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Original Research Article

Evaluation of Ginsenoside Rg1 as a Potential Antioxidant for Preventing or Ameliorating Progression of Atherosclerosis

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Abstract

Purpose: *To determine whether Rg1 inhibits H_2O_2-induced injury in human umbilical vein endothelial cells (HUVECs), an injury often regarded as a key early event in the development of atherosclerosis.*
Methods: *Cell viability of HUVECs treated with Rg1 and/or H_2O_2 was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Lactate dehydrogenase (LDH) release, lipid peroxidation, and reserved oxidase were detected using different available kits. The apoptosis pathway involved in the effect of Rg1 was also evaluated.*
Results: *Exposing HUVECs to 100 μmol/L H_2O_2 significantly decreased cell viability (78.12 ± 1.78 %), nitric oxide production, and nitric oxide synthase, superoxide dismutase, and glutathione activities, but markedly increased malondialdehyde content (from 26.87 ± 3.97 to 45.84 ± 3.50 nmol/mg of protein) and LDH release (from 8.63 to 31.42 %) ($p < 0.05$). These results were accompanied by a decrease in mitochondrial membrane potential and up-regulation of Bid and caspase-3, -8, and -9 mRNA expressions. However, pretreatment with different Rg1 concentrations (4, 8, and 16 μmol/L) markedly attenuated these changes ($p < 0.05$).*
Conclusion: *Rg1 may protect HUVECs against H_2O_2-induced injury via the anti-oxidative and anti-apoptosis mechanisms, which could be applied potentially for the prevention of endothelial cell dysfunctions associated with atherosclerosis.*

Keywords: *Ginsenoside Rg1; Human umbilical vein endothelium, Oxidative damage; Atherosclerosis.*

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INTRODUCTION

The endothelium is a thin layer of cells that line the interior surface of blood vessels, forming an interface between the circulating blood in the lumen and the rest of the vessel wall [1]. Endothelial injury or the loss of proper endothelial function is a hallmark for vascular diseases and is often regarded as a key early event in the development of atherosclerosis (AS) [2].

Numerous studies have shown that oxidative stress, which is defined as excessive production of reactive oxygen species (ROS), is one of the main causes of endothelial cell injury [3]. ROS are continuously generated in cells as products of cellular oxidation–reduction processes and as the mechanisms of biophylaxis [4]. Hydrogen peroxide (H_2O_2) is one of the most important ROS that can easily penetrate the plasma membrane and can affect neighboring cells and H_2O_2-producing cells. It plays a central role in

vascular pathophysiology and has been extensively used in studying the oxidative injury model of endothelial cell *in vitro* [5].

Panax ginseng is a medicinal plant cultivated in many oriental countries. *P. ginseng* has been used for thousands of years as a traditional medicine and a daily supplement in China. Numerous active ingredients of *P. ginseng* have been isolated and characterized. Among these, ginsenoside Rg1 is one of the most active and abundant steroid saponins that shares structural similarity with many steroid hormones [6]. Gingenoside Rg1 can attenuate oxidative damage in the liver of thioacetamide-treated rats [7]. In addition, several studies have shown that Rg1 has protective effects on the central nervous and endocrine systems [8]. However, the effects of ginsenoside Rg1 on regulating the free-radical scavenging system and protecting endothelial cells against H_2O_2-induced oxidative damage are still unknown. Therefore, the present study aims to investigate the protective effect of Rg1 on H_2O_2-induced injury in human umbilical vein endothelial cells (HUVECs).

EXPERIMENTAL

Cell culture

HUVECs were obtained from the College of Medicine of Nanchang University. Fetal calf serum (FCS), Dullbecco's modified Eagle's medium (DMEM), and serum-free medium were purchased from Gibco (Invitrogen, USA). Ginsenoside Rg1 was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

The HUVECs were incubated in DMEM medium supplemented with 10% FCS, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 0.29g/L L-glutamine and then maintained at 37 °C in a humidified atmosphere with 5% CO_2. The culture media were changed every 2 days. Upon reaching confluence, the cells were harvested by treatment with 0.25% trypsin and 0.02% EDTA for 1 min and then replanted.

Determination of cytotoxicity of ginsenoside Rg1 on HUVECs

When cells were 80 % confluent, the media were removed and the fresh serum-free media with different concentrations of Rg1 (1, 4, 16, 64, and 250 μmol/L) were added and incubated for 24h. Thereafter, the media containing Rg1 were discarded and the cells were washed twice with PBS. Then, cells were incubated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, USA) (final concentration 0.5 mg/ml) for 4 h at 37 °C. The media were removed from each well and 200 μL of DMSO was added. The absorbances were measured at 490 nm using a Thermo Scientific Multiskan MK3 Microplate Reader (Thermo Fisher, USA).

Effect of ginsenoside Rg1 on viability of H_2O_2-induced HUVECs

Based on preliminary findings, 100 μmol/L of H_2O_2 was selected to establish the oxidative stress model in the present study. HUVECs were randomly divided into the control, H_2O_2 and four ginsenoside Rg1 groups. In the ginsenoside Rg1 groups, the cells were pre-treated with different final concentrations of ginsenoside Rg1 (1, 4, 16, and64 μmol/L) for 24 h, followed by co-culture with 100 μmol/L of H_2O_2 for another 8 h. Finally, the effects of Rg1 on the H_2O_2-induced cell injury in HUVECs were measured through MTT assay as described above.

Determination of lactate dehydrogenase (LDH) release, nitric oxide (NO) production, malondialdehyde (MDA) content, superoxide dismutase (SOD), glutathione (GSH), and nitric oxide synthase (NOS) activities

LDH release (%), NO production, MDA concentration, and SOD, GSH, and NOS activities were determined using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, China). LDH was released from injured cells and used as an index of cell injury. LDH release (%) was calculated as in Eq 1. The MDA levels were evaluated by the thiobarbituric acid reacting substance (TRARS) methods. Briefly, MDA reacts with thiobarbituric acid at an acidic high temperature and form a stable chromophoric production. The absorbance of the production was determined at 532 nm and expressed as nmol/mg protein[9]. The activities of NOS were spectrophotometrically measured with commercially-available kits based on the oxidation of oxyhaemoglobin to methaemoglobin by nitric oxide. NO production was tested by measuring the accumulation of nitrites, a stable product of NO metabolism in the supernatant of the cells [10]. In brief, the supernatant of the cells was mixed with the Griess reagent (1 percent sulfanilamide, 0.1 percent *N*-l-naphathyletylenediamine dihydrochloride and 2.5 percent phosphoric acid) at room temperature for 10 min. Nitrite products in the supernatants were determined by measuring absorbance at 540 nm. The assay of SOD activity was based on the ability of SOD to inhibit the oxidation of hydroxylamine by O^{2-} produced from the xanthine–xanthine oxidase system. One enzyme

unit (U) of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition in the rate of nitro-blue tetrazolium (NBT) reduction at 560 nm and was expressed as U/mg protein[11]. GSH was measured based on the fact that 5, 5-dithiobis 2-nitrobenzoic acid reacts with GSH to generate 2-nitro-5-thiobenzoic acid which is yellow. Hence, the GSH concentration in the assay mixture can be determined by measuring absorbance at 412 nm [11].

LDH release (%) = {(LDHs)/(LDHs + LDHc)}100 … (1)

where LDHs is the release of LDH in the supernatant and LDHc is the release of LDH from the cell lysate.

Flow cytometry analysis

The apoptosis of HUVECs was assessed by an annexin-V/propidium iodide binding assay (Biosciences, USA) according to the manufacturer's instructions. The results were interpreted as follows: cells negative for both PI and Annexin-V-FITC staining were considered as live cells; PI-negative and Annexin-V-FITC-positive stained cells were considered in early apoptosis; PI positive and Annexin-VFITC-positive-stained cells were considered in late apoptosis or secondary necrosis.

Measurement of mitochondrial membrane potential (ΔΨm)

Rhodamine 123, a cationic fluorescent dye whose mitochondrial fluorescence intensity decreases quantitatively with the dissipation of the mitochondrial membrane potential, was used to evaluate perturbations in mitochondrial membrane potential [12]. The HUVECs were cultured as described above and divided into the control, H_2O_2, and Rg1 groups. Subsequently, the cells were harvested, washed twice with cold phosphate-buffered saline, and then stained with 1 μmol/L Rhodamine 123 for 30 min in the dark at room temperature. Fluorescence was measured via flow cytometry at an excitation wavelength of 485 nm.

Quantitative real-time (qRT)-PCR) for mRNA expression

Total RNAs were isolated from HUVECs using Trizol reagent (Invitrogen, Carlsbad, USA) and were determined by using spectrophotometer (Beckman Instrument, California, USA). One μg of RNA was reverse-transcribed with PrimerScript™ RT-PCR kit (TaKaRa code: DRR014A) according to the manufacturer's protocol. Quantitative real-time PCR was performed to determine the expression levels of genes such as Bid, as well as caspase-3, -8, -9 using SYBR Premix Ex Taq™ (TaKaRa Code: DRR041A) in the ABI 7900HT Real-Time PCR system (Applied Biosystems, USA). The following PCR conditions were used: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 1 min. The florescence signals were detected with an ABI 7900HT Version 2.3 sequence detection system (Applied Biosystems, USA). The cycle threshold (Ct) values for each gene were determined and the gene expression data were normalized to the endogenous control β-actin. The relative mRNA expression levels were calculated via the comparative threshold cycle (ΔCt) method, where ΔCt is the difference in Ct value between the target gene and β-actin. Primers used for qRT-PCR are listed in Table 1.

Statistical analysis

Data are expressed as mean ± SD (n ≥ 3). Statistical comparisons were performed with SPSS 17.0. One-way ANOVA and Student's *t*-test were carried out to determine statistical significance. $P < 0.05$ was considered statistically significant.

RESULTS

Cytotoxicity of ginsenoside Rg1 on HUVEC

The cytotoxicity of Rg1 on HUVEC was excluded by analyzing its effects on the proliferation of HUVEC. No significant difference was found in the cell viability between cells treated with 1 and 4 μmol/L of Rg1 and the control ($p < 0.05$). By

Table 1: Sequences of primers used in qRT-PCR

Gene	Forward	Reverse	Product size (bp)
β-actin	AGTTGCGTTACACCCTTTCTTG	CACCTTCACCGTTCCAGTTTT	152
Bid	AGTCACACGCCGTCCTTGCT	GCTGTGACCACATCAAGCTTTAG	157
Caspase-3	TGTGAGGCGGTTGTGGAAGAGT	AATGGGGGAAGAGGCAGGTGCA	182
Caspase-8	TGTCCTTCCTGAGGGAGCTGCT	TGAGCCCTGCCTGGTGTCTGAA	115
Caspase-9	TGGAGGATTTGGTGATGTCGAGCA	ATCTGGCTCGGGGTTACTGCCA	97

contrast, 16, 64, and 250 μmol/L of Rg1 significantly promoted the proliferation of HUVECs ($p < 0.05$), however, the survival rate of 64 μmol/L and 250 μmol/L was no significant difference. Therefore, 1, 4, 16, and 64 μmol/L of Rg1 were used for subsequent studies.

Protective effect of ginsenoside Rg1 on the H_2O_2-injury HUVEC

Cell viability was significantly decreased when the HUVECs were exposed to H_2O_2 ($p < 0.05$). The cell survival rate was approximately 78.12% ± 1.78% in the H_2O_2 group. After pretreatment with Rg1 for 24 h prior to oxidative injury, cell viability was improved in a concentration-dependent manner compared with the H_2O_2 group. In addition, after pretreatment with 4, 16, and 64 μmol/L Rg1, the cell survival rates were approximately 80.19 ± 1.54, 83.47 ± 3.24, and 90.30 ± 2.33%, respectively. These results suggest that Rg1 protected HUVECs against oxidative stress.

Effect of Rg1 on representative indicators of H_2O_2-induced cell injury

As shown in Fig 1A, LDH release was 8.63 % in the control group, and a dramatic increase (31.42 %) was observed after exposure to 100 μmol/L H_2O_2. However, LDH releases were markedly decreased after pre-treatment with different concentrations of Rg1 (1, 4, 16, and 64 μmol/L) for 24 h compared with those in the H_2O_2 group ($p < 0.05$, Fig. 1A).

The MDA concentration in the control group was 26.87 ± 3.97 nmol/mg protein. It was significantly increased in the H_2O_2 group (45.84 ± 3.50 nmol/mg protein) ($p < 0.05$, Fig. 1B). By contrast, MDA concentrations decreased markedly in the Rg1 groups compared with those in the H_2O_2 group ($p < 0.05$).

As shown in Fig. 1 (C and D), the NO level and NOS activity in HUVECs (25.71 ± 5.00 μmol/L and 1.58 ± 0.36 U/mg of protein, respectively) significantly decreased in the H_2O_2 group compared with those in the control group (41.99 ± 5.01 μmol/L and 2.49 ± 0.54 U/mg protein, $p < 0.05$). However, pre-treatment with Rg1 triggered a distinct, dose-dependent increase in NO level and NOS activity than that in H_2O_2 group ($p < 0.05$).

Treating HUVECs with 100 μmol/L of H_2O_2 significantly decreased SOD and GSH activities (44.23 ± 6.04 U/mg and 98.16 ± 11.07 U/mg of protein, respectively) compared with those in the control group (70.60 ± 4.89 U/mg and 115.34 ± 18.93 U/mg of protein, respectively) ($p < 0.05$, Figs. 1E and 1F). However, pre-incubation with Rg1 for 24 h attenuated the changes in SOD and GSH activities ($p < 0.05$).

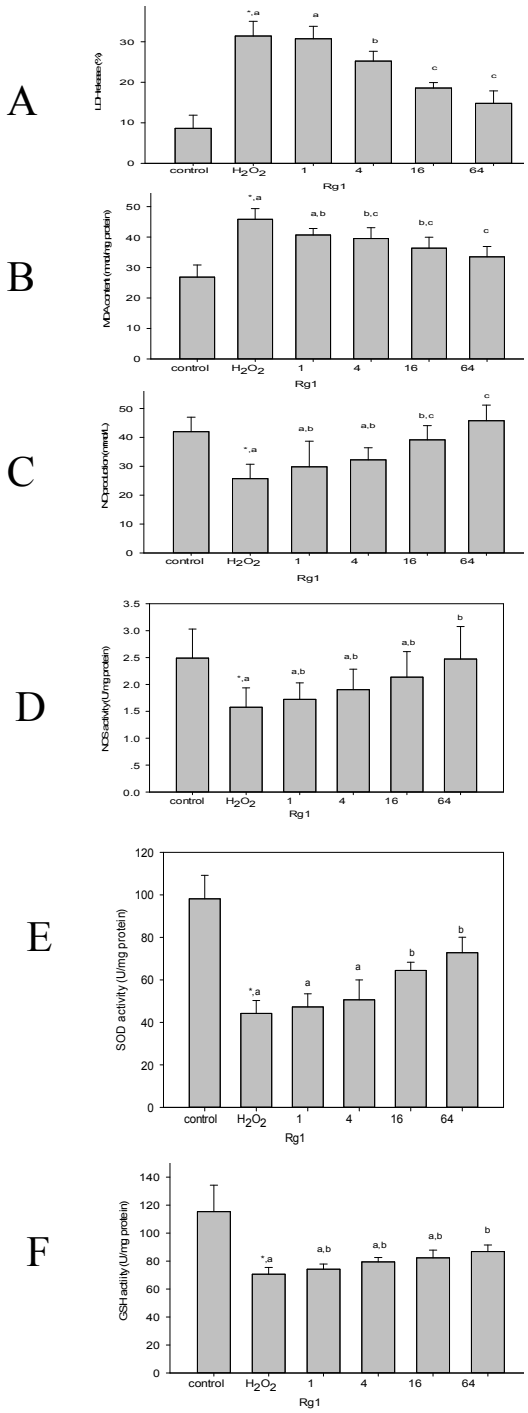

Fig 1: Effect of Rg1 on mediators related to oxidative stress in HUVECs. Asterisks (*) indicate statistically significant differences between control and H_2O_2 groups ($p < 0.05$), while letters a, b, and c indicate statistically significant differences between H_2O_2 and Rg1 groups ($p < 0.05$).

These results suggest that Rg1 protects HUVECs against H_2O_2-induced cell injury via the anti-oxidative system.

Effect of Rg1 on H_2O_2-induced apoptosis in HUVECs

The amount of cells that stepped into early apoptosis increased from 0.1% in the control group to 34.0% in the H_2O_2 group (Fig. 2).. However, the percentages of apoptosis decreased to 20.0% and 10.9% in the groups treated with 4 and 64 μmol/L Rg1, respectively. These results suggest that Rg1 elicits a protective effect on H_2O_2-induced apoptosis in HUVECs. This effect is particularly important in maintaining normal physiological functions and preventing the formation of atherosclerotic plaques.

Effect of Rg1 on ΔΨm

Apoptosis may be initiated through mitochondrial-induced pathways. The changes in ΔΨm reflect the initial cell apoptotic phenomenon. In the present study, ΔΨm was measured using flow cytometry. As shown in Figs. 3A and 3B, a more substantial decrease in ΔΨm was observed in the H_2O_2 group than in the control group. However, the two peaks shown in Figs. 3C and 3D suggest that a high concentration of Rg1 could attenuate the decline in ΔΨm.

Effect of Rg1 on mRNA expression

Depolarization of mitochondrial membrane induces the expression of several anti-apoptotic and pro-apoptotic proteins, most notably Bid, that translocate from the mitochondria into the cytosol, thereby activating caspase-3, 8, 9 kinase. In the current study, the mRNA expressions of Bid and caspase-3, -8, and -9 were significantly up-regulated in the H_2O_2 group compared with those in the control group. However, different concentrations of Rg1 suppressed H_2O_2-induced up-regulations. This result indicates that the protective mechanism of Rg1 on H_2O_2-induced injury in HUVECs may be involved in the anti-apopotic system (Fig. 4).

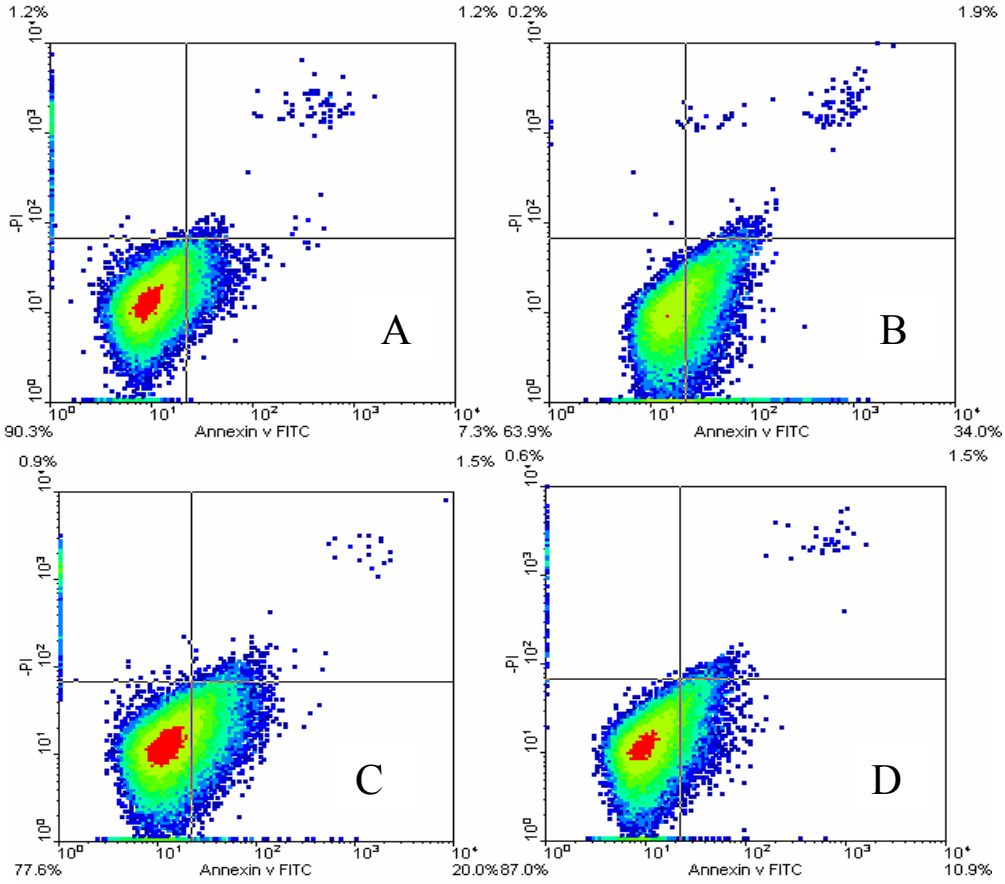

Fig 2: Bivariate graphs illustrating FITC-PI fluorescent resolution of HUVECs. Quadrant analysis of fluorescence intensity of gated cells in Annexin V-FITC and PI channels was based on 16,000 events. A: control; B: H_2O_2 group; C: ginsenoside Rg1 group (4 μmol/L); D: ginsenoside Rg1 group (64 μmol/L)

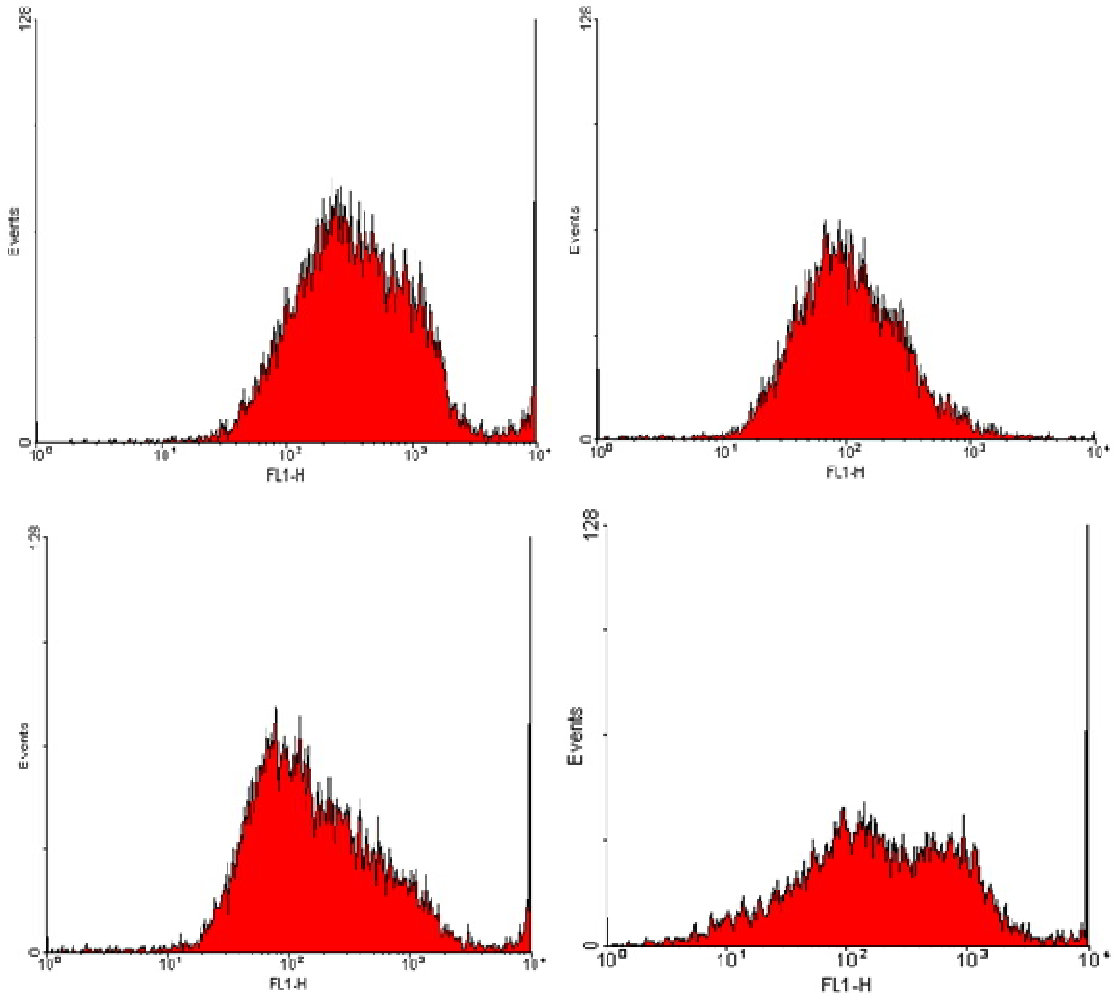

Fig 3: Effect of Rg1 on the mitochondrial membrane potential. $\Delta\psi_m$ depolarization was detected using flow cytometry with Rh123 staining. A: control; B: H_2O_2 group; C: ginsenoside Rg1 group (4 μmol/L); D: ginsenoside Rg1 group (64 μmol/L).

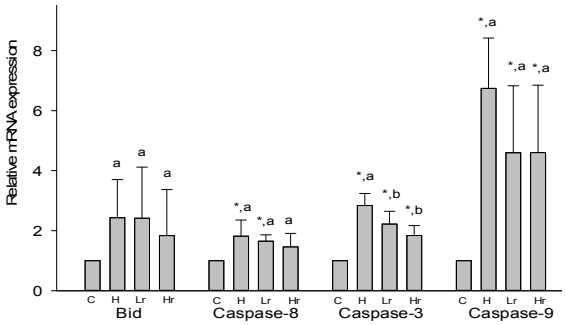

Fig 4: Effect of Rg1 on mRNA expression of Bid, enos, and caspase-3, -8, and -9 in HUVECs. ***Note:*** C = control; H = H_2O_2 group; Lr = low ginsenoside Rg1 concentration group, Pretreatment with 4 μmol/L Rg1 for 24 h prior to H_2O_2 (100 μmol/L, 8 h) treatment. Hr = high ginsenoside Rg1 concentration group, pretreatment with 64 μmol/L Rg1 for 24 h prior to H_2O_2 (100 μmol/L, 8 h) treatment. Asterisks (*) indicate statistically significant differences between the control and H_2O_2 groups ($p < 0.05$), whereas a, b, and c indicate statistically significant differences between the H_2O_2 and Rg1 groups ($p < 0.05$).

DISCUSSION

Ginsenoside Rg1, one of the major active molecules from *P. ginseng*, may attenuate the oxidative damage in rat liver cells [7]. However, the effects of ginsenoside Rg1 on regulating the free-radical scavenging system and protecting endothelial cells against H_2O_2-induced oxidative damage are still unknown. To investigate the protective effect of Rg1 on endothelial cells against H_2O_2-induced oxidative damage, indicators of oxidative stress-related cell injury (LDH, MDA, NO, NOS, SOD, and GSH) were measured in the present study. Lipid peroxidation is one of the main phenomena in free-radical-mediated cell damage. MDA is a by-product of lipid peroxidation induced by excessive ROS, which has been widely used as a biomarker of oxidative stress. A significant increase in MDA production is accompanied by increased LDH release [13]. NO, a diatomic free radical with a half-life of a few seconds, is one of the most important mediators in regulating

endothelial functions. It is synthesized by NOS enzymes in which NO and L-citrulline are two products, and L-arginine is the substrate [14]. Within the vasculature, NO induces vasodilatation, prevents platelet adhesion to endothelial cells, inhibits platelet aggregation, controls smooth muscle cell proliferation and migration, maintains an endothelial cell barrier function, and regulates apoptosis [15]. At higher levels, O^{2-} reacts with NO to form a cytotoxic peroxynitrite (ONOO−) and decrease NO production, which plays a critical role in endothelial cell damage [16]. Several studies have reported that the down-regulation of NO production is implicated in the pathogenesis and clinical course of all known cardiovascular diseases and is also related to future risks of adverse cardiovascular events [17]. In the present study, the NO production, NOS activity, and mRNA expression of eNOS were significantly decreased in the H_2O_2 group ($p < 0.05$). However, these H_2O_2-induced decreases were significantly inhibited in a dose-dependent manner after pretreatment with different Rg1 concentrations prior to H_2O_2 addition. SOD and GSH play a pivotal role in preventing ROS-induced cellular damage. Therefore, intracellular ROS can be effectively eliminated by the combined action of SOD and GSH, providing a repairing mechanism for oxidized membrane components [18]. In the present study, the activities of SOD and GSH in HUVECs significantly decreased after exposure to H_2O_2, indicating impairment of antioxidant defenses. Nonetheless, pre-incubating HUVECs with Rg1 significantly attenuated these H_2O_2-induced cellular events and enhanced endogenous antioxidant preservation. These results suggest that Rg1 protects HUVECs against oxidative stress through the anti-oxidation system, which is beneficial for the prevention and treatment of AS.

Apoptosis is a highly ordered cell death process that may be initiated by mitochondrial inducing pathways [19]. In other words, the mitochondria play a major role during apoptosis induction, leading to the organized degradation of cellular structure and results in the formation of apoptotic bodies [20]. Recent evidence suggests that the opening of the mitochondrial permeability transition (MPT) pore is a critical event in the process leading to apoptosis. The opening of the MPT pore can cause the dissipation of the inner ΔΨm and culminate in the disruption of outer membrane integrity, which leads to the liberation of inter-membrane proteins from the mitochondrion [21]. In addition, Bid is a pro-apoptotic Bcl-2 protein containing the only BH3 domain that can induce the opening of the mitochondrial voltage-dependent anion channel. This opening also results in the release of some pro-apoptotic factors from the mitochondria. Once in the cytosol, these apoptogenic proteins activate caspase proteases, amplifying the apoptotic process [22].

Although many potential stimuli may initiate apoptosis, these signals seem to converge on the caspase pathway to execute the final phases of the apoptotic process [23]. Caspase-8 and caspase-9 have been linked to the mitochondrial death pathway and are initiator caspases that act upon caspase-3. In some cell lines, caspase-3 is the effector caspase that plays a direct role in the proteolytic cleavage of cellular proteins responsible for the progression to apoptosis. The sequential activation of caspase-3, -8, and -9 finally induce the occurrence of apoptosis [24].

In the current study, upon exposure to 100 μmol/L H_2O_2, a decrease in ΔΨm and an increase in the mRNA expression of Bid and caspase-3, -8, and -9 were clearly observed compared with the control. High concentrations of Rg1 ameliorated the decrease in ΔΨm (Fig. 3) and the upregulation of H_2O_2-induced mRNA expression . These results suggest that the protective mechanism of Rg1 on H_2O_2-injured HUVECs may be involved in the anti-apoptosis system initiated by mitochondrial-induced pathways.

CONCLUSION

In conclusion, ginsenoside Rg1 can protect HUVECs against H_2O_2-induced injury by regulating the indicators related to oxidative stress, enhancing anti-oxidant enzyme activity, and suppressing the over-expression of Bid and caspase-3, -8, and -9. The protective mechanism of Rg1 on H_2O_2-induced injury in HUVECs may be a caspase-dependent, anti-apoptotic mechanism involved in mitochondrial-induced pathways. The results of the present study provide important information for the treatment and prevention of oxidative stress-induced atherosclerosis.

CONFLICT OF INTEREST

The authors declare that there are not conflicts of interest.

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