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Original Research Article

Grape seed polyphenol extract mitigates oxidative damages induced by benzopyrene in human aortic endothelial cells by maintaining the AhR and Nrf2 signals balance

Man Zheng[1,2,3,4], Fenglei Zhang[3], Xiang Gong[5], Haitao Yuan[1,2,3,*]

[1]*Department of Cardiology, Shandong Provincial Hospital, Shandong University, Jinan, Shandong 250021, PR China*
[2]*Department of Cardiology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, Shandong 250021, PR China*
[3]*JiNan Key Laboratory of Cardiovascular Disease, Jinan, Shandong 250021, PR China*
[4]*Department of Cardiology, Dongying People's Hospital (Dongying Hospital of Shandong Provincial Hospital Group), Dongying, Shandong 257091, PR China*
[5]*Shenzhen Haichuang Biotechnology Co., Ltd., Shenzhen, 518052, PR China*

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**** Corresponding Author:***
Tel: +860536-81923133
Fax: +860536-81923133
yuanhaitao@sdfmu.edu.cn

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Abstract

Objective: To investigate the protective effects of grape seed polyphenol extract (GSPE) against oxidative damage to human aortic endothelial cells (HAECs) induced by polycyclic aromatic hydrocarbons (PAHs), with a focus on its antioxidative mechanisms and potential regulation of Nrf2 and AhR signaling pathways.

Materials and Methods: GSPE was prepared and characterized for antioxidant activity. A cell model of benzopyrene (BaP)-induced oxidative stress in HAECs was established. Cell viability, apoptosis, reactive oxygen species (ROS) and malondialdehyde (MDA) levels, antioxidant enzyme activity [Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GSH-Px)], mitochondrial function (membrane potential and ATP production), and Nrf2/AhR signaling pathway components were assessed using CCK-8 assay, flow cytometry, fluorescence probes, spectrophotometric analysis, and Western blotting.

Results: GSPE significantly reversed BaP-induced decreases in HAEC viability and inhibited apoptosis. It suppressed BaP-triggered ROS and MDA overproduction, restored antioxidant enzyme activity, and improved mitochondrial function (e.g. membrane potential and ATP levels). Mechanistically, GSPE modulated the Nrf2 and AhR pathways, maintaining their balanced activation to counteract oxidative stress.

Conclusion: GSPE exerts protective effects against BaP-induced oxidative damage in vascular endothelial cells by scavenging ROS and MDA, enhancing antioxidant defenses, preserving mitochondrial integrity, and regulating Nrf2/AhR signaling. These findings highlight GSPE as a potential therapeutic agent for mitigating cardiovascular risks associated with BaP exposure.

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Introduction

Atherosclerosis, a primary contributor to cardiovascular diseases, is a multifaceted process influenced by various factors (Liu et al. 2022). In addition to the accumulation of vascular lipids, oxidative stress can be regarded as a pivotal proatherogenic factor (Khan et al. 2024). Oxidative damage in vascular endothelial cells has been implicated in both the initiation and progression of atherosclerosis. Furthermore, there is increasing evidence that long-term exposure to polycyclic aromatic hydrocarbons (PAHs), is associated with an elevated risk of atherosclerosis (Iwano et al. 2005). PAHs constitute a family of compounds known for their toxicity to humans; among them, benzo(α)pyrene (BaP) is recognized as an environmental carcinogen (Zhang et al. 2024). Some PAHs are classified as or suspected to be potent mutagens, carcinogens, or teratogens. Sources of exposure to these chemicals include automobile emissions, industrial processes, tobacco smoke, cooking methods, and consumption of barbecued foods. Epidemiological studies have also revealed significant correlations between exposure to PAHs and the incidence of dysfunction in vascular endothelial cells (Lv et al. 2022).

Moreover, numerous studies have indicated that PAHs can induce dioxin-like responses through the activation of the aryl hydrocarbon receptor (AhR). The AhR protein plays a crucial role in the toxicological pathways associated with dioxin-like compounds including PAHs (Deval et al. 2024; Dubiel et al. 2023). This ligand-dependent transcription factor responds to various ligands and is critical not only for mediating toxicity but also for regulating immune function, cardiovascular physiology, and xenobiotic metabolism (Gassmann et al. 2010). The interaction between PAHs and AhR can influence the expression of numerous genes and elicit diverse biological or toxic effects. Additionally, cytochrome P450 (CYP) 1A-a xenobiotic-metabolizing enzyme-is regulated by AhR activation (Xie et al. 2024).

In our daily lives, the regular consumption of fresh fruits and natural products can significantly reduce the risk of illness due to their high content of antioxidants that scavenge free radicals. Recently, there has been a growing interest in utilizing natural antioxidants for the prevention and recovery from cardiovascular diseases (Sheng et al. 2023). It is well-established that polyphenols isolated from natural products exhibit a wide range of bioactivities, including antioxidant (Wang et al. 2024a), anti-aging (Zhuang et al. 2024), and anti-inflammatory effects (Om et al. 2022). Grape (*Vitis vinifera L.*) seeds are particularly rich in antioxidant flavonoids, flavonoid glycosides, and polyphenols. Extracts derived from grape seeds are widely employed as metabolic regulators and the reactive oxygen species (ROS) scavengers. Modern pharmacological studies have also demonstrated that flavonoids and polyphenols extracted from grape seeds possess antibacterial (Omoya and Momoh 2023), antiviral (Brandin et al. 2007), and antifungal properties (Saniewska 2004). Furthermore, flavonoids isolated from grape seeds have shown potent anticancer effects (Jifu et al. 1999).

However, the potential of grape seed extracts in mitigating atherosclerosis induced by PAHs has been infrequently investigated. In this study, we hypothesize that grape seed polyphenol extract (GSPE) may confer therapeutic benefits against PAHs-induced damages to vascular endothelial cells by mitigating oxidative stress. Understanding the relationship between the antioxidative effects of GSPE and its underlying mechanisms involved in protecting vascular endothelial cells could support its potential use as a therapeutic agent against atherosclerosis.

Materials and Methods

Reagents and cells

Fresh grape seeds (3.3 kg) were provided by Shenzhen Haichuang Biotechnology Co., Ltd. Endothelial Basal Medium-2 (EBM-2) and fetal bovine serum (FBS) were purchased from Invitrogen (Thermo Fisher Scientific, MA, USA). The chemicals utilized in the current study were of analytical-reagent grade and sourced from Aldrich or Adamas without further purification. All ELISA kits were obtained from Beyotime (Beyotime Institute of Biotechnology, Jiangsu, China).

Human aortic endothelial cells (HAECs) were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in EBM-2 cell culture medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were maintained in a humidified incubator at 37°C with 5% CO_2.

Preparation of the GSPE

Fresh grape seeds were ground and subdivided into five aliquots of 200 g each. These aliquots were individually suspended in ethanol solutions at concentrations of 15%, 30%, 45%, 60%, and 75% for a 24 h period. Following centrifugation at 3000 g for 10 min, the supernatants were collected and freeze-dried. This process yielded five fractions designated as GSE-1 to GSE-5, which were subsequently evaluated for their protective activity in a cell model of HAECs exposed to BaP.

The GSE-3 (45% ethanol extract), which exhibited the highest cell viability-promoting activity, was selected for further purification. The use of ethanol at varying concentrations is a well-established strategy for the sequential extraction of polyphenols based on their polarity and molecular weight. Generally, lower ethanol concentrations (e.g. 15-30%) are more polar and favor the extraction of higher molecular weight compounds such as tannins and highly polymerized proanthocyanidins. Conversely, higher ethanol concentrations (e.g. 60-75%) are less polar and more efficient at extracting medium to low molecular weight flavonoids and phenolic acids. The intermediate concentration of 45% ethanol is known to provide a balanced polarity optimal for extracting a wide spectrum of oligomeric proanthocyanidins (OPCs), which are renowned for their potent antioxidant activity (Nie et al. 2023). Therefore, the superior protective effect observed in the GSE-3 fraction is likely attributable to its enriched content of these bioactive OPCs.

This selected GSE-3 fraction was subsequently dissolved in 50% methanol and heated at 90°C for a duration of 4 hr. Subsequently, the samples were cooled and centrifuged at 4000 g for 5 min. The resulting dry extract was further dissolved in a solution of 1.2 M HCl in a mixture of 50% methanol and water, followed by heating at 90°C for an additional 4 hr. Thereafter, the soluble portion of the solution was collected, freeze-dried, and stored as total grape seed polyphenol extract (TGSPE).

The TGSPE was dissolved in a 10% ethanol solution and subsequently loaded onto a Sephadex G-10 chromatography column (3.0 × 80 cm), which had been equilibrated with the same 10% ethanol solution. A stepwise elution was conducted using increasing concentrations of ethanol (10%, 15%, 30%, 50%, and 75%) at a flow rate of 2.0 ml/min. Each eluate (200 ml) was collected, resulting in five fractions designated as TGSPE-1 to TGSPE-5, which were then freeze-dried. Following this process, the protective activity of TGSPE-1 to TGSPE-5 against BaP exposure in HAECs was assessed.

The TGSPE-3 significantly enhanced the viability of HAECs and is referred to as grape seed polyphenol extract (GSPE) in the current study. GSPE was subsequently purified using Reversed-phase high-performance liquid chromatography (RP-HPLC) on an Agilent 1200 HPLC system equipped with a Zorbax SB C-18 column

(4.6 × 250 mm, 5 μm). The elution solvent system consisted of water (solvent A) and methanol (solvent B). GSPE was separated through gradient elution from 10% to 45% solvent B over a period of 25 min at a flow rate of 1.0 ml/min. The detection wavelength was set at 310 nm, and the column temperature was maintained at 18°C. The structures of the grape seed polyphenols (GSPs) were characterized by analysis using ^{1}H-NMR, ^{13}C-NMR, and ESI-MS techniques (Liu et al. 2021).

Cell vitality detection

The cell viability was assessed using the CCK-8 assay method (Fan et al. 2024). After seeding the HAECs at a density of 2 × 10^4 cells per well in a 6-well culture plate overnight, the HAECs were treated with BaP at a concentration of 10 μM for an additional 6 hr. Following this treatment, the HAECs were collected and washed twice with cold phosphate-buffered saline (PBS), and then, incubated with GSPs, TGSPE, or GSPE at a concentration of 10 μg/ml for 12 hr. Subsequently, cell viability was measured, and the results were compared to those of the control group, which was set as having a vitality of 100%. Resveratrol served as a positive control compound in this experiment.

ROS levels, and SOD, GSH-Px activity detection

After seeding the HAECs at a density of 2 × 10^4 cells per well in a 6-well culture plate overnight, the HAECs were treated with BaP at a concentration of 10 μM for an additional 6 hr. Following this treatment, the HAECs were collected and washed twice with cold PBS, and then, incubated with GSP-1 or GSPE at a concentration of 10 μg/ml for 12 hr. Subsequently, intracellular ROS level and antioxidant enzyme activities-including glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) were quantified using appropriate commercial ELISA kits according to the manufacturer's protocol.

Mitochondrial transmembrane potential (ΔΨm) and cellular apoptosis detection

After seeding the HAECs at a density of 2 × 10^4 cells per well in a 6-well culture plate overnight, the HAECs were treated with BaP at a concentration of 10 μM for an additional 6 hr. Following this treatment, the HAECs were collected and washed twice with cold PBS, and then, incubated with GSP-1 or GSPE at a concentration of 10 μg/ml for 12 hr. Subsequently, the HAECs were harvested, and the ΔΨm as well as cell apoptosis were assessed using previously established methods (Jie et al. 2018; Liu et al. 2021).

Western blotting

After seeding the HAECs at a density of 2 × 10^5 cells/well in a 6-well culture plate overnight, the HAECs were treated with BaP at a concentration of 10 μM for an additional 6 hr. Following this treatment, the HAECs were collected and washed twice with cold PBS, and then, incubated with GSP-1 or GSPE at a concentration of 10 μg/ml for 12 hr. Subsequently, the HAECs were harvested, and western blotting was conducted following the methodology previously reported(Liu et al. 2017). The protein bands were quantified, and densitometric analysis of the blot bands was performed using Image *J* software.

Statistical analysis

All data are presented as mean ± standard deviation (SD) from three independent biological replicates (n = 3). Statistical analyses were conducted using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA). For comparisons between two groups, an unpaired two-tailed Student's t-test was applied. A p-value of less than 0.05 was deemed statistically significant for all analyses.

Results

GSPE enhances HAECs viability under BaP exposure

In this study, we isolated antioxidant compounds from grape seeds using a bioactivity-guided isolation method in HAECs cell model exposed to BaP (Figure 1).

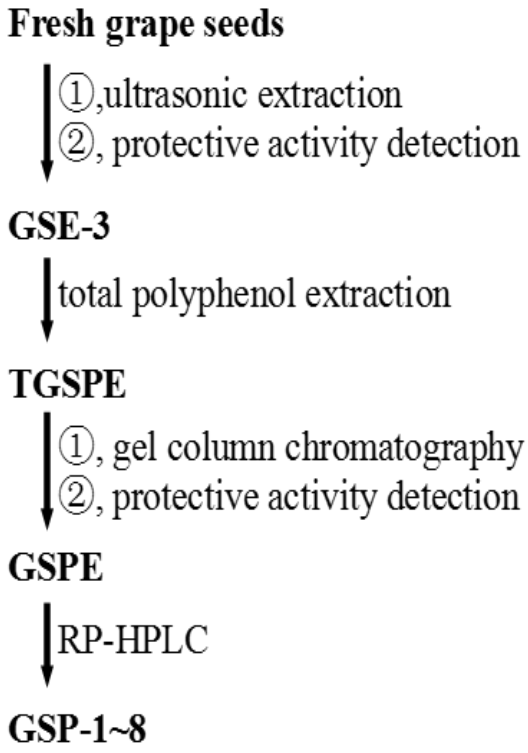

Figure 1. Preparation process of GSPE

The cell viability in the blank control group was established as 100%. In comparison to the blank control group, the cell viability in the BaP group exhibited a significant decrease of 20.7% ($p<0.01$), indicating that HAECs experienced substantial damages following BaP exposure. However, subsequent treatment with GSPE or TGSPE resulted in a notable reversal of the decline in cell viability induced by BaP. Specifically, when compared to the BaP group, cell viability increased by 13.4% ($p<0.05$) and 22.1% ($p<0.01$) in the TGSPE and GSPE treated groups, respectively (Figure 2a).

Interestingly, the current results indicate that GSPE exhibits significantly greater efficacy in enhancing cell viability compared to TGSPE and GSE-3. Subsequently, we conducted a more detailed analysis and isolation of the active ingredients in GSPE utilizing RP-HPLC. Ultimately, eight grape seed polyphenols (GSPs; Figure 3) were isolated and their structures were identified. In this study, we also assessed the potential of these GSPs to enhance cellular vitality. Compared to the BaP-induced group, cell viability was effectively increased by 15.1% ($p<0.01$), 14.2% ($p<0.05$), and 13.9% ($p<0.05$) in the GSP-1, GSP-3, and GSP-4 groups respectively, indicating that GSPs exhibit protective activity and significantly attenuate BaP-induced damages in HAECs. Notably, GSP-1 demonstrated the highest promotion of cell viability; however, GSPE showed superior cell viability enhancement at an equivalent dose, suggesting a synergistic effect among the GSPs involved. Furthermore, we explored potential molecular mechanisms with both GSP-1 and GSPE in HAECs.

GSPE protects HAECs against oxidative damage induced by BaP

In the current study, we assessed intracellular levels of ROS and the activity of antioxidant enzymes. Notably, compared to the control group, BaP exposure resulted in a significant increase in intracellular ROS and MDA levels by 273.2% and 190.2% respectively ($p<0.01$; Figure 2b and 2c). However, this overproduction of intracellular ROS induced by BaP was markedly attenuated following treatment with GSPE and GSP-1. Specifically, relative to the BaP group, intracellular ROS and MDA levels were reduced by 23.2% and 36.6% respectively ($p<0.01$) in the GSPE group and by 11.7% ($p<0.05$) and 28.4% respectively ($p<0.01$) in the GSP-1 group.

To investigate whether GSPE enhances enzyme activity in HAECs exposed to BaP, we conducted the ELISA assays to measure the activities of SOD and GSH-Px. As illustrated in Figures 2d, 2e and 2f, BaP exposure resulted in a significant decrease in SOD, GSH-Px and CAT activities by

21.5% ($p<0.01$), 19.7% ($p<0.01$) and 30.28 ($p<0.01$), respectively, compared to the control group. However, treatment with GSP-1 significantly increased SOD, GSH-Px and CAT activities by 17.3% ($p<0.01$), 12.4% ($p<0.05$) and 18.1 ($p<0.05$), respectively, when compared to the BaP group. Similarly, GSPE treatment enhanced SOD, GSH-Px and CAT activities by 18.9% ($p<0.01$), 15.3% ($p<0.01$) and 28.5% ($p<0.01$), respectively, relative to the BaP group. In summary, both GSP-1 and GSPE effectively reversed the decline in antioxidative enzymes activity induced by BaP exposure. Here, the GSPE demonstrated superior antioxidant activity compared to GSP-1 at an equivalent dose, suggesting a synergistic effect among the involved GSPs.

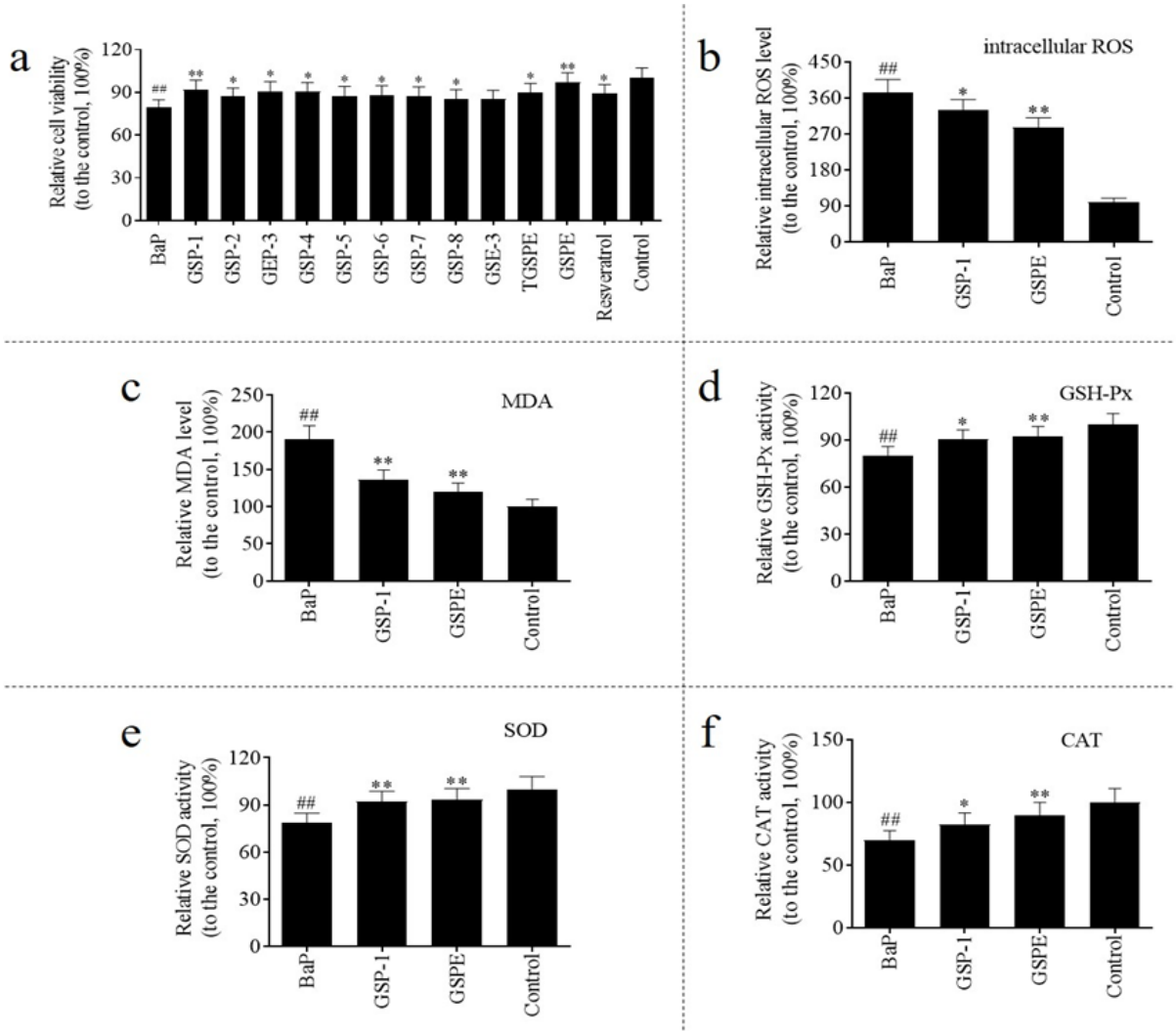

Figure 2. Grape seed polyphenol extract (GSPE) attenuates benzopyrene (BaP)-induced Oxidative Stress in human aortic endothelial cells (HAECs). (a) GSPE inhibits the cell viability decline induced by BaP; Following treatment according to the experimental design, the cell viability was assessed using the CCK-8 assay method; (b, c) GSPE inhibits the overproduction of reactive oxygen species (ROS) and malondialdehyde (MDA) induced by BaP; (d-f) GSPE mitigates the decline in Superoxide Dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GSH-Px) activity caused by BaP exposure. Following treatment according to the experimental design, intracellular ROS and MDA levels as well as SOD, CAT and GSH-Px activities were quantified using appropriate commercial ELISA kits in accordance with the manufacturers' protocols. The results are expressed as mean ± standard deviation (S.D.) from three replicate wells or three independent experiments. $^{\#\#}p<0.01$ vs control group; and $^{*}p<0.05$ and $^{**}p<0.01$ compared to BaP group.

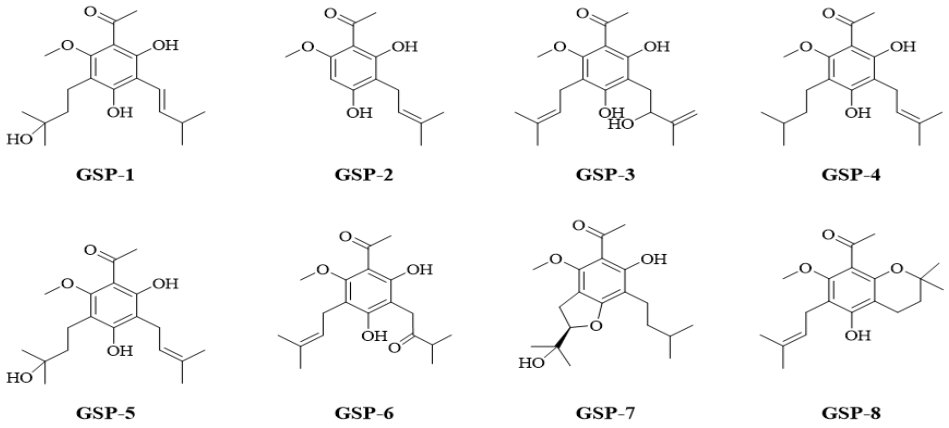

Figure 3. Structures of GSP-1 to GSP-8

GSPE protects HAECs against the collapse of mitochondrial membrane potential induced by BaP

As illustrated in Figure 4a, compared to the control group (Control group, with a ΔΨm decline of 4.01%), there was a significant increase of 13.7% in cells exhibiting ΔΨm decline within the BaP-induced group ($p < 0.01$). However, this BaP-induced decline in ΔΨm was mitigated following treatment with GSPE. In comparison to the BaP group (BaP group, with a ΔΨm decline of 17.7%), HAECs exhibited reductions in ΔΨm decline by 21.6% ($p<0.01$) and 11.3% ($p<0.05$) in the GSPE and GSP-1 groups, respectively. Notably, GSPE effectively reversed the BaP-induced decrease in ΔΨm among HAECs, demonstrating a protective effect against BaP-induced mitochondrial dysfunction.

As shown in Figure 4b, compared to the control group, BaP treatment resulted in a significant release of Cyt c protein from mitochondria, evidenced by a marked up-regulation of cytosolic Cyt c levels and a down-regulation of mitochondrial Cyt c levels. However, GSPE effectively reversed this BaP-induced release of Cyt c. Furthermore, GSPE treatments suppressed the BaP-induced up-regulation of relative expression levels of Bax and Bcl-2 (Figure 4c). Collectively, these results further indicate that GSPE exerts protective effects against BaP-induced oxidative damage potentially through regulation of mitochondrial function via modulation of Cyt c transfer and expression levels of Bcl-2 family proteins.

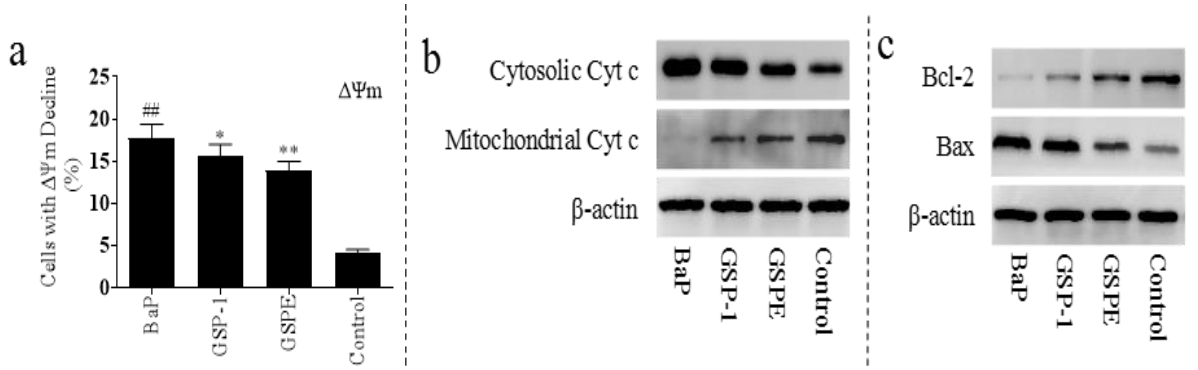

Figure 4. GSPE attenuates BaP-induced mitochondrial dysfunction. (a) GSPE mitigates the decline of mitochondrial membrane potential induced by BaP. Following treatment as the experimental design, HAECs were collected, and mitochondrial transmembrane potential was assessed; (b) GSPE inhibits the release of cytochrome c (Cyt c) from mitochondria into the cytosol induced by BaP. The levels of Cyt c in both mitochondrial and cytosolic fractions were determined via western blotting; (c) GSPE suppresses disturbances in Bcl-2 family protein expression caused by BaP. The expressions of Bcl-2 and Bax proteins were evaluated through western blotting, with β-actin serving as an internal control. The results are expressed as mean ± standard deviation (S.D.) from three replicate wells or three independent experiments. $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs control group; $^{*}p<0.05$, $^{**}p<0.01$ compared to BaP group.

GSPE inhibits BaP-induced apoptosis in HAECs

In the current study, cell apoptosis was assessed using an Annexin V-FITC/PI assay via flow cytometry (Figure 5a). Compared to the control group (total apoptosis and necrosis: 0.4%), BaP-induced apoptosis in HAECs increased by 40-fold ($p < 0.01$). However, when compared to the BaP group (total apoptosis and necrosis: 16.0%), there was a reduction in HAECs apoptosis by 12.4% following GSP-1 treatment (total apoptosis and necrosis: 14.1%, $p < 0.05$), and by 45.9% after GSPE treatment (total apoptosis and necrosis: 8.7%, $p < 0.01$). The results regarding cell apoptosis also provide direct evidence for the protective activity of GSPE against BaP-induced damage in HAECs.

To confirm the anti-apoptotic effect of GSPE on BaP-induced apoptosis in HAECs, we assessed the protein expressions of caspase-8, caspase-9, and their cleaved forms. As illustrated in Figure 5b, BaP exposure resulted in a significant increase in the levels of cleaved caspase-8 and -9 compared to the control group. Concurrently, there was a notable decrease in total caspase-8 and -9 expression following BaP treatment relative to controls. However, GSPE treatment led to a significant reduction in both cleaved caspase-8 and -9 levels. Additionally, GSPE administration restored the inhibition of caspase-8 and -9 protein expression induced by BaP exposure. These findings further indicate that GSPE may inhibit BaP-induced apoptosis in HAECs through modulation of the caspase-dependent pathway as well as regulation of Cyt c release and Bcl-2 family protein expression.

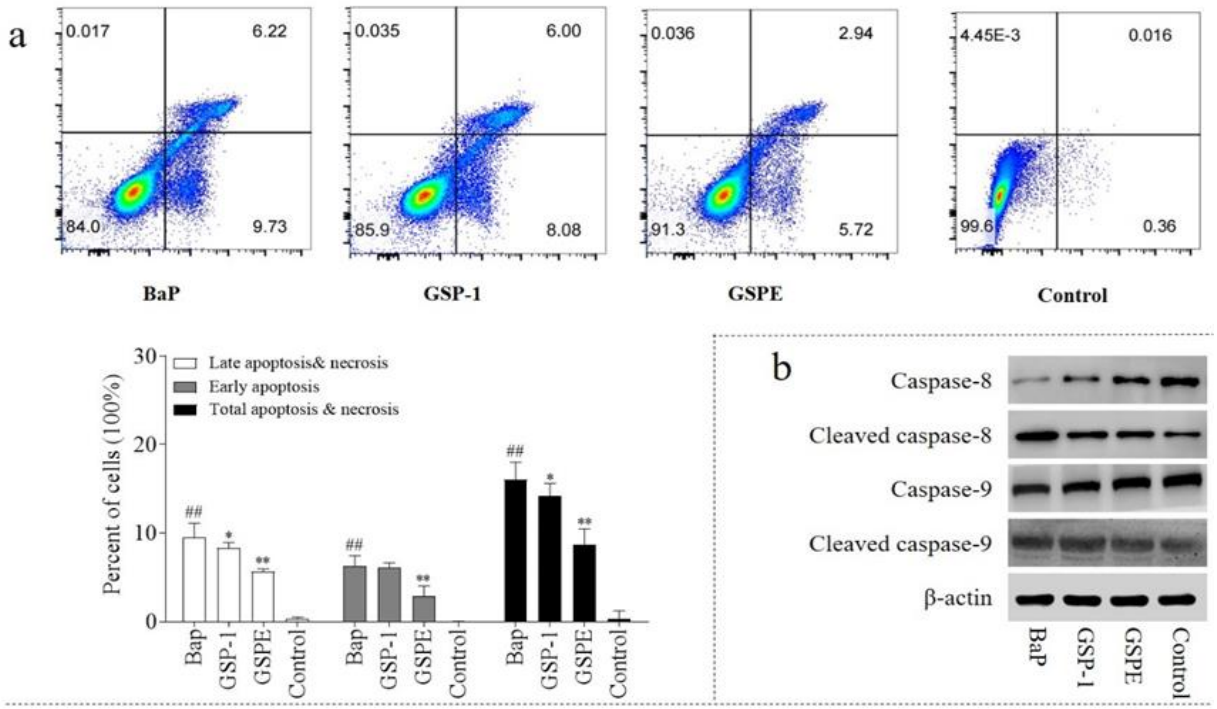

Figure 5. GSPE attenuates BaP-induced apoptosis in HAECs. (a) Following the experimental design, HAECs were collected after treatment, and cell apoptosis was assessed using the Annexin V-FITC/PI staining assay. Cells in the lower right quadrant (Annexin V^+/PI^-) represent early apoptotic cells, and those in the upper right quadrant (Annexin V^+/PI^+) represent late apoptotic cells. (b) Effects of GSPE on Caspase protein expression. The expressions of caspase 8 and 9, as well as their cleaved forms, were detected using western blotting. β-actin was employed for normalization purposes. The results are expressed as mean ± standard deviation (S.D.) from three replicate wells or three independent experiments. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs control group; $^{*}p<0.05$ and $^{**}p<0.01$ compared to BaP group.

GSPE inhibits the nuclear translocation of Nrf2 and the activation of AhR induced by BaP in HAECs.

In the current study, western blot analysis was performed to assess the expression levels of cytoplasmic Nrf2, NQO1, and HO-1, with results presented in Figure 6a. Exposure to BaP significantly suppressed the expression of NQO1 and HO-1 proteins while concurrently inducing nuclear translocation of Nrf2. In contrast to BaP exposure, treatment with GSPE notably reversed both the inhibition of NQO1 and HO-1 protein expression as well as the nuclear translocation of Nrf2 induced by BaP.

As illustrated in Figure 6b, BaP exposure significantly upregulated AhR expression, and similar results were observed for CYP1A1. Clearly, the AhR signaling pathway in HAECs was activated by BaP exposure. Interestingly, the activation of the AhR signal induced by BaP was markedly inhibited by GSPE treatment. The findings indicated that GSPE treatments reversed the inhibition of AhR and CYP1A1 expression caused by BaP exposure. Furthermore, these results suggested that GSPE exerted protective effects against BaP-induced oxidative damage in HAECs, potentially through the regulation of protein expressions within both the AhR and Nrf2 signaling pathways.

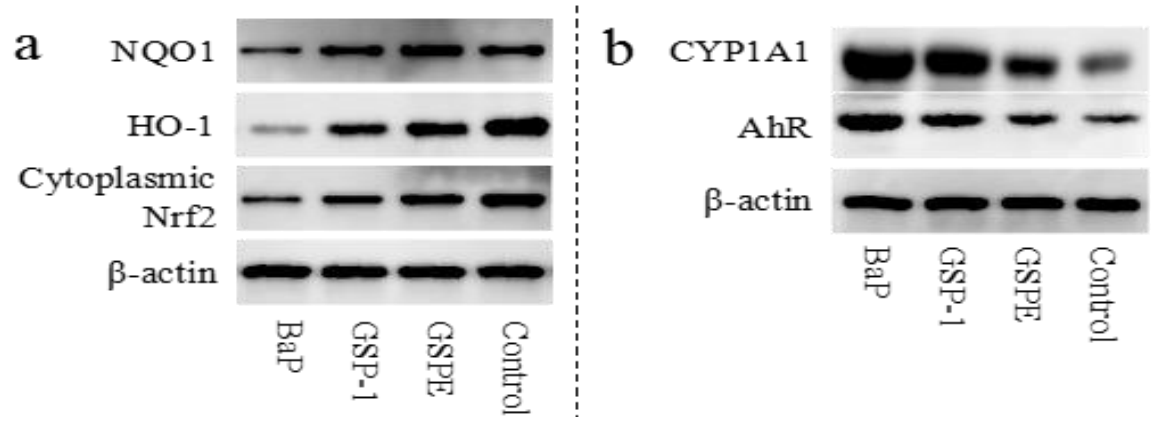

Figure 6. GSPE inhibits BaP-induced nuclear translocation of Nrf2 and activation of AhR in HAECs. (a) GSPE suppresses BaP-induced nuclear translocation of Nrf2 in HAECs; (b) GSPE inhibits BaP-induced activation of AhR in HAECs. Following the experimental design, HAECs were harvested after treatment, and the protein levels of NQO1, HO-1, Nrf2, CYP1A1, and AhR were assessed using western blotting. β-actin was utilized as a loading control for normalization. The results are expressed as mean ± standard deviation (S.D.) from three replicate wells or three independent experiments. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs control group; $^{*}p<0.05$ and $^{**}p<0.01$ compared to BaP group.

Discussion

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed organic pollutants, with evidence linking chronic exposure to an increased risk of cardiovascular diseases including atherosclerosis. Oxidative damage to vascular endothelial cells induced by PAHs is a significant contributor to the pathogenesis and progression of atherosclerosis (Zhang et al. 2024). Consequently, the administration of exogenous antioxidants represents a promising strategy for protecting vascular endothelial cells against oxidative stress. Benzo[α]pyrene (BaP), a major PAH found in tobacco smoke, fossil fuel combustion, and barbecued foods, is one of the most potent and extensively studied PAHs, often used as a toxicological prototype for this class of pollutants (Lu et al. 2023).

Grape seeds, rich in antioxidants and long used as dietary supplements, were investigated in this study for their potential

to mitigate BaP-induced oxidative stress in HAECs.

Exposure to PAHs is a recognized risk factor for inflammatory and oxidative cardiovascular diseases like atherosclerosis. Previous studies have established that BaP induces ROS production and contributes to oxidative stress (Wang et al. 2023), which along with subsequent inflammatory chemokine production, are considered critical mediators in both the initiation and exacerbation of atherosclerosis (Khan et al. 2024). Dietary phytochemicals including polyphenols and glucosinolates from fruits and vegetables, are known for their health-promoting properties, with several identified as potential inhibitors of atherosclerosis (Bernis et al. 2012). This study demonstrated that GSPE significantly enhanced HAEC viability compromised by BaP exposure. Notably, GSPE exhibited a more potent effect than its subfraction TGSPE or the isolated compound GSP-1, suggesting a synergistic interaction among the constituent polyphenols in the crude extract.

The decline in antioxidant enzyme activity associated with oxidative damage is also implicated in atherosclerosis, highlighting the importance of activating cellular antioxidant defenses (Bernis et al. 2012). Superoxide dismutase (SOD) is crucial for scavenging superoxide anion radicals, while the glutathione (GSH) redox system, particularly glutathione peroxidase (GSH-Px), protects cells by reducing peroxides and facilitating hydrogen peroxide decomposition (Wang et al. 2024c). Our findings confirm that BaP exposure significantly suppressed SOD, GSH-Px, and CAT activities, and increased ROS and MDA levels. Both GSPE and GSP-1 effectively reversed these trends, with GSPE showing superior efficacy, further supporting the notion of synergistic effects within the extract.

Free radical overproduction often damages mitochondria, leading to the collapse of the mitochondrial membrane potential ($\Delta\Psi m$). The mitochondrion-dependent pathway plays a crucial role in cellular responses to oxidative stress (Ko et al. 2005). Mitochondria are a primary source of ROS, and alterations in cellular redox status can facilitate cell death. The release of cytochrome c (Cyt c) from mitochondria, mediated by ROS, is a central event in initiating the apoptotic cascade (Ko et al. 2005). The Bcl-2 family proteins, such as Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic), regulate this process; their relative expression levels determine cell fate. Overexpression of Bcl-2 can protect against various apoptotic stimuli by reducing intracellular ROS (Liu et al. 2024). In this study, GSPE significantly mitigated the BaP-induced $\Delta\Psi m$ collapse, Cyt c release, and dysregulation of Bax/Bcl-2 expression, indicating its protective role against mitochondrial dysfunction.

Mitochondrial permeability transition (MPT) is a pivotal step in apoptosis, characterized by $\Delta\Psi m$ depolarization, release of pro-apoptotic factors like Cyt c, and activation of the caspase cascade (Wang et al. 2024b). Apoptosis is an orderly process essential for removing damaged cells, with the caspase cascade, regulated by molecules like Bcl-2 family proteins, being vital (Wang et al. 2024b). Oxidative stress often triggers the mitochondrial pathway, activating caspase-9 and subsequently other caspases, leading to apoptosis. Released Cyt c can also facilitate procaspase-8 activation (Jun et al. 2023). Our results showed that BaP induced significant apoptosis in HAECs, accompanied by increased levels of cleaved caspase-8 and -9. GSPE treatment markedly reduced apoptosis and modulated the expression and cleavage of these caspases, suggesting inhibition of BaP-induced apoptosis via caspase-dependent pathways, likely initiated by the prevention of mitochondrial dysfunction.

Oxidative stress triggers defensive responses including the activation of genes that facilitate ROS detoxification and

promote cell survival (Bernis et al. 2012). The Nrf2 signaling pathway is a critical cellular defense mechanism against oxidative stress. Under stress, Nrf2 translocates to the nucleus and activates antioxidant genes like HO-1 and NQO1 (Han et al. 2012). Conversely, the aryl hydrocarbon receptor (AhR) mediates biological responses to PAHs like BaP (Dubiel et al. 2023). AhR activation plays a critical role in BaP-induced oxidative damage (Deval et al. 2024), often leading to the upregulation of cytochrome P450 enzymes like CYP1A1 (Xie et al. 2024). PAHs can activate both AhR/XRE and Keap1/Nrf2/ARE pathways(Tiem and Giulio 2011). Our study found that BaP exposure induced nuclear translocation of Nrf2 and upregulated AhR and CYP1A1 expression. GSPE treatment effectively reversed these BaP-induced alterations, indicating that its protective effects involve the modulation of both the Nrf2 and AhR signaling pathways.

In summary, the present study demonstrates that GSPE confers protection against BaP-induced oxidative stress and apoptosis in HAECs through a multi-faceted mechanism. This includes mitigating mitochondrial dysfunction, inhibiting caspase-dependent apoptosis, and concurrently modulating both the Nrf2 and AhR signaling pathways. While our findings are consistent with prior research highlighting the potent antioxidant capacity of GSPE in various models (Cadiz-Gurrea et al. 2017; El Ayed et al. 2017), they significantly extend this understanding by providing novel evidence of a dual-target effect on the Nrf2 and AhR pathways in HAECs, a mechanism not previously reported. The superior efficacy of the crude extract over its isolated components further suggests a synergistic interaction among its constituent polyphenols. Collectively, this work elucidates the potential of GSPE as a dietary supplement and identifies a novel therapeutic strategy for combating BaP-induced endothelial injury, which underlies the development of atherosclerosis.

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Conflicts of interest

The authors state that the research was carried out with no commercial or financial relationships that might be seen as a potential conflict of interest.

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Authors' Contributions

Man Zheng: concept and design, data collection, writing the article

Fenglei Zhang: data collection, analysis and interpretation

Xiang Gong: statistical analysis

Haitao Yuan: writing the article, critical revision of the article, final approval of the article, overall responsibility

Data availability statement

The study's primary contributions are contained within the article or Supplementary Material. For additional information, contact the corresponding author.

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