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Original Research Article

Does grape seed extract potentiate the inhibition of platelet reactivity in the presence of endothelial cells?

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ABSTRACT

Purpose: Numerous studies have suggested that grape seed extract (GSE) confers vascular protection due to the direct effect of its polyphenol content on endothelial cells. The aim of the study was to determine whether GSE confers vascular protection through the direct effect of its polyphenol content on endothelial cells.

Material/methods: After incubation with GSE-treated human umbilical vein endothelial cells (HUVECs), blood platelet reactivity was evaluated with regard to the expression of CD62P and the activated form of GPIIbIIIa in ADP-stimulated platelets.

Results: Lower concentrations of GSE were found to enhance the antiplatelet action of HUVECs: 1 μg/ml GSE reduced platelet reactivity by about 10%. While platelet reactivity was not altered by HUVECs incubated with higher concentrations of GSE, HUVEC proliferation was significantly reduced by GSE of up to 10 μg gallic acid equivalent/ml.

Conclusions: The results of the study show that low doses of GSE potentiate the inhibitory action of HUVECs on platelet reactivity, which may account, at least partially, for the protective effects of grape products against cardiovascular diseases. In contrast, high concentrations of GSE significantly impair endothelial cell proliferation *in vitro*.

1. Introduction

Cardiovascular disease (CVD) is a leading cause of death in many economically developed nations. Although some of the major risk factors for CVD are not "modifiable" – age, sex, genetic predisposition – aspects of diet and lifestyle are recognized as major risk factors modulating the outcome of CVD [1]. Recent findings have highlighted the importance of oxidative stress, vascular inflammation and endothelial dysfunction in the development of CVD [2]. Under physiological conditions, the endothelial cells prevent blood coagulation in vessels *via* production of nitric oxide and prostacyclin (PGI_2), both of which are potent inhibitors of platelet activation. The endothelial cells also produce an ecto-ADPase (also known as CD39 or NTPDase-1), which degrades extracellular adenosine 5′-diphosphate (ADP) resulting in decreased platelet reactivity [3]. Homeostasis of the vascular endothelium, in terms of both metabolic and physiological activities, is subject to fine-tuning by individual nutrients or nutrient derivatives [4,5]. Grape skin or seeds are very rich source of polyphenols, especially flavanols with proanthocyanidins [6], and other compounds with antioxidant and anti-inflammatory activities resembling those of resveratrol [7,8]. Many studies confirm the cardioprotective effects of grape seeds, demonstrating reduction of blood pressure, lower levels of oxidized low-density lipoprotein (ox-LDL), and enhanced nitric oxide (NO) production [9–12]. Recent studies have provided evidence that red grape components are a source of antioxidant compounds that ameliorate the viability and function of endothelial progenitor cells (EPC) [13]. Extracts from purple grape skins and seeds inhibit platelet function and platelet-dependent inflammatory responses [14], but the mechanism involved in these beneficial effects of grape extracts on platelet activation remains unresolved. Vitseva et al. [15] report a decrease in aggregation, a marked decrease in

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superoxide release, a significant increase in radical-scavenging activity, and enhanced platelet NO when platelets were incubated with seed or skin extract.

The present study examines the influence of the polyphenol-rich grape seed extract (GSE) on the viability and antiplatelet properties of human endothelial cells under *in vitro* conditions. Seed extract at lower concentration of polyphenolic compounds was found to increase the antiplatelet action of human umbilical vein endothelial cells (HUVECs) after stimulation with ADP. HUVECs incubated with higher concentrations of GSE did not demonstrate altered platelet reactivity, and up to 10 μg/ml GSE (as gallic acid equivalent) significantly reduced HUVEC proliferation.

2. Material and methods

2.1. Reagents

DMSO, (+)catechin, gallic acid, vanillin, ADP, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma (St. Louis, MO, USA). All other chemicals were reagent-grade products purchased from POCh S.A. (Gliwice, Poland). Materials for flow cytometry (anti-CD61PerCp, anti-CD62PE, PAC-1-FITC, CellFix and others) were obtained from Becton Dickinson (San Diego, CA, USA).

2.2. Characteristics of GSE

Grape (*Vitis vinifera* L.) seed extract (OMNIVIR®) was purchased from C.E. Roeper GmbH, Germany. For chemical analysis, the extract was diluted to 10 mg/ml with 10% DMSO. The total phenolic content of the extract was determined colorimetrically according to Folin–Ciocalteau [16] and expressed as milligrams gallic acid equivalent per gram of extract (mg GAE/g). Total flavanol content was estimated using the vanillin–H_2SO_4 method [17] and was expressed as milligrams (+)catechin equivalents per gram of extract (mg CE/g). Total proanthocyanidin content was determined after acid depolymerisation to the corresponding anthocyanidins as described by Rosch et al. [18]. The proanthocyanidin content (mg cyanidin/g of extract) was calculated using the molar extinction coefficient of cyanidin (ε = 17,360 L mol^{-1} cm^{-1}) and its molar mass (287 g mol^{-1}). The total flavonoid concentration was measured using a colorimetric assay [19] and was expressed as milligrams of (+)-catechin equivalent per gram of extract.

The composition of the GSE was determined spectrophotometrically (Table 1). The extract was found to comprise 41% phenolic compounds by weight, 81% of which were flavonoids. In total, flavanols (monomers and proanthocyanidins) constituted 27.2% of the extract by weight and proanthocyanidins (oligomers and polymers of flavanols) only 6.9% [6,10]. HPLC analysis of the same GSE samples published in an earlier report revealed that the extract contained monomers such as (+)-catechin and (−)-epicatechin, dimers such as procyanidin B1 and B2, and a trimer: procyanidin C1 [20].

Table 1
Composition of grape seed extract.

Component	Concentration (mg/g)
Total phenolics	410 ± 2
Flavonoids	333 ± 3
Flavanols	272 ± 6
Proanthocyanidins	69 ± 1

Data presented as mean ± SE, n = 5. The composition of grape seed extract was determined by spectrophotometric methods.

2.3. Maintenance of HUVEC culture

HUVECs and all reagents needed for cell culture were purchased from Cascade Biologics (Portland, or, USA). The HUVECs were cultured according to the manufacturer's instructions and the cells underwent 2–6 passages.

In flow cytometry studies, HUVECs were first transferred to 24-well plates at 75 × 10^3 cells/well and grown further at 37 °C for 24 h under a humidified atmosphere of 5% CO_2 in air.

For measurement of eNOS expression, HUVECs (1 × 10^6) were placed in 75 cm^2 culture bottles (NUNC, Roskilde, Denmark) and grown for 48 h. The culture medium was then replaced and stock solutions of polyphenol extract from grape seeds were added to give final concentrations of 0, 0.25, 1, 2.5, 5 and 10 μg gallic acid equivalent/ml. After 24 h the culture medium was discarded and the HUVEC monolayer was rinsed four times with PBS. The cells in PBS were then scraped and centrifuged (250 × g, 6 min, +4 °C). Finally, 1 ml of fenozole was added to the cell pellet for RNA isolation [21].

2.4. Effect of polyphenol extract from grape seeds on HUVEC proliferation

HUVECs were seeded on 96-well plates (10,000 cells/well) and grown further at 37 °C for 24 h in a humidified atmosphere of 5% CO_2 in air. They were then exposed to polyphenol extract at final concentrations of 0.1, 0.5, 1, 5, 10, 25, 50, 100 and 200 μg gallic acid equivalent/ml grape extract for 24 h. After this incubation, the polyphenol-containing culture medium was carefully removed and replaced with fresh medium, and the cells were cultured for a further 24 h. Proliferative activity was then determined by MTT assay [22]. The cells were treated with the MTT reagent for 2 h. The MTT-formazan crystals were dissolved in 20% SDS, 50% DMF, pH 4.7 and the absorbance at 570 nm was measured using a Victor3 multifunctional ELISA-plate reader (Perkin-Elmer, MA, USA).

2.5. Determination of eNOS expression

RNA was isolated using a Total RNA Prep Plus Minicolumn Kit (A&A Biotechnology, Poland) based on the RNA isolation method developed previously [21]. For real-time PCR normalization, UV absorbance was used to determine the amount of RNA added to a cDNA reaction. PCRs were then set up using cDNA derived from the same amount of input RNA; isolated RNA has an $A_{260/280}$ ratio of 1.6–1.8. For the reverse transcriptase (RT) reaction, an Enhanced Avian HS RT-PCR Kit (Sigma, St. Louis, MO, USA) was used according to the manufacturer's protocol. The cDNA was used immediately or stored at −20 °C. Before the quantitative analysis of gene expression by real-time PCR, the parameters were checked using qualitative PCR. The reaction mixture for PCR amplification consisted of a cDNA template, 0.5 μM of each primer, 10× AccuTaq Buffer, 0.5 U of AccuTaq LA DNA Polymerase Mix, 0.2 mM each dNTP, and water to a final volume of 20 μl. A negative control (sample without a cDNA template) was included in each experiment. The primer sequences for both genes, target-eNOS and reference *GAPDH*, were designed using Primer3 online software (Steve Rozen, Helen J. Skaletsky (1998) Primer3. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html). Real-time PCR was performed on the corresponding cDNA synthesized from each sample. For real-time PCR the MX3005P™ System (STRATAGEN, Santa Clara, CA, USA) was used. The gene eNOS and a reference gene *GAPDH* were amplified in parallel for each sample in separate wells during the same PCR procedure. GAPDH was utilized as an internal positive control and as a normalizer for correcting the expression data. For