

Grape seed extract ameliorates tumor necrosis factor- α -induced inflammatory status of human umbilical vein endothelial cells

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Abstract

Background Inflammation has played a key role in the causation of atherosclerosis. However, the effects of grape seed extract (GSE) on the pro-inflammatory intracellular signaling, enzyme activity, and inflammatory mediators of endothelial cells have not been sufficiently studied, and less information exists on the comparison between GSE and vitamin C, a well-known antioxidant compound, on their anti-inflammatory properties.

Purpose We investigated the effects of GSE and vitamin C on the cell viability, oxidative stress, monocyte adhesion,

the expression of nuclear factor- κ B inhibitor (I κ B), inter-cellular adhesion molecule-1 (ICAM-1) and cyclooxygenase-2 (COX-2), and the production of prostaglandin E₂ (PG E₂) in TNF- α -treated human umbilical vein endothelial cells (HUVECs).

Methods Cell viability was measured by MTT assay. The adhesion of THP-1 to HUVECs was evaluated by cell adhesion assay. The oxidized nucleoside 8-hydroxydeoxyguanosine (8-OHdG) (an indicator of oxidative damage to DNA), ICAM-1, and PG E₂ were measured by ELISA. I κ B and COX-2 expression were evaluated by western blot analysis.

Results TNF- α (10, 20, and 50 ng/mL), GSE (50 and 200 μ g/mL), or vitamin C (100 μ M) did not affect cell viability. GSE (50–100 μ g/mL) attenuated TNF- α (20 ng/mL)-induced 8-OHdG production, THP-1 adhesion, the expression of I κ B degradation, ICAM-1 and COX-2, and the production of PGE₂ in a dose-dependent manner. Vitamin C (100 μ M) also showed significant antioxidative and anti-inflammatory effects.

Conclusions GSE effectively ameliorates TNF- α -induced inflammatory status of HUVECs. The findings of the present study suggest that consumption of GSE may be beneficial to inflammatory atherosclerosis.

Keywords Inflammation · Grape seed extract · Adhesion · Cyclooxygenase-2 · Prostaglandin E₂ · Endothelial cell

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Introduction

Inflammatory process has been found to play a key role in the causation of atherosclerosis, including initiation, progression (atheroma formation), and plaque rupture [1, 2]. The formation of atherosclerosis starts with the adherence of leukocytes to the vascular endothelial cells mediated by

adhesion molecules, such as P-selectin, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1). These molecules can be expressed by endothelial cells upon proatherogenic stimuli, including oxidized low-density lipoprotein, oxygen free radicals, as well as pro-inflammatory cytokines secreted by macrophages and T cells, such as tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), and interleukin-6 (IL-6) [3, 4]. Moreover, cytokines can activate pro-inflammatory intracellular signaling, such as nuclear factor-kappa B (NF- κ B) [5, 6], and stimulate the expression of cyclooxygenase-2 (COX-2) and production of prostaglandin E₂ (PG E₂), which leads to further cell adhesion [7], increase in endothelial permeability [8, 9], and instability of plaque [10].

Recently, clinical studies have demonstrated that systemic markers of inflammation are strong predictors of cardiovascular disease [2, 11, 12]. Vitamin C, which is a well-known strong antioxidant and also has anti-inflammatory properties, has been widely used to improve atherosclerosis [13, 14]. However, the results of the clinical studies were disappointing [15]. In contrast, several epidemiological studies have shown an inverse correlation between the dietary consumption of flavonoids and mortality from cardiovascular disease [16, 17]. Like vitamin C, flavonoids play an important role in the scavenging of oxygen free radicals and inhibition of inflammation [14]. Flavonoids, accounting for a major portion of polyphenols, are present abundantly in fruits, vegetables, nuts, and seeds and divided into six major subgroups: chalcones, flavonols, flavanone, flavones, anthocyanidins, and isoflavonoids [18]. After emergence of French paradox [19], grape seed extract (GSE), a popular commercial product that contains high quantity of flavonoids, especially anthocyanidins, has prompted the research interest in its effects on vascular protection [14, 20]. However, the effects of GSE on the pro-inflammatory intracellular signaling, enzyme activity, and inflammatory mediators of human umbilical vein endothelial cells (HUVECs) have not been sufficiently studied, and less information exists on the comparison between GSE and vitamin C on their anti-inflammatory properties.

In the present study, we investigated the effects of GSE and vitamin C on the cell viability, oxidative stress, monocyte adhesion, the expression of nuclear factor- κ B inhibitor (I κ B), ICAM-1 and COX-2, and the production of PGE₂ in TNF- α -treated HUVECs.

Methods

Cell culture of HUVECs

Human umbilical vein endothelial cells (HUVECs) were cultured as previously described [21]. Cells were seeded at

a density of 1×10^5 per 75-cm² flask in medium 199 (Gibco, Grand Island, New York, USA), supplemented with 20 mM HEPES, 100 μ g/mL endothelial cell growth substance (Collaborative Research Inc, Waltham, MA), and 20% fetal calf serum (Gibco, Grand Island, New York, USA). The cultures were maintained at 37 °C with a gas mixture of 5% CO₂ and 95% air. Subcultures were performed with trypsin–EDTA. All media were supplemented with 5 U/mL heparin, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. Medium was refreshed every third day. The endothelial cells were identified by the presence of factor VIII-related antigen (Histoset Kit, Immunolok, Carpinteria, CA, USA) and a typical “cobblestone” appearance. Endothelial cells of the third to fifth passages in the actively growing condition were used for experiments.

Cell culture of THP-1 cells

THP-1 monocytes (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 (Gibco, Grand Island, New York, USA) containing 10% fetal bovine serum, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM L-glutamine, pH 7.2. The cultures were maintained at 37 °C with a gas mixture of 5% CO₂ and 95% air.

MTT assay for cell viability

Cell viability was measured by a quantitative colorimetric assay using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), which shows mitochondrial activity of living cells. Cells in a 24-well plate were treated with TNF- α (Sigma Chemical Co., St. Louis, MO, USA) (10–20–50 ng/mL), GSE (Puritan's Pride, Oakdale, NY, USA) (50–200–400 μ g/mL), or vitamin C (Sigma–Aldrich, St. Louis, MO, USA) (100 μ M) for 24 h and then incubated with MTT for 4 h. The amount of MTT product was then determined by measuring the absorbance at 590 nm using an ELISA reader.

Cell adhesion assay

HUVECs were cultured in a 48-well plate containing M199 with 10% fetal bovine serum and treated with TNF- α (20 ng/mL) alone or in the presence of GSE (50–100 μ g/mL) or vitamin C (100 μ M) for 6 h. THP-1 cells were labeled with 2',7'-bis-(carboxyethyl)-5(6')-carboxyfluorescein acetoxymethyl ester (BCECF/AM) (Sigma–Aldrich, St. Louis, MO, USA) for 1 h and then seeded onto confluent HUVECs. The number of adherent THP-1 cells was measured by an ELISA reader with 485 nm excitation and 535 nm emission.

ELISA for 8-OHdG, ICAM-1, and PGE₂

In experiments, HUVECs were treated with TNF- α (20 ng/mL) alone or in the presence of GSE (50–100 μ g/mL) or vitamin C (100 μ M) for 6 h. Then supernatants were collected for the detection of 8-OHdG (Cosmo Bio Company LTD., Tokyo, Japan), ICAM-1 (Bender Med-Systems Inc, Burlingame, CA, USA), and PGE₂ (NEOGEN Corporation, Lexington, KY, USA) using ELISA according to the manufacturers' instructions.

Western blot analysis for I κ B- α and COX-2

The I κ B- α and COX-2 antibodies (Upstate Biotechnology, Lake City, NY, USA) were used for analyses. HUVECs were washed twice with ice-cold phosphate-buffered saline and lysed in 200 μ L of homogenization buffer (10 mM Tris-HCl at pH 7.4, 2 mM EDTA, 1 mM EGTA, 50 mM NaCl, 1% TritonR X-100, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1:100 v/v proteinase inhibitor cocktail) at 4 °C for 30 min. Cell lysates were then ultracentrifuged at 12,000 g for 30 min at 4 °C, and the supernatants were used as the cell extracts. The cell extracts were subjected to 12% SDS-polyacrylamide gel and then transferred to the polyvinylidene fluoride (PVDF) membrane with a semi-dry blot apparatus (Bio-Rad) at 3 mA/cm² of the gel in transfer buffer (25 mM Tris at pH 8.3, 192 mM glycine, and 20% methanol) at room temperature for 30 min. The free protein-binding sites on the PVDF membrane were blocked by incubation with 5% nonfat milk in Tris/Tween-buffered saline (TTBS) (20 mM Tris at pH 7.4, 0.15 M NaCl, and 0.2% Tween-20) at 25 °C for 2 h. The membrane was

immunoblotted with 0.1 μ g/mL primary antibody in TTBS buffer containing 3% nonfat milk at 4 °C overnight and then with secondary antibody conjugated with peroxidase (1:1,000) at 25 °C for 1 h. Immunoblots were developed using an enhanced chemiluminescence system. The luminescence was visualized on X-ray film.

Data analysis

Data were obtained from at least three separate experiments and presented as mean \pm SEM. All statistical data were obtained by analysis of variance followed by Student's *t* test. A *P* value <0.05 was considered statistically significant.

Results

Effect of TNF- α , GSE, and vitamin C on cell viability

TNF- α (10, 20, and 50 ng/mL), GSE (50 and 200 μ g/mL), or vitamin C (100 μ M) alone did not affect the cell viability of HUVECs. GSE (400 μ g/mL) alone reduced the cell viability (Fig. 1).

Effect of GSE and vitamin C on 8-OHdG production

8-OHdG, a good indicator of ROS-induced DNA damage, was employed to detect oxidative stress. The 8-OHdG production in HUVECs was significantly increased by TNF- α (20 ng/mL). The TNF- α -induced 8-OHdG production was significantly reduced by GSE (50 μ g/mL) and vitamin C (100 μ M) (Fig. 2).

Fig. 1 Effect of TNF- α , GSE, and vitamin C on cell viability in HUVECs. TNF- α (10, 20, and 50 ng/mL), GSE (50 and 200 μ g/mL), and vitamin C (100 μ M) did not affect the cell viability. GSE (400 μ g/mL) alone reduced the cell viability. Cells were treated with TNF- α , GSE, or vitamin C for 24 h. The cell viability was determined by MTT assay as described in “Methods”. Data are mean \pm SEM (*n* = 4). **P* < 0.05 vs. control

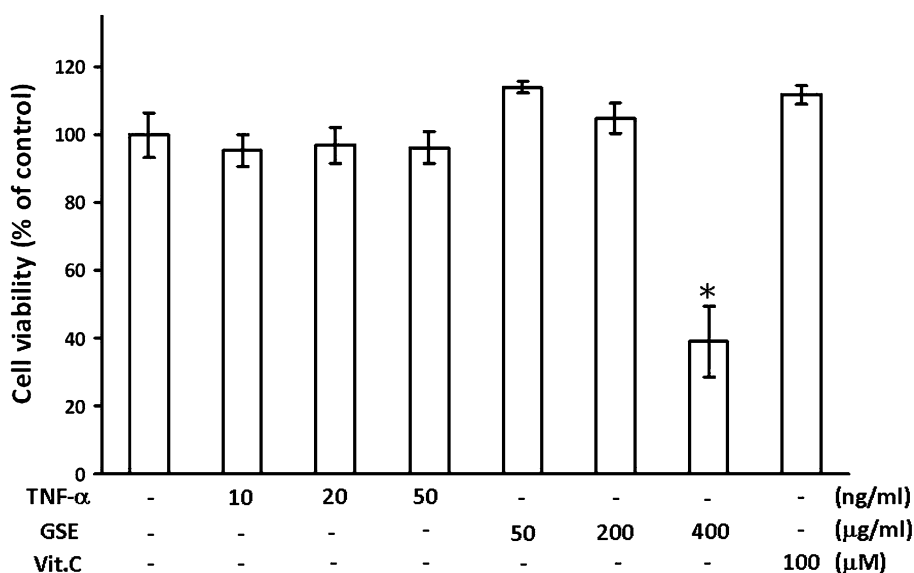
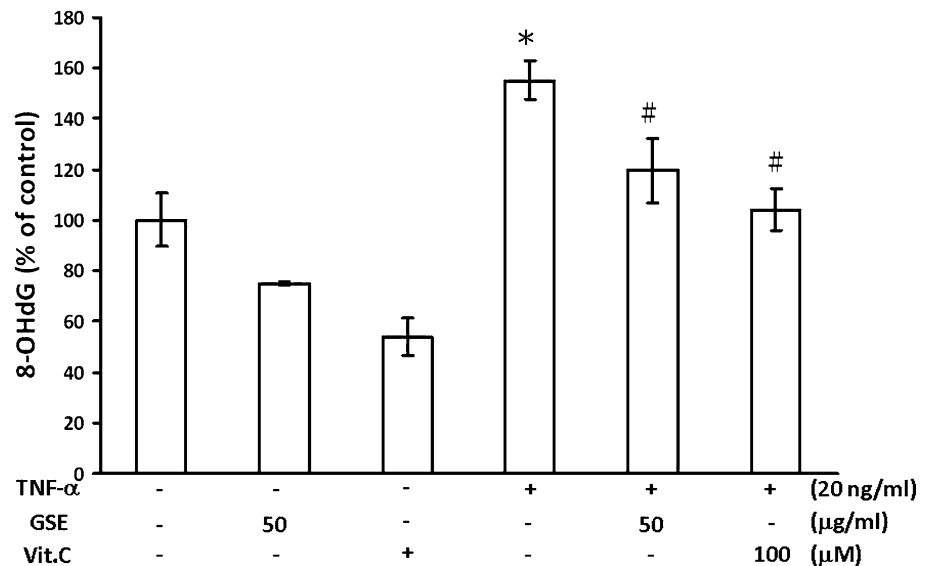


Fig. 2 Effect of GSE and vitamin C on 8-OHdG production in HUVECs. The 8-OHdG production in HUVECs was significantly increased by TNF- α (20 ng/mL). The TNF- α -induced 8-OHdG production was reduced by GSE (50 μ g/mL) and vitamin C (100 μ M). Cells were treated with TNF- α alone or in the presence of GSE or vitamin C for 6 h. The measurement of 8-OHdG was performed by ELISA as described in “Methods”. Data are mean \pm SEM ($n = 4$). * $P < 0.05$ vs. control, # $P < 0.05$ vs. TNF- α (20 ng/mL)



Effect of GSE and vitamin C on THP-1 adhesion

TNF- α (20 ng/mL) significantly increased the adhesion of THP-1 to HUVECs. The TNF- α -induced adhesion of THP-1 to HUVECs was decreased by GSE (50 and 100 μ g/mL) in a dose-dependent manner. The inhibitory effect of vitamin C (100 μ M) on TNF- α -induced THP-1 adhesion to HUVECs was also prominent (Fig. 3).

Effect of GSE and vitamin C on I κ B expression

TNF- α (20 ng/mL) caused a significant decrease in I κ B expression in HUVECs. GSE (50 and 100 μ g/mL) showed a dose-dependent manner in attenuating the degradation of I κ B- α . The attenuating effect of vitamin C (100 μ M) on

TNF- α -induced I κ B degradation was also prominent (Fig. 4).

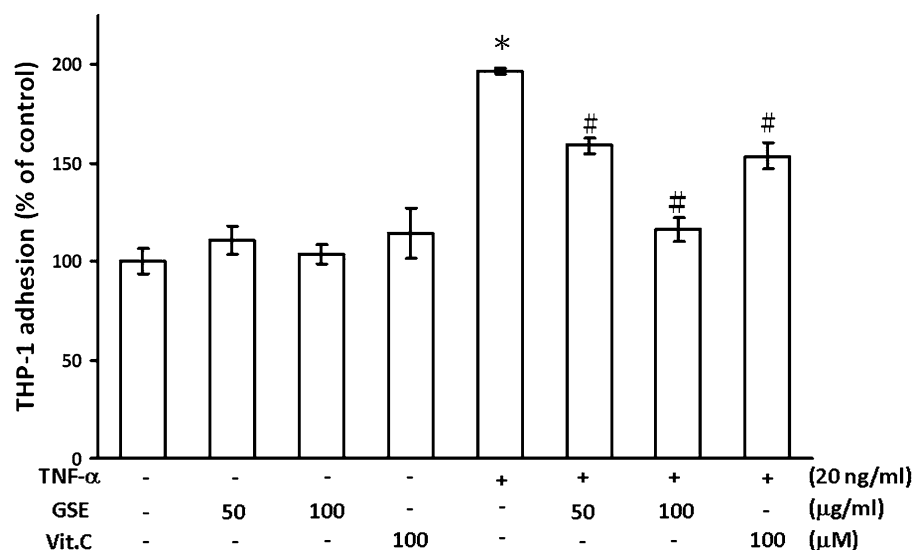
Effect of GSE and vitamin C on ICAM-1 expression

TNF- α (20 ng/mL) significantly increased ICAM-1 expression in HUVECs. The TNF- α -induced ICAM-1 expression was significantly reduced by GSE (50 μ g/mL) and vitamin C (100 μ M) (Fig. 5).

Effect of GSE and vitamin C on COX-2 expression

TNF- α (20 ng/mL) caused a significant increase in COX-2 expression in HUVECs. GSE (50 and 100 μ g/mL) showed a dose-dependent manner in inhibiting COX-2

Fig. 3 Effect of GSE and vitamin C on THP-1 adhesion to HUVECs. TNF- α (20 ng/mL) increased the adhesion of THP-1 to HUVECs. The TNF- α -induced THP-1 adhesion was decreased by GSE in a dose-dependent manner. Cells were treated with TNF- α alone or in the presence of GSE or vitamin C for 6 h. The THP-1 adhesion was determined by cell adhesion assay as described in “Methods”. Data are mean \pm SEM ($n = 4$). * $P < 0.05$ vs. control, # $P < 0.05$ vs. TNF- α (20 ng/mL)



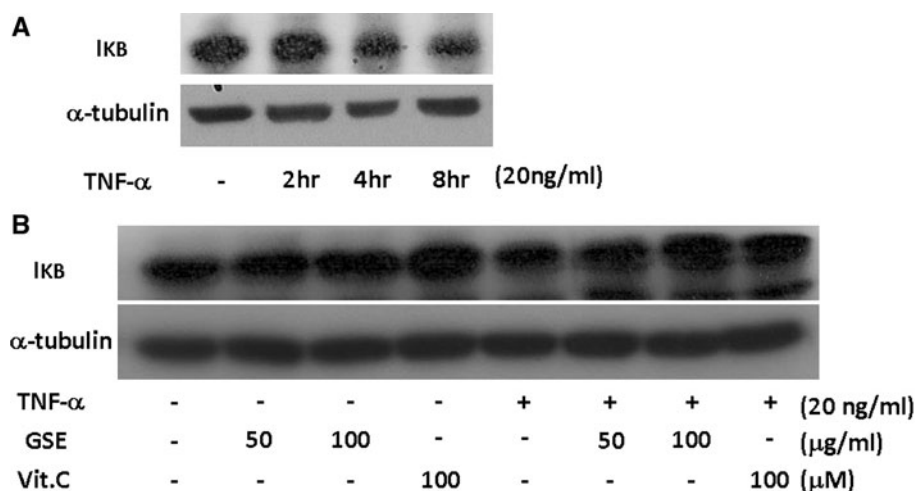
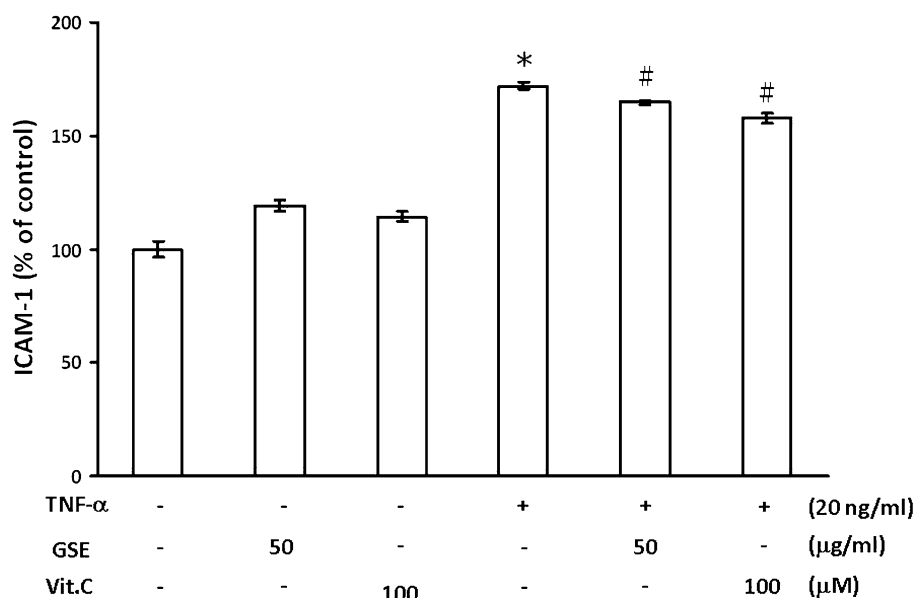


Fig. 4 Effect of GSE and vitamin C on I κ B expression in HUVECs. **a** After treatment of TNF- α (20 ng/mL), the protein level of I κ B rapidly decreased. **b** The expression of I κ B was decreased by TNF- α (20 ng/mL) and increased by GSE (50 and 100 μ g/mL) and vitamin C (100 μ M). GSE showed a dose-dependent manner in attenuating the

TNF- α -induced degradation of I κ B. Cells were treated with TNF- α alone or in the presence of GSE or vitamin C for 6 h. Western blotting to identify I κ B was performed as described in “Methods”. The data are from a representative experiment of three experiments with similar results

Fig. 5 Effect of GSE and vitamin C on ICAM-1 expression in HUVECs. TNF- α (20 ng/mL) increased the expression of ICAM-1. The TNF- α -induced ICAM-1 expression was reduced by GSE (50 μ g/mL) and vitamin C (100 μ M). Cells were treated with TNF- α alone or in the presence of GSE or vitamin C for 6 h. The measurement of ICAM-1 was performed by ELISA as described in “Methods”. Data are mean \pm SEM ($n = 4$). * $P < 0.05$ vs. control, # $P < 0.05$ vs. TNF- α (20 ng/mL)



expression. The inhibitory effect of vitamin C (100 μ M) on TNF- α -induced COX-2 expression was also prominent (Fig. 6).

Effect of GSE and vitamin C on PGE₂ production

TNF- α (20 ng/mL) significantly increased PGE₂ production in HUVECs. The TNF- α -induced PGE₂ production was significantly reduced by GSE (50 μ g/mL) and vitamin C (100 μ M) (Fig. 7).

Discussion

The cardinal findings of this study indicated that in HUVECs, GSE (50–100 μ g/mL), a complex mixture containing mainly active ingredients of flavonoids, significantly attenuated TNF- α -induced 8-OHdG production, THP-1 adhesion, the expression of I κ B degradation, ICAM-1 and COX-2, and the production of PGE₂.

TNF- α , a potent cytokine that is predominantly produced by macrophages, has been reported to increase the

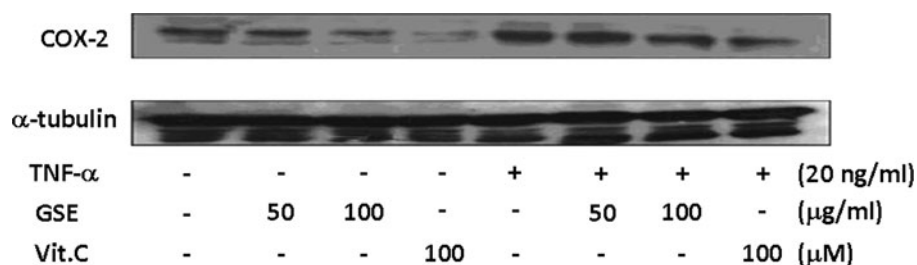
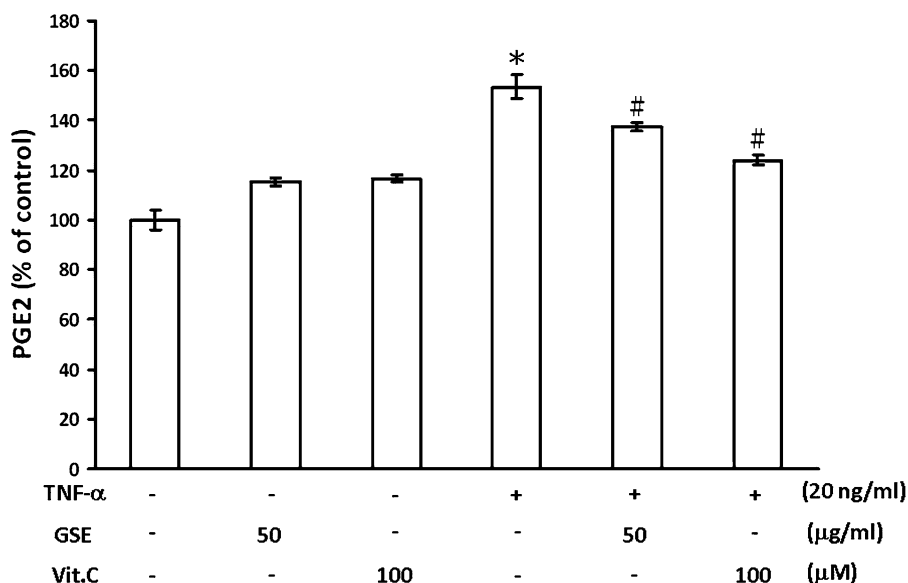


Fig. 6 Effect of GSE and vitamin C on COX-2 expression in HUVECs. The expression of COX-2 was increased by TNF- α (20 ng/mL). GSE showed a dose-dependent manner in inhibiting TNF- α -induced COX-2 expression. Cells were treated with TNF- α

alone or in the presence of GSE or vitamin C for 6 h. Western blotting to identify COX-2 was performed as described in “Methods”. The data are from a representative experiment of three experiments with similar results

Fig. 7 Effect of GSE and vitamin C on PGE₂ production in HUVECs. TNF- α (20 ng/mL) increased the production of PGE₂. The TNF- α -induced PGE₂ production was reduced by GSE (50 μ g/mL) and vitamin C (100 μ M). Cells were treated with TNF- α alone or in the presence of GSE or vitamin C for 6 h. The measurement of PGE₂ was performed by ELISA as described in “Methods”. Data are mean \pm SEM ($n = 4$). * $P < 0.05$ vs. control, # $P < 0.05$ vs. TNF- α (20 ng/mL)



production of reactive oxygen species (ROS) such as superoxide anion via NADPH oxidase in endothelial cells, subsequently activating NF- κ B to enter the nucleus and induce pro-inflammatory gene expression [22]. Without stimulation, NF- κ B is present in the cytoplasm as an inactive complex bound by I κ B (an inhibitor of NF- κ B); on stimulation, phosphorylation and degradation of I κ B occur, and NF- κ B gets unmasked and translocated into the nucleus [23]. 8-OHdG has been known as an indicator of oxidative damage to DNA and thought to be related to cellular apoptosis in HUVECs [24]. In the present study, we also demonstrated that 8-OHdG could efficiently evaluate the status of oxidative stress in TNF- α -induced inflammatory process of HUVECs. As to the inhibitory effect of GSE on NF- κ B activity, it has been demonstrated in LLC-PK₁ tubule cells [25], but not yet in HUVECs. Using I κ B degradation as an indicator of NF- κ B activation, we found that the protein level of I κ B rapidly degraded after TNF- α treatment; the rapid decrease in I κ B expression was similar to that observed in high glucose-induced apoptosis in HUVECs [26].

TNF- α is also thought to cause initiation of atherosclerosis by up-regulating the expression of adhesion molecules on the endothelial cell surface [22]. The leukocyte adhesion includes rolling adhesion (mediated by selectins), firm adhesion (facilitated by VCAM-1 and ICAM-1), and then transmigration through endothelial cell junctions (governed by platelet/endothelial cell adhesion molecule-1), which prompts the progression of atherosclerosis to the next stage [4]. ICAM-1 is much closer to the endothelial junctions and thought to play a more important role than VCAM-1 in transmigration [4]. The in vitro studies conducted to evaluate the regulatory effects of GSE on adhesion molecules were few [27, 28]. One study showed that pretreatment with grape seed proanthocyanidin extract decreased the adherence of Jurkat T cells to TNF- α -treated HUVECs, down-regulated VCAM-1 expression but not ICAM-1 expression [27]. Another study also revealed selective inhibition of advanced glycation end products-induced VCAM-1 by GSPE [28]. In contrast, GSE, being a mixture of polyphenols in our study, effectively decreased ICAM-1 expression. The exact mechanism of the different

results between studies was not clear. The distinctive regulatory pathways of VCAM-1 and ICAM-1 expression might contribute to part of the explanation [29, 30].

Previous study reported that COX-2 expression could be induced in TNF- α -treated HUVECs via activation of p38 MAP kinase [7]. COX-2, via converting arachidonic acid to prostaglandins (PGE₂ and PGI₂), may enhance cell adhesion [7], increase endothelial cell permeability [9], up-regulate monocyte-derived macrophages [31], and lead to plaque rupture [10], although some COX-2 inhibitors were reported to increase the risk of cardiovascular events [32]. Flavonoids, such as kaempferol and quercetin, inhibited COX-2 expression in cytokines-treated HUVECs in a concentration-dependent manner at 5–50 μ M [33]. In our study, GSE also showed a dose-dependent effect on the inhibition of COX-2 expression. We did not compare the potency between different flavonoids; however, we provided the comparison results of GSE and vitamin C as a reference for the readers.

Vitamin C is a well-known strong antioxidant and also has anti-inflammatory properties. Although vitamin C therapy failed to show cardiovascular benefits in most of the clinical observational and prospective studies [15], its role as one of the combination agents might be rational [34, 35]. The single concentration of vitamin C (100 μ M) chosen for reference in the present study, though above the ranges of physiological concentrations (means around 30–40 μ M) [21, 36], was based on the experience of our previous study [37] and the study conducted by Rössig et al. [38], which showed that the concentration of vitamin C (100 μ M) effectively inhibited TNF- α (50 ng/mL)-induced apoptosis in HUVECs.

Grape seeds, which consist of lipid, protein, carbohydrates, and 5–8% phenols by weight, contain two-thirds of the extractable phenols of the grapes [18, 39]. The phenols are essentially flavonoids, which are referred to as monomeric flavan-3-ols (molecular weight about 290) [40], with polymerization in the range of 1–20 [41], consisting of about 8% monomers, 70% oligomers (dimers to heptamers), and 22% polymers (above heptamers) [42]. The antioxidant effects of GSE have been extensively studied [18]. In vitro studies showed that GSE reduced oxidized low-density lipoprotein [43] and was an even more potent scavenger of oxygen free radicals when compared with vitamin C and vitamin E [44]. In animal studies, GSE decreased free radical-induced lipid peroxidation in aged rats [45] and attenuated the development of atherosclerosis in cholesterol-fed rabbits [46]. In human studies, GSE reduced postprandial antioxidant levels in smokers [47] and improved flow-mediated dilatation in high-risk subjects [48].

The GSE in our study was a mixture of polyphenols, containing actually one portion of GSE and three portions

of citrus flavonoids from sweet oranges (*Citrus sinensis*), the major component of which is hesperidin, a flavanone glycoside with molecular weight about 610 [40]. This commercial product has been analyzed and demonstrated to contain flavonoids and condensed tannins [49]. Like vitamin C (100 μ M), the concentrations of GSE in this study was also above the physiological range.

It has been reported that the physiological concentrations of polyphenols are presumed not to exceed 10 μ M [14, 50–52]. Therefore, it has been questioned that the physiological concentrations of polyphenols are too low for antioxidative actions [53]. However, the polyphenols in food could be turned into more active metabolites via digestive and hepatic activity after the intestinal absorption. Therefore, the actual total plasma concentration of polyphenols is thought to be substantially higher due to the presence of metabolites, which are usually unable to be detected by present measurements [51, 52].

An example of more powerful effects of active metabolites was that the concentration of quercetin metabolites (sulfate/glucuronide) (1 μ M) had comparable antioxidant and anti-apoptotic effects on high glucose-treated HUVECs in comparison with vitamin C (100 μ M) and quercetin (the aglycone) (10–50 μ M) [37]. Another example was that oral consumption of vitamin C (2 g/d) and GSE (2 g/d) (1 g of polyphenols) could improve flow-mediated vasodilatation in humans [48, 54]. According to these two human studies, taking into account of the comparable power of vascular protection between vitamin C and polyphenols, the estimated plasma concentrations were 75 and 50 μ M, respectively [14, 50]. Since the estimated plasma concentration of polyphenols (50 μ M) was thought about 10 times higher than the parent polyphenols, it seemed that the active metabolites significantly contribute to the vascular protection [14]. In the present study, we were not able to indicate the exact chemical compositions of GSE that contribute their biological effects. For quality control, further studies are needed to identify the beneficial effects of its specific components.

In conclusion, we demonstrate that GSE effectively ameliorates TNF- α -induced inflammatory status of HUVECs. The findings of the present study suggest that the consumption of GSE may be beneficial to inflammatory atherosclerosis.

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