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The protective effects of a novel synthetic β-elemene derivative on human umbilical vein endothelial cells against oxidative stress-induced injury: Involvement of antioxidation and PI3k/Akt/eNOS/NO signaling pathways

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ABSTRACT

Antioxidant therapy is considered as promising strategy for treating oxidative stress-induced cardiovascular disease. Bis (β-elemene-13-yl) glutarate (BEG) is a novel β-elemene derivative. Herein, we examined the antioxidant activity of BEG on human umbilical vein endothelial cells (HUVECs) after injury with hydrogen peroxide (H_2O_2) and investigated the mechanism involved. HUVECs were divided into the following groups: control group (untreated cells); treated groups (cells treated with 0.1, 1, 10 μmol/L of BEG); positive control group (cells treated with 0.1 mM Vitamin E); model group (cells treated with 0.5 mM H_2O_2 alone). Cells were pre-incubated with or without BEG for 24 h and then incubated for a further 2 h with 0.5 mM H_2O_2. Our results showed that BEG significantly reduced H_2O_2 induced loss in endothelial cell viability, reactive oxygen species (ROS) production, reduced lactate dehydrogenase (LDH) release, and malonyldialdehyde (MDA) level in a concentration-dependent manner. Also, BEG increased the cellular the superoxide dismutase (SOD) activity. Moreover, we found that H_2O_2 decreased Akt and eNOS phosphorylation, which perhaps, indirectly reduced nitric oxide (NO) production. These effects induced by H_2O_2, however, were reduced by pre-treatment with BEG. BEG effects were inhibited by a PI3K inhibitor (wortmannin) and eNOS inhibitor (L-NAME). In conclusion, the present study demonstrated that BEG has antioxidant activity. Furthermore, BEG reduced H_2O_2-induced endothelial cells injury by the involvement of antioxidation and PI3K/Akt/eNOS/NO signaling pathways.

1. Introduction

Oxidative stress occurs from an imbalance between the oxidant and the antioxidant defense systems subsequent to physiologic stressors, disease, or environmental factors [1–3]. Substantial evidence suggests that increase oxidative stress plays an essential role in the pathogenesis of cardiovascular diseases including atherosclerosis, and hypertension [4]. Nitric oxide (NO) plays an important role in maintaining cardiovascular homeostasis [5]. NO wields many vaso-protective effects such as vasodilatation, anti-inflammatory and anti-aggregation actions to suppress the lipid oxidation and vascular smooth muscle proliferation [6]. Endothelial nitric oxide synthase (eNOS) has been discovered to be phosphorylated by some kinases such as PKA, Akt, AMPK, PKC, Ca2/calmodulin-dependent kinase II, etc [7]. Furthermore, studies have reported that the best-characterized pathway that regulates eNOS phosphorylation is the phosphatidylinositol 3-kinase (PI3K)-Akt pathway [7,8]. β-elemene, a sesquiterpene compound isolated from the essential oil of Curcuma Wenyujin, is a traditional Chinese medicine (TCM) [9]. β-elemene has been approved by the China Food and Drug Administration for human cancer treatment [10]. Recently, studies in endothelial cells damaged by oxidative stress have demonstrated that β-elemene exerts moderate antioxidant activity against oxidative injury and in vivo, it inhibits neointimal hyperplasia after vascular injury [11]. Furthermore, it prevents H_2O_2-induced monocyte-endothelial cells interactions in vitro and reduces atherosclerosis development in vivo [12,13]. Our previous studies showed that β-elemene derivatives possess antioxidant activities more than β-elemene and the positive control vitamin E in HUVECs damaged by H_2O_2 [14,15].

Though β-elemene (Fig. 1A) has limited bioavailability due to poor water solubility, rapid clearance and short half-life, the introduction of

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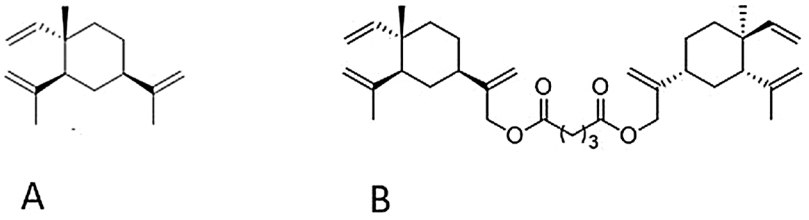

Fig. 1. Chemical structure of β-elemene (A) and Bis (β-elemene-13-yl) glutarate (B).

a hydrophilic group such as oxygen or nitrogen-containing groups into the skeleton of b-elemene could favorably impact its water solubility and activity [14]. Bis (β-elemene-13-yl) glutarate (Fig. 1B), a novel β-elemene derivative, was designed and synthesized to increase the antioxidant activity of the parent compound and to improve its pharmacological properties. In this study, we examine its antioxidant activity, and the potential antioxidant mechanisms involved in oxidative stress-induced HUVECs damage.

2. Materials and methods

2.1. Materials

Hydrogen peroxide (H_2O_2), 3-(4,5-dimethyl– thiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT), dimethylsulfoxide (DMSO) were purchased from (Nanjing KeyGen Biotech. Co.Ltd, Nanjing, China). Nitric oxide (NO), lactate dehydrogenase (LDH), malondialdehyde (MDA) and superoxide dismutase (SOD) commercial kits were obtained from Jiancheng Bioengineering Institute (Nanjing, China). 2,7-dichlorofluorescein diacetate (DCFH-DA), BCA protein assay kit, phenylmethylsulfonyl fluoride (PMSF) and Ripa lysis buffer were purchased from Beyotime (Jiangsu, China). Quantitative RT-PCR kit and SYBR Green Premix Ex Taq were obtained from Takara Biomedical Inc (Japan). NG-nitro-L-arginine methyl ester (L-NAME), and PI3K inhibitor wortmannin were from Sigma (MO, USA). eNOS, phospho-eNOS (Ser1177), Akt, p-Akt (serine 473) and β-actin antibodies and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody were purchased from Santa Cruz Biotechnology Inc (USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (MD, USA).

2.2. Chemical synthesis and physicochemical properties of BEG

The chlorinated *β*-elemene **2** was prepared by chlorination of *β*-elemene **1** with NaClO, followed by treatment with CH_3COONa to give the acylated compound **3**. Then **3** was undergone alkaline hydrolysis to produce 13-*β*-elemol **4**, which was further reacted with glutaric anhydride in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimidehydrochloride (EDCI)/4-*N*,*N*-dimethylaminopyridine(DMAP) to yield the target compound **5** (Fig. 2).^{1}H NMR (300 MHz, $CDCl_3$) δ 5.74 (dd, J = 17.7, 10.5 Hz, 2H), 5.00 (s, 2H), 4.95 (s, 2H), 4.86 (d, J = 3.7 Hz, 2H), 4.81 (s, 2H), 4.76 (s, 2H), 4.59 (s, 4H), 4.52 (s, 2H), 3.39 (s, 2H), 2.00 – 1.90 (m, 4H), 1.64 (s, 6H), 1.60 – 1.50 (m, 3H), 1.46 – 1.32 (m, 6H), 0.93 (s, 6H). HRMS (ESI) calculated for $C_{33}H_{49}O_4$ [M + H]$^+$ 509.3625, found 509.3612.

2.3. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were obtained from the center for new drug safety evaluation and research, school of pharmacy, China pharmaceutical university and maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, 100 U/ml streptomycin. Cells were incubated at 37 C with 95% humidity and 5% CO_2. The medium was changed every 2–3 days until the cells reached (~90) confluence and cells between 4 and 8 passages were used for the experiments. HUVECs were divided into the following experimental groups: 1) control group, untreated cells; 2) treated group, cells pretreated with different concentrations of BEG (0.1, 1 and 10 μmol/L) for 24 h; 3) positive control group, cells incubated with Vitamin E 0.1 mM for 24 h; and 4) model group, cells incubated with 0.5 mM H_2O_2 alone for 2 h. The treated and positive control groups were further induced by incubation with 0.5 mM H_2O_2 for 2 h, while the control group was kept untreated. In western blot experiments, HUVECs were pre-incubated with eNOS for 1 h and PI3k/Akt inhibitors. H_2O_2 (0.5 mM) and Vitamin E (0.1 mM) concentrations were determined from the pilot study by our research group and based on similar methods described previously [12,14 and 16].

2.4. MTT assay of HUVECs

MTT cell assay was performed to study the cytotoxic effect of BEG in HUVECs. Cells were seeded in 96-well plates at a density of 5×10^3cells/well for 24 h. After 24 h incubation, cells were treated with different concentrations of BEG (0.1, 1, 10, 50, 100, 500 and 1000 μmol/L) and the control group was treated only with DMEM for 24 h at 37 °C in 5% CO_2 incubator. MTT solution (50 μL) was added to each group and cells were incubated at 37 °C for a further 4 h. After 4 h incubation, DMEM containing MTT solution was discarded. Cells were then dissolved by adding (200 μL) DMSO to each well and the solutions were mixed thoroughly for 5 min. Finally, the absorbance was determined at 570 nm with a microplate spectrophotometer (BD

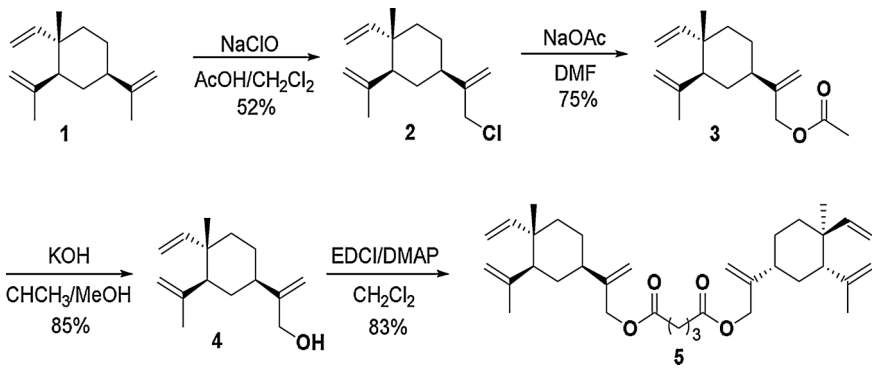

Fig. 2. Chemical synthesis of Bis (β-elemene-13-yl) glutarate.

Bioscience, San Jose, CA, USA). The absorbance of untreated cells was regarded as 100% of cell survival. Cell Viability = (treated viable cells) / (non-treated control viable cells) × 100%.

2.5. Cell viability assay of HUVECs damage by H_2O_2

To study the protective effects of BEG in HUVECs damaged by oxidative stress, MTT cell assay was used as described above with some modification. Cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After 24 h incubation, HUVECs were pre-treated with different concentrations of BEG (0.1, 1, 10 μM) and 100 μM Vitamin E (positive control) for 24 h at 37 °C in 5% CO_2 incubator. Then, these cells were treated with 0.5 mM H_2O_2 for further 2 h. The model group cells were incubated with 0.5 mM H_2O_2 alone for 2 h while the control group was kept untreated.

2.6. Measurement of LDH, NO, SOD and MDA

The contents of LDH, NO, SOD and MDA were determined by following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute Nanjing, China). All procedures entirely described previously [11,17]. The levels of LDH, NO, SOD and MDA were expressed as unit's micromoles per liter supernatant, units per liter supernatant, per milligram protein and nanomoles per milligram protein, respectively. The absorbance of LDH, NO, SOD and MDA were measured at wavelengths of 440, 550, 550 and 530 nm respectively. Each experiment was performed in triplicate.

2.7. Reactive oxygen species (ROS) assay

Intracellular ROS generation was investigated using ROS detection kit following the manufacturer instructions. Briefly, HUVECs were incubated with different concentrations of BEG for 24 h and then stimulated with 0.5 mM H_2O_2 for further 2 h, the control group was left untreated. After that, HUVECs were labeled with 10 μM DCFH-DA and incubated at 37 °C for 30 min. After three times washes with PBS, the DCFH fluorescence of the cells from individual cells was measured at excitation and emission wavelengths, 485 nm and 530 nm respectively, using a fluorescence microplate reader (Tecan, Männedorf, Switzerland). ROS fluorescent intensity index was expressed as the percentage of the control group fluorescence intensity. Each experiment was performed in triplicate.

2.8. Quantitative real-time PCR analysis of mRNA expression

Quantitative real-time PCR was used to detect eNOS mRNA expression in each group. HUVECs were treated with BEG (0.1, 1, 10 μM) for 24 h and then stimulated with (0.5 Mm) H_2O_2 for further 2 h. Total RNA was extracted by trizol reagent according to the manufacturer instructions (Invitrogen). cDNAs were synthesized with Prime Script RT Master Mix kit (Takara, Japan) following the manufacturer instructions. cDNAs were used for PCR amplification using SYBR Premix Ex TaqTM (Takara, Japan) according to the manufacturer instructions on ABI Prism 7900 Sequence Detection System (Applied Biosystems, CA, USA). β-actin was used as an internal control. Primer sequences were as follows: 5′-AAG ATC TCC GCC TCG CTC A-3′(sense) and 5′-GCT GTT GAA GCG GAT CTT A-3′(antisense) for eNOS; 5′-CGC AAA GAC CTG TAC GCC AAC-3′ (sense) and 5′-CAC GGA GTA CTT GCG CTC AGG-3′(antisense) for β-actin.

2.9. Western blot analysis

Cells were treated as described above. HUVECs were washed twice with ice-cold PBS and then lysed by using RIPA lysis buffer containing 1% PMSF at 4 °C. The lysates were centrifuged at 12,000*g* at 4 °C for 15 min to remove the insoluble materials. Supernatants were collected and the protein concentration of each sample was determined using a BCA Protein Assay kit (Pierce, US) according to the manufacturer instructions. The lysates were denatured by boiling them in the SDS sample buffer. Then equal amounts of protein (50 g) from each group were loaded onto 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 1 in 5% phosphate buffered saline (PBS) containing 0.1% (v/v) tween 20 and 5% non-fat dry milk and then incubated overnight at 4 °C with primary antibody against eNOS or phospho-eNOS or Akt, or phospho-Akt Ser 473, and β-actin. The dilution factor for the primary antibodies against 1:500 eNOS, 1:500 phospho-eNOS,1: 1000 Akt, 1: 1000 p-Akt and 1: 1000 β-actin. After washing, the membranes were incubated with the anti-IgG secondary antibody (1:2000) conjugated to horseradish peroxide for 1 h at room temperature. Then the membranes were washed in TBST for 30 min and exposed to enhanced chemiluminescence reagents. Densitometric analysis was performed to quantify the signal intensity. β-actin was used as a standard reference. The relative density of each protein band was normalized to β-actin.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 7.00 (GraphPad Software, Inc.). Data were expressed as means ± S.D from three independent experiments. The ANOVA test was used to determine the statistical significance. The P value ($p < 0.05$) was considered significant among all the analysis.

3. Results

3.1. Cytotoxic effect of BEG in HUVECs

Cytotoxicity of BEG in HUVECs was investigated using MTT assay. Our results indicated that incremental doses of BEG treatment for 24 h did not produce any obvious cytotoxic effect (Fig. 3A). Therefore, doses of 0.1 μmol/L, 1 μmol/L, and 10 μmol/L of BEG were used in the subsequent experiments for examining its possible protective role against H_2O_2 induced toxicity.

3.2. Effect of BEG on H_2O_2 induced HUVECs damage

In order to evaluate whether BEG protects HUVECs against oxidative stress, cell viability was measured by MTT assay. As shown in (Fig. 3B), the control group did not show any significant difference while the model group incubated with H_2O_2 alone for 2 h decreased the cell viability to 63%. In the treated group, treatment of BEG reduced the cell growth inhibition caused by H_2O_2 and increased the cell viability in a dose-dependent manner. The cell viability was restored up to 97.6667% by pretreatment with 10 μmol/L of BEG, which was almost superior to the positive control group (100 μM Vit-E).

3.3. Effect of BEG on H_2O_2 induced LDH leakage in HUVECs

Increased LDH release from HUVECs into the culture medium also reflects cells damage. Therefore, LDH leakage was investigated to further confirm the protective effect of BEG on H_2O_2 induced HUVECs damage. For this purpose, cells were pretreated with different concentrations of BEG (0.1, 1, 10 μmol/L) and Vit E 100 μM for 24 h followed by further treatment with 0.5 mM H_2O_2 for 2 h. As shown in (Fig. 4A), cells treated with 0.5 mM H_2O_2 alone for 2 h significantly increased cells LDH leakage into the medium, while pretreatment of HUVECs with various concentrations of BEG inhibited H_2O_2 -induced LDH leakage in a dose-dependent manner.

3.4. Antioxidant effect of BEG against H_2O_2 induced HUVECs damage

SOD activity and MDA content were evaluated to reflect the level of

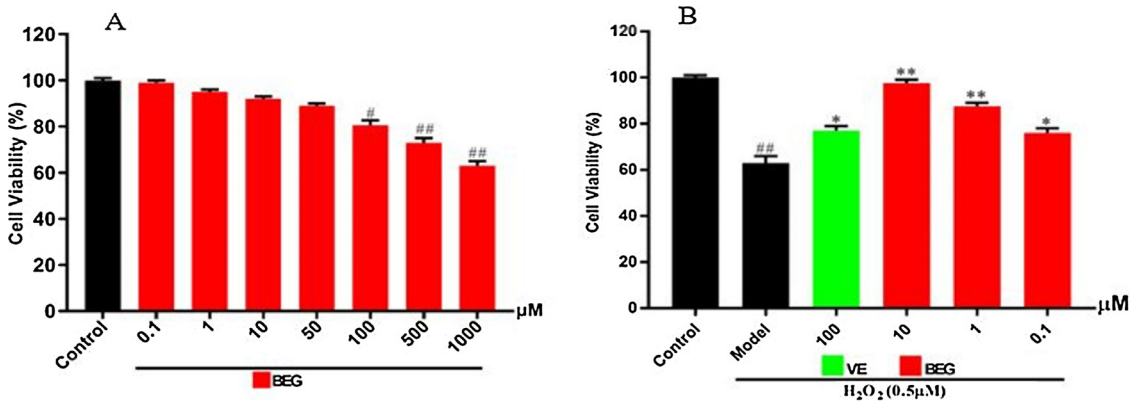

Fig. 3. Effect of BEG on cell viability. (A) Concentration-dependent effect of BEG on cell viability of HUVECs incubated for 24 h. (B) The protective effect of BEG in HUVECs after H_2O_2 induced cells damage. MTT assay was used to evaluate the cell viability. Values are presented as means ± SD, n = 3. ##Greater significant difference from the control group at $P < 0.01$. *Significant difference from the model group at $P < 0.05$. **Greater significant difference from the model and VE groups at $P < 0.01$.

oxidative damage in HUVECs and to study the antioxidant effect of BEG. As shown in (Fig. 4B and C) pre-incubation with H_2O_2 alone for 2 h significantly increased intracellular MDA level and decreased the intracellular SOD activity compared to the control group. While treatment with the indicated concentrations of BEG and Vit-E results indicated that H_2O_2 -induced oxidative stress injury was significantly attenuated by pretreatment with BEG in dose-dependent manner.

3.5. Effect of BEG on ROS production induced by H_2O_2

The intracellular ROS generation was evaluated by the dichlorofluorescein (DCFH) assay. Effect of BEG on ROS level in H_2O_2-treated cells is shown in Fig. 5A & B. Results indicated that BEG reduced the ROS generation in HUVECs damage by H_2O_2 in a dose-dependent manner. Cells treated with 0.5 mM H_2O_2 alone for 2 h increased the intracellular ROS concentration compared with the control group ($P < 0.01$). However, pre-incubation with different concentrations of BEG significantly reduced H_2O_2 -induced ROS overproduction. ROS production in cells treated with H_2O_2 (model group) reduced from 260.9 ± 19.44 to 191.7 ± 2.64, 173.6 ± 2.476, and 133.1 ± 6.10 in the cells treated with BEG (0.1, 1, 10 μmol/L) respectively. These results suggest that BEG significantly reduced ROS overproduction in HUVECs after H_2O_2 induced oxidative stress in a dose-dependent manner.

3.6. Effect of BEG on NO release in H_2O_2 -treated HUVECs

NO is a molecule that has obtained identification as an important

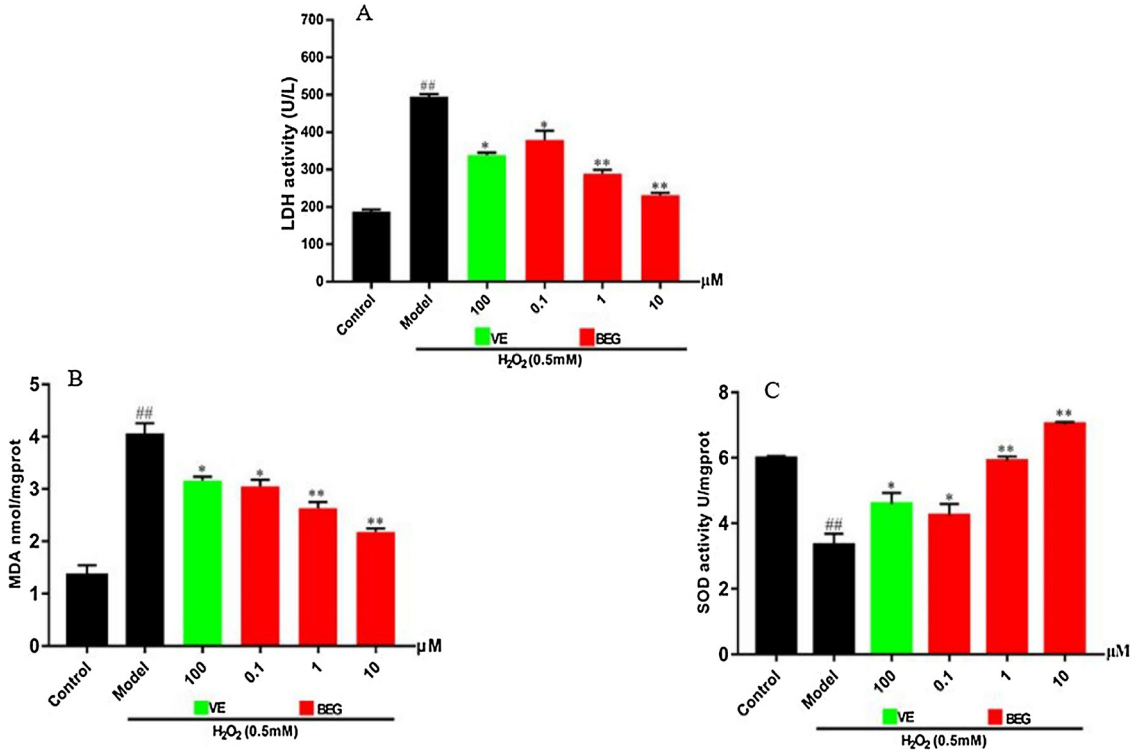

Fig. 4. Antioxidant Effect of BEG against H_2O_2 induced HUVECs damage. (A) Concentration-dependent effect of BEG on LDH leakage. (B) MDA level and (C) SOD activity in endothelial cells after H_2O_2 induced cells damage. Values are presented as means ± SD, n = 3. ##Greater significant difference from the control group at $P < 0.01$. *Significant difference from the model group at $P < 0.05$. **Greater significant difference from the model group at $P < 0.01$.

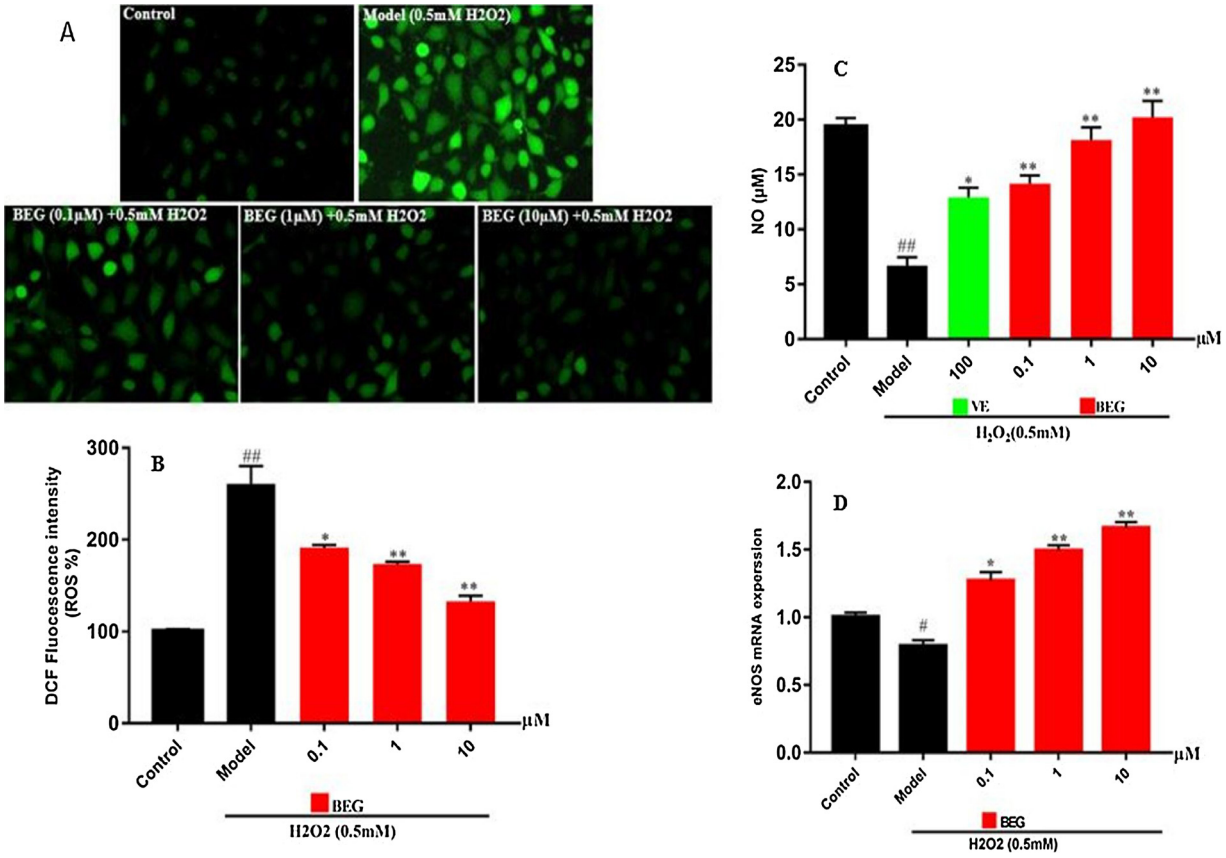

Fig. 5. Effect of BEG on ROS production NO and eNOS mRNA in endothelial cells after H_2O_2 induced cell damage. (A) Intracellular ROS generation in HUVECs was determined by measuring DCFH fluorescence. (B) ROS fluorescent intensity index was presented as the percentage of the control group. (C) Effect of BEG on HUVECs NO release (D) RT-PCR analysis. Effect of BEG on eNOS mRNA expression. Values are presented as means ± SD, n = 3. ##Greater significant difference from the control group at P < 0.01. *Significant difference from the model group at P < 0.05. **Greater significant difference from the model group at P < 0.01.

modulator of cardiovascular diseases, and one of the hallmarks of endothelial dysfunction is a reduction in the bioavailability of NO, which is catalyzed by eNOS [18]. In this study, NO release was assessed. As shown in Fig. 5C, NO production was significantly decreased in the model group compared to the control group ($P > 0.01$). Meanwhile, pre-incubation with indicated concentrations of BEG and Vit-E for 24 h and further incubation for 2 h with H_2O_2 significantly improved NO production in a dose-dependent manner.

3.7. Effect of BEG on decreased Akt/eNOS expression induced by H_2O_2 in endothelial cells

To determine the preliminary molecular mechanisms accounting for the protective effects of BEG on HUVECs against H_2O_2, we investigated the gene expression of eNOS mRNA (Fig. 5D) and the protein expressions of Akt and eNOS (Fig. 6). As shown in (Fig. 5D), treatment with 0.5 mM H_2O_2 for 24 h decreased eNOS mRNA expression compared with the control group. While eNOS mRNA expression was effectively improved by pre-treatment with different concentrations of BEG (0.1, 1, 10 μmol/L) for 24 h and further incubation with 0.5 mM H_2O_2 for 2 h in a dose-dependent manner. We also observed that the expression of eNOS and Akt proteins were enhanced significantly by treatment with BEG in a dose-dependent manner compared with model group alone. These results showed that BEG protects HUVECs from H_2O_2 induced damage and improved the expression of eNOS mRNA (Fig. 5D), Akt, and eNOS proteins (Fig. 6).

3.8. BEG exerts protective effects on endothelial cells through Akt/eNOS pathways

We further examined the underlying mechanisms by which increased eNOS modulates specific cellular functions. Previous studies found that ROS regulates eNOS activity and expression via PI3K/Akt signaling pathway. Akt stimulation leads to eNOS activation and increased the phosphorylation of eNOS (p-eNOS) that in turn increase NO generation. In this study, we found that, in comparison to other experimental groups, BEG treated group up-regulated the Akt phosphorylation and the total Akt level ($P < 0.05$ *vs* groups 2 and 5) and improved phosphorylation of eNOS ($P < 0.05$ *vs* groups 2 and 4) and total eNOS levels ($P < 0.05$ vs groups 2 and 3). Furthermore, these effects were inhibited by PI3K inhibitor wortmannin and eNOS inhibitor L-NAME ($P < 0.01$*vs* BEG + H_2O_2 group). Therefore, these results further demonstrated that the Akt/eNOS signaling pathway was involved in the BEG-induced protective effects on HUVECs as shown in Fig. 7.

4. Discussion

Oxidative stress influences various biological functions of vascular endothelial cells and plays a critical role in the pathophysiology of cardiovascular diseases [19]. High concentrations of oxidative stress, such as H_2O_2 is associated with increased vascular endothelial cell injury [20]. Therefore, there are increasing interests to use antioxidant agents against ROS stimulus in cardiovascular disease, which has been demonstrated in studies of vitamin C, sphingosine 1-phosphate and propofol [21–23]. An abnormality in ROS production and the subsequent decrease in vascular NO bioavailability have been long

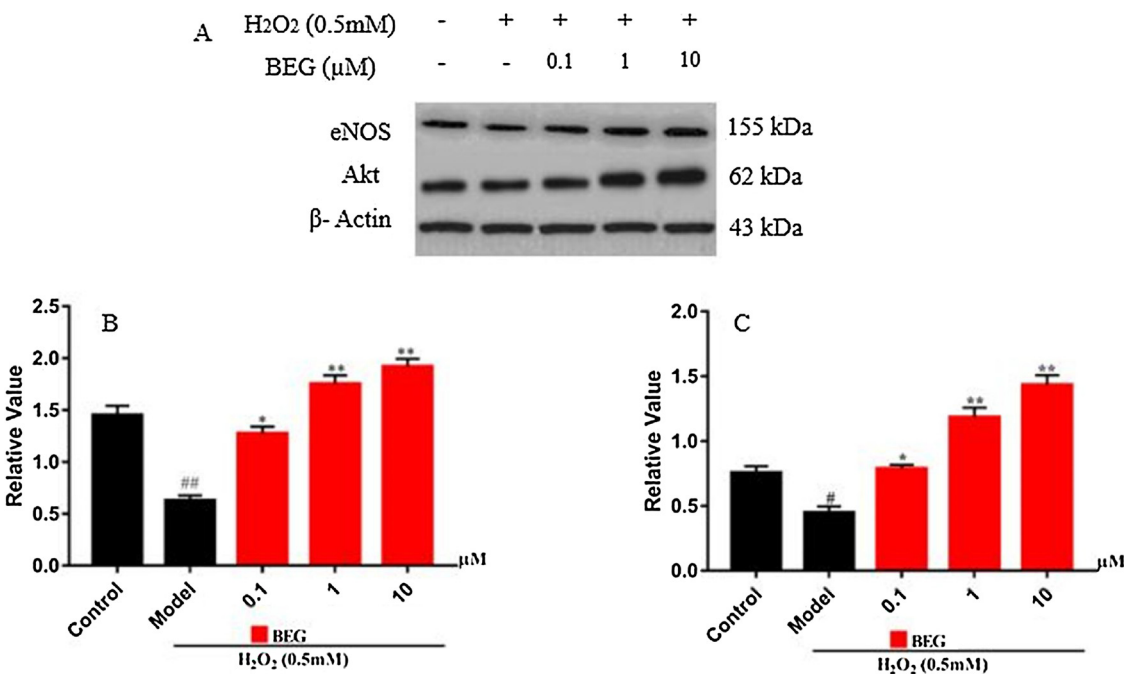

Fig. 6. Effect of BEG on decreased Akt/eNOS expression induced by H_2O_2 in endothelial cells.
(A) Concentration-dependent effect of BEG on Akt and eNOS protein expressions in endothelial cells after H_2O_2 induced damage as determined by Western blot analysis. (B) Relative levels of eNOS protein expression. (C) Relative levels of Akt protein expression. Optical densities were achieved after normalization to β-actin. Values are presented as means ± SD, n = 3. #Significant difference from the control group at $P < 0.05$. ##Greater significant difference from the control group at $P < 0.01$. *Significant difference from the model group at $P < 0.05$. **Greater significant difference from the model group at $P < 0.01$.

suggested to be the common pathogenic mechanism of endothelial dysfunction that leads to various cardiovascular risk factors such as hypertension, atherosclerosis, diabetes mellitus [25]. The present study aimed to examine the antioxidant activity of BEG on vascular endothelial cells damaged by oxidative stress and to investigate the involved mechanisms. Results presented here indicated that BEG increased the survival rate of endothelial cells and possesses significant protective effects against H_2O_2-induced damage. Moreover, cell viability was diminished to approximately 60% when exposed to 0.5 mM H_2O_2, while it was enhanced significantly by pre-treatment with BEG (Fig. 3B). These findings indicated that BEG can effectively prevent oxidative damage induced by H_2O_2 and increase the survival rate of endothelial cells.

Lipid peroxidation is one of the fundamental consequences of free radical-induced cell damage [24]. MDA is a byproduct of lipid peroxidation mediated by ROS overproduction and is commonly used as an

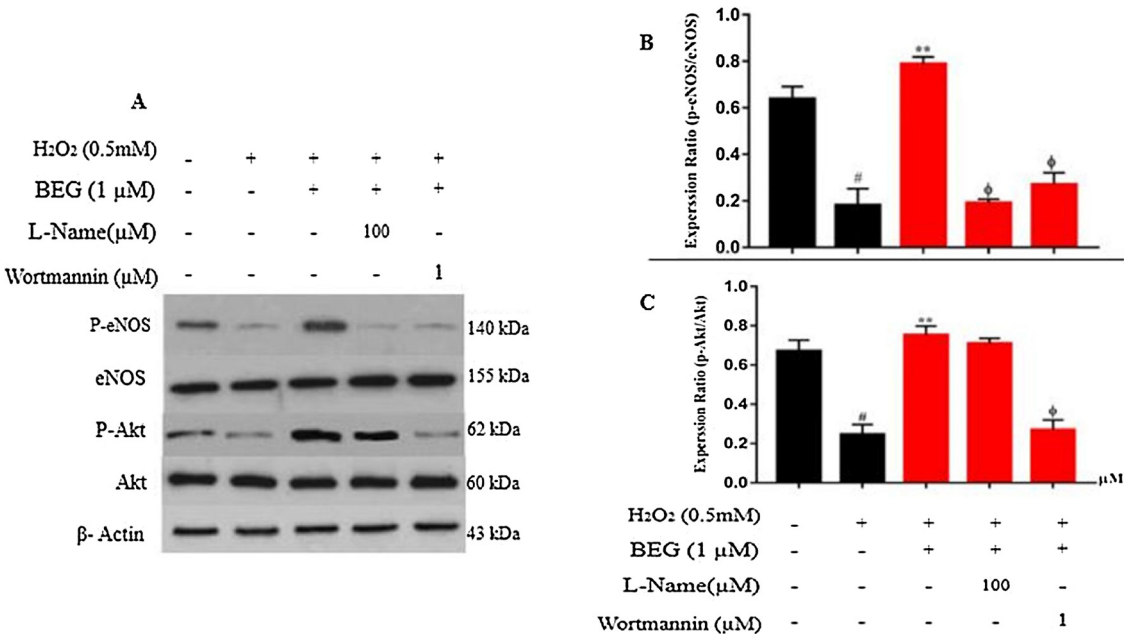

Fig. 7. Involvement of Akt/eNOS pathways in the protective effect of BEG on endothelial cells after H_2O_2 induced damage. (A) Western blot analysis for phospho-eNOS (p-eNOS), eNOS, phospho-Akt (p-Akt), and Akt (B, C). Densitometric data are presented as means ± SD, n = 3. #Significant difference from the control group at $P < 0.05$. **Greater significant difference from the model group at $P < 0.01$. ΦGreater significant difference from the BEG and Control groups at $P < 0.01$.

oxidative stress biomarker [26]. In the present study, MDA level significantly increased in the H_2O_2 damage group while in BEG pre-treated groups, MDA level reduced significantly in a dose-dependent manner (Fig. 4B). From this, we speculate that BEG can attenuate the cell damage caused by H_2O_2. As we know that cells are often armed with several antioxidant agents for the protection against free-radicals. SOD, among others enzymatic and non-enzymatic antioxidants, perform a critical role in preventing cellular injury caused by oxidative stress [27]. Therefore, the intracellular ROS can be effectively eliminated by the combined action of SOD, and other endogenous antioxidants, which provide a repairing mechanism against increased oxidative stress. Our results showed that the activity of SOD in the H_2O_2 damage group was significantly decreased compared with the untreated group (Fig. 4C). It indicates that the antioxidant ability of endothelial cells was decreased by H_2O_2 treatment. However, BEG treated group significantly improved SOD activity, suggesting that BEG strengthens the ability of SOD against free radicals to protect the endothelial cells from oxidative damage induced by H_2O_2. In addition, the effect of BEG was superior to the positive control group vitamin E (100 μmol/L) in preventing oxidative damage in endothelial cells induced by H_2O_2. Previously, we demonstrated that some of the β-elemene derivatives have cytoprotective effects and antioxidant activity against oxidative stress-induced vascular endothelial cells damage [14]. Consistent with previous reports, our results indicated that BEG can successfully inhibit H_2O_2 -induced endothelial cells injury and possess considerable antioxidant activity.

Accumulative evidence has shown that H_2O_2 can cause endothelial cell injury by inducing ROS generation through different pathways [28–30]. To confirm the ability of BEG to scavenge the intracellular ROS generation induced by H_2O_2, endothelial cells were pre-treated with different concentrations of BEG and further incubation for 2 h with 0.5 mM H_2O_2. Non-toxic fluorescence probe dichlorofluorescein (DCFH) was used to confirm ROS production considering its sensitivity for reliable measurement [31]. Incubation of endothelial cells with H_2O_2 increased the intracellular ROS production that is significantly inhibited by pre-incubation with BEG in a concentration-dependent manner. These findings, convincingly suggest that a significant inhibition of H_2O_2 -induced ROS generation by BEG may participate in restoring the viability of endothelial cells. Moreover, nitric oxide (NO) plays a fundamental role in maintaining the vasculature homeostasis, inhibiting the aggregations of blood platelets and preventing conglutination between endothelial cells and between blood cells and endothelial cells [32]. Furthermore, abnormal production of NO is associated with increased oxidative stress and consequently impaired endothelium-dependent vasodilation. There have been several studies demonstrated that down-regulation of NO production is implicated in the clinical course and pathophysiology of all known cardiovascular diseases [33]. As shown in Fig. 5C, our results indicated that NO production was significantly decreased in the model group compared to the control group. On the other hand, pre-incubation with indicated concentrations of BEG and Vit-E significantly improved NO production in a dose-dependent manner. eNOS expression is the main factor for NO production in vascular endothelial cells. As described above, H_2O_2 -induced oxidative damage is a typical model of inducing vascular endothelial cell damage. This model helps in studying the pathology process of cardiovascular diseases and to investigate the novel compounds that may protect blood vessels. The present screenings of eNOS mRNA and protein levels showed that BEG in a dose-dependent manner improves eNOS expression in endothelial cells and leads to the protection of endothelial cells. In contrast, eNOS expression was down-regulated by treatment with 0.5 mM H_2O_2 alone as shown in Fig. 5D. In regards with eNOS expression regulatory mechanism, it has been described that an increased ROS generation in endothelial cells inhibits eNOS expression [34,35].

A large body of evidence has shown that Akt/eNOS-related signaling pathway has been involved in vascular endothelial biology. Akt stimulation induces the phosphorylation of the Akt. The phosphorylated Akt leads to activation of its downstream eNOS substrate and therefore improved NO production, which is an essential molecule for maintaining the biological functions of endothelial cells [4]. Here in this study, our results showed that H_2O_2 decreased expression of eNOS mRNA, eNOS, Phospho-Akt and NO release in endothelial cells indicating that sequences of beneficial physiological activities are mediated by ROS/Akt/eNOS signaling pathways. However, BEG pretreatment successfully reversed H_2O_2 induced cell damage and activates the Akt/eNOS pathways (Fig. 6). To further investigate the association between the Akt/eNOS pathway and the antioxidant effect of BEG, we assessed the effect of PI3k/Akt inhibitor (wortmannin) and eNOS inhibitor (L-NAME) on the antioxidant action of BEG. In agreement with previous studies showing that co-treatment of Wortmannin and L-NAME significantly blunted the phosphorylation of Akt and eNOS respectively in endothelial cells [36,37]. Our current study showed that co-treatment of BEG and L-NAME and Wortmannin reduced eNOS and Akt phosphorylation significantly in HUVEs respectively, under oxidative stress conditions (Fig. 7). Though L-NAME did not reduce the effect of BEG on Akt phosphorylation level, it significantly eradicated the antioxidant effect of BEG on H_2O_2 treated endothelial cells. Conversely, our study found that BEG-induced eNOS phosphorylation was blunted by the PI3K/Akt inhibitor wortmannin in endothelial cells co-treated with H_2O_2. We found that BEG induced protection of HUVECs and multiple biological functions of endothelial cells were significantly reduced, suggesting that these mechanisms are implicated in the protective role of BEG against endothelial cells injury. In light of these findings, we speculate that BEG has the protective effects on human umbilical vein endothelial cells against oxidative stress-induced injury in a dose-dependent manner and it works through PI3k/Akt/eNOS/NO signaling pathways.

5. Conclusion

This study demonstrated that BEG has antioxidant activity and superior to the positive control (vitamin E). BEG offers protection against oxidative stress induced by H_2O_2 by inhibiting ROS production, which in turn inactivates downstream signaling events through PI3K/Akt/eNOS/NO pathways. In this regards, BEG might be considered as a future candidate for developing a novel therapeutic agent in the prevention of oxidative stress-induced cardiovascular diseases.

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