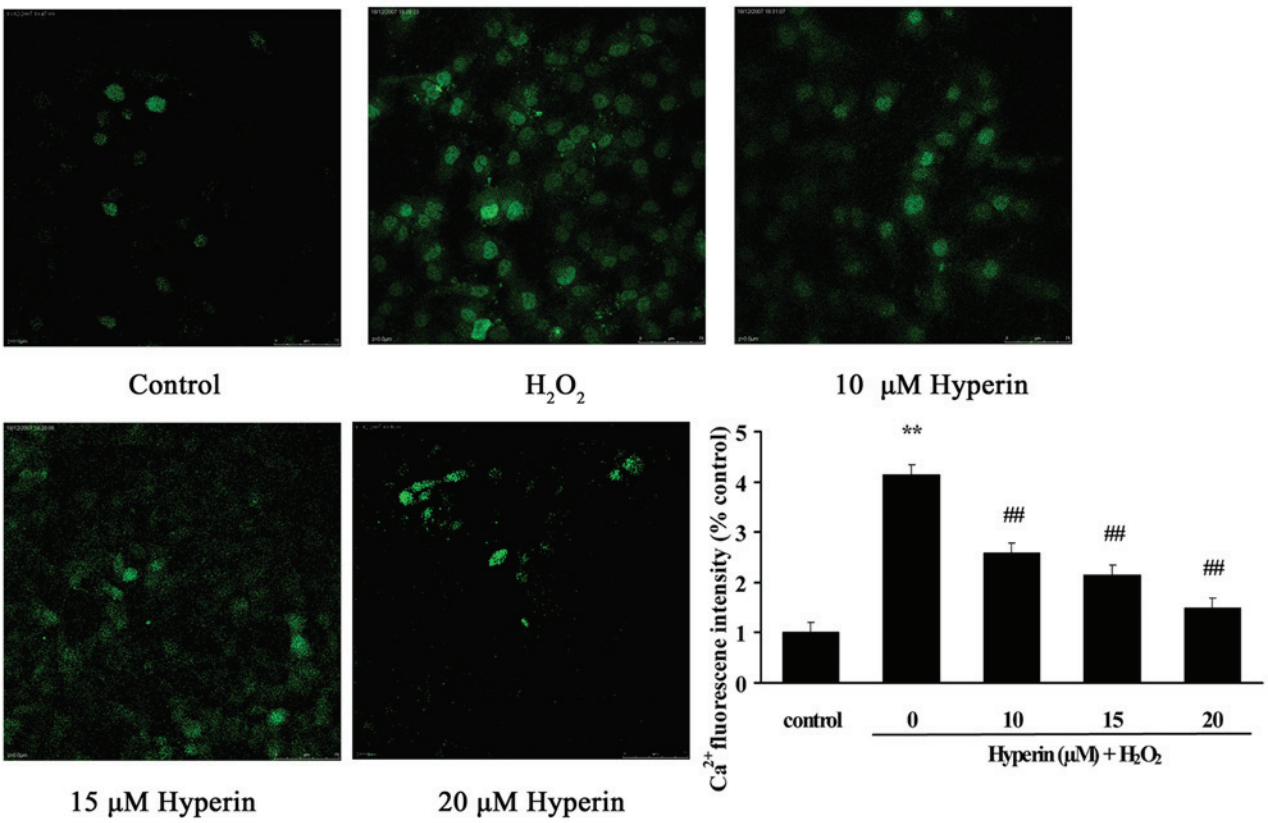

Figure 4. Changes in the Ca^{2+} content in the human umbilical vein endothelial cells was measured with the specific fluorescent probe, Fluo-3/AM. The data are presented as the mean ± standard deviation ($^{**}P<0.01$ vs. control; $^{\#\#}P<0.01$ vs. H_2O_2 treatment). Magnification, x100. H_2O_2, hydrogen peroxide.

Discussion

Apocynum venetum L., known as Luobuma in China, is a traditional Chinese herb exhibiting diverse activities, including inhibition of platelet aggregation and myocardial ischemia/reperfusion injury, hypotension and antioxidative effect, and is widely used for the prevention of cardiovascular diseases. Chemical studies have demonstrated that flavonoids were rich in *A. venetum*, including quercetin, kaempferol, rutin and hyperoside (10). Increasing evidence has demonstrated

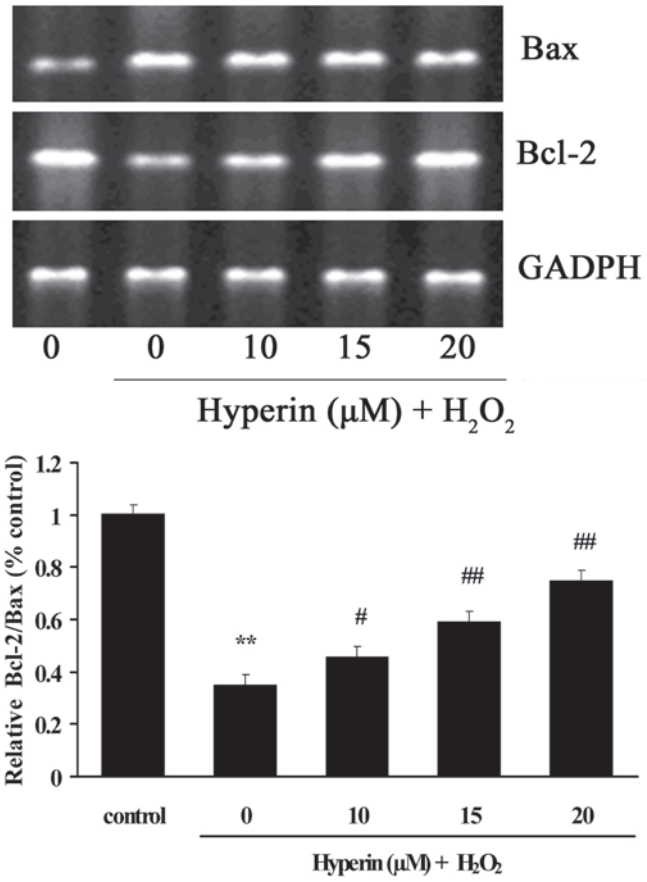

Figure 5. mRNA expression levels of Bcl-2 and Bax as determined by reverse transcription-polymerase chain reaction. A representative agarose gel showing the mRNA levels is shown (top) and the relative expression of Bcl-2/Bax is shown (bottom). The data are presented as the mean ± standard deviation (**P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. H_2O_2 treatment). Bcl, B cell lymphoma; Bax, Bcl-2 associated X protein; H_2O_2, hydrogen peroxide.

that hyperoside exhibited anti-inflammatory, antioxidative and cellular protective effects. The present study demonstrated the protective effects of farrerol against oxidative stress-induced apoptosis in HUVECs.

A previous study demonstrated that the elevation of intracellular Ca^{2+} concentration was involved in the induction of mitochondrial dysfunction and leads to mitochondria-dependent apoptosis (18). Chen *et al* (19) also reported that hyperoside can inhibit Ca^{2+} influx in dissociated neonatal rat brain cells (19). The present study found that hyperoside exhibited the inhibition of H_2O_2-induced increase of intercellular Ca^{2+} concentration in HUVECs, suggesting a protective effect of hyperoside on H_2O_2-induced HUVEC injury.

The Bcl-2 protein family is essential in the mitochondrial apoptosis pathway. This protein family can be divided into two categories: i) Anti-apoptotic members, including Bcl-2 and Bcl-xl; ii) pro-apoptotic members, including Bax and Bak. Numerous previous studies have demonstrated that the ratio of Bcl-2/Bax determines the fate of cells to apoptosis or survival. The decrease of Bcl-2/Bax induced the increase of mitochondrial permeability and the release of cytochrome *c*, which resulted in the activation of caspase-3 and apoptosis (20-24). The present study revealed that hyperoside increased the mRNA and protein expression levels of Bcl-2 and decreased the expression of Bax in the H_2O_2-induced HUVECs, indicating the antiapoptotic effects of hyperoside on H_2O_2-induced apoptosis of HUVECs.

Caspase components are central in the execution of apoptosis (25). Caspase-3, which mediates apoptosis for both extrinsic and intrinsic pathways, is cleaved and activated in the process of apoptosis. The present study detected the effect of hyperoside on the expression of cleaved csspase-3 by western blotting. The result indicated that hyperoside inhibited the increased expression of cleaved caspase-3 induced by H_2O_2 in HUVECs, which demonstrated the antiapoptotic effect of hyperoside on oxidative stress-induced HUVEC apoptosis.

Increasing evidence has indicated that exposure of the cells to H_2O_2 induces the activation of the members of the mitogen-activated protein kinase (MAPK) pathway, including extracellular signal-regulated kinases (ERK) 1/2, c-jun NH2-terminal kinases (JNK) and p38 kinase (26). Among these kinases, the JNK and p38 pathways are commonly considered to be apoptotic, whereas ERK1/2 is associated with protection from apoptosis (27). The present research indicated that the hyperoside regulated the activation of p38 MAPK induced by H_2O_2 in HUVECs, which demonstrated that the antiapoptotic effect of hyperoside was likely associated with its regulation of the p38 MAPK signaling pathway.

In conclusion, the present study demonstrated that hyperoside protected HUVECs from H_2O_2-induced apoptosis, which was likely associated with the regulation of hyperoside on intracellular Ca^{2+} content, expression of apoptosis-associated proteins (Bcl-2, Bax and Cleaved caspase-3) and activation of the p38 MAPK. The findings suggested that hyperoside

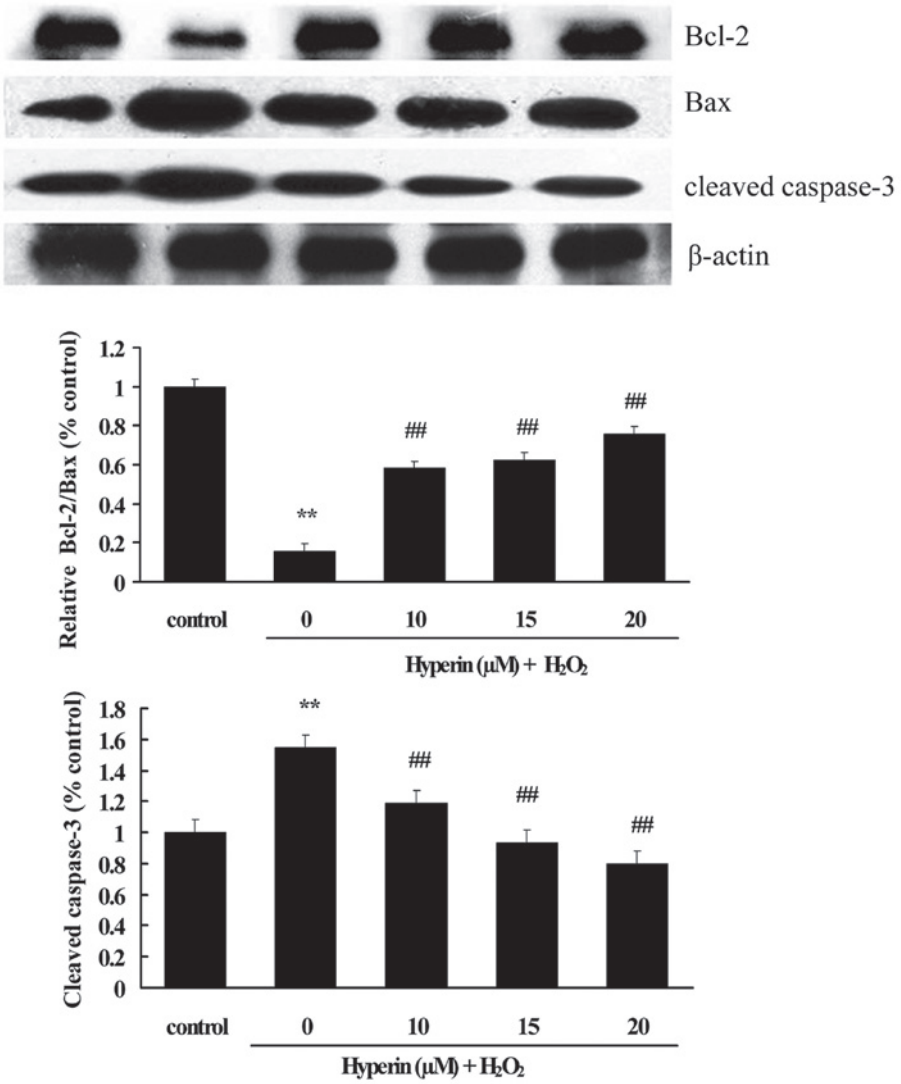

Figure 6. Expression levels of Bcl-2, Bax and cleaved caspase-3 were assessed by western blotting. The data are presented as the mean ± standard deviation (**P<0.01 vs. control; $^{\#\#}$P<0.01 vs. H_2O_2 treatment). Bcl-2, B-cell lymphoma; Bax, Bcl-2-associated X protein.

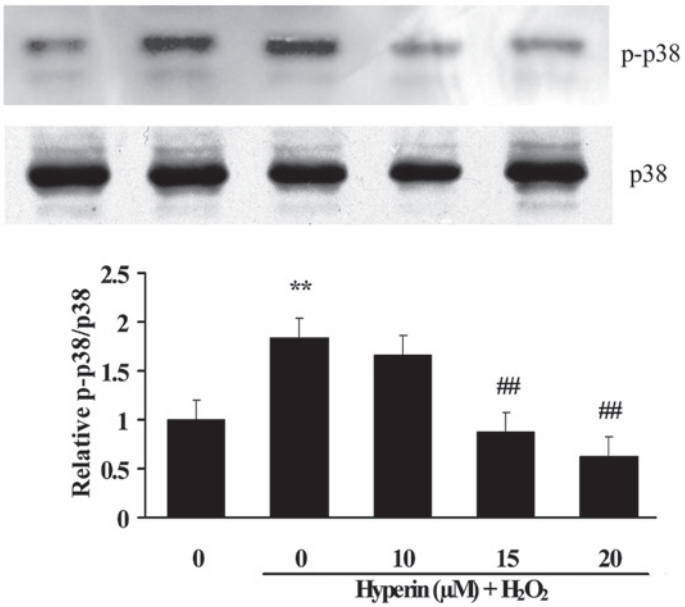

Figure 7. Expression levels of p38 and p-p38 were determined by western blotting. The data are presented as the mean ± standard deviation (**P<0.01 vs. control; $^{\#\#}$P<0.01 vs. H_2O_2 treatment). p-, phosphorylated.

protected cells from oxidative stress-induced injury, which was likely associated with the prevention of cardiovascular diseases.

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