European Journal of Pharmaceutics and Biopharmaceutics 80 (2012) 176–184

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Red grape skin and seeds polyphenols: Evidence of their protective effects on endothelial progenitor cells and improvement of their intestinal absorption

Francesca Felice [a], Ylenia Zambito [b,*], Giacomo Di Colo [b], Claudio D'Onofrio [c], Catia Fausto [c], Alberto Balbarini [a], Rossella Di Stefano [a]

[a] Cardiothoracic and Vascular Department, University of Pisa, Pisa, Italy
[b] Department of Pharmaceutical Sciences, University of Pisa, Pisa, Italy
[c] Department of Fruit Science and Plant Protection of Woody Species "G. Scaramuzzi", University of Pisa, Pisa, Italy

ARTICLE INFO

Article history:
Received 13 March 2011
Accepted in revised form 7 September 2011
Available online 24 September 2011

Keywords:
Chitosan
Endothelial progenitor cells
Red grape skin and seed
Red wine

ABSTRACT

Scope: To evaluate the ability of grape skin and seeds to protect endothelial progenitor cells (EPC) from oxidative stress induced by hyperglycemia (HG) compared to red wine (RW) and prepare innovative pharmaceutical systems for the oral administration of red grape extract allowing the overcoming of its poor intestinal absorption.

Methods and results: Human EPC were characterized by expression of cell surface markers. Cells were incubated with different concentrations of total polyphenols from grape components or RW in the presence or absence of HG. Cell viability, migration, adhesion, and reactive oxygen species (ROS) production were assayed. Intestinal permeation of polyphenols was studied in the absence or presence of a quaternary ammonium-chitosan conjugate (N^+(60)-Ch). Grape components and RW increased EPC viability, adhesion and migration, and prevented the HG effect ($P < 0.01$). ROS production induced by HG was significantly reduced only by grape seed extract and RW ($P < 0.01$). N^+(60)-Ch acted as an effective enhancer of polyphenol permeability across the excised rat intestine.

Conclusions: Red grape components are a source of antioxidant compounds that ameliorate EPC viability and function, while preventing endothelial dysfunction. The use of polycationic chitosan derivatives can promote the absorption of polyphenols across intestinal epithelium, thus increasing their bioavailability and potential therapeutic value in atherosclerosis.

1. Introduction

Cardiovascular diseases (CVD) are the leading cause of death and disability in the Western world [1].

Endothelial dysfunction, induced by elevated levels of oxidized low-density lipoprotein (Ox-LDL) levels, free radicals, shear stress, hypertension, toxins after smoking, or combination of these and other risk factors, gradually promotes vascular smooth muscle cells (SMC) proliferation, platelet aggregation, fibrinolysis, and monocyte adhesion, leading to formation of occlusive atherosclerotic plaque [2]. Angiogenesis, that is, the ability of the organism to spontaneously develop new blood vessels, has also been recognized as an important process for the progression of atherosclerotic plaques, which largely determines the severity of CVD [3].

Recent studies have provided increasing evidence that cardiovascular diseases, such as atherosclerosis, restenosis after injury and myocardial regeneration after infarction, not only depend on cells formerly residing in the location of the vascular or myocardial insult but are also influenced by bone marrow-derived cells [4–6]. Moreover, angiogenesis is due not only to the sprouting of pre-existing vessels but also to stem cells mobilized from the bone marrow capable of assuming endothelial phenotype [7]. These cells first described as endothelial progenitor cells (EPC) [5] circulate into peripheral blood and significantly contribute to neovascularization and re-endothelialization as part of the process of vascular repair [8]. Several studies have reported decreased EPC numbers in coronary disease and in patients at elevated risk of

Abbreviations: Ch, chitosan; CHD, coronary heart disease; CVD, cardiovascular diseases; DS, degree of substitution of N^+-Ch by pendant chains; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cells; GS, grape seed; GSE, grape seed extract; *n*, mean number of quaternary ammonium groups in N^+-Ch pendant chains; N^+-Ch, quaternary ammonium-chitosan conjugates; N^+(60)-Ch, N^+-Ch with DS = 60%, *n* = 1.7; Ox-LDL, oxidized low-density lipoproteins; PBMC, peripheral blood mononuclear cells; ROS, reactive oxygen species; RW, red wine; S, grape skin; SE, grape skin extract; SMCs, smooth muscle cells; TMC, N-trimethylchitosan; VE-Cad, VE-cadherin; VEGF, vascular endothelial grow factor; vWF, vonWillebrand factor; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate; KDR, vascular endothelial grow factor receptor 2.

* Corresponding author. Department of Pharmaceutical Sciences, University of Pisa, Via Bonanno 33, 56126 Pisa, Italy. Tel.: +39 050 2219657; fax: +39 050 2219660.

E-mail address: zambito@farm.unipi.it (Y. Zambito).

0939-6411/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2011.09.002

cardiovascular disease [9]. Moreover, EPC display a dysregulated proliferation and adhesion in subjects with diabetes [10].

Strategies that can prevent or ameliorate EPC number and function are currently of special clinical interest.

In 1992, Renaud and de Lorgeril [11] observed that despite a high intake of saturated fats, a significantly lower mortality rate for coronary heart disease (CHD) was present in France compared with other Northern European countries, a phenomenon known as the "French paradox". For years, red wine was thought to have beneficial effects on cardiovascular health, due to its antioxidant properties. A lot of studies show that red wine polyphenols contribute to the prevention of endothelial dysfunction, by increasing endothelial nitric oxide synthase (eNOS) expression [12], inhibition of LDL [13], inhibition of SMC proliferation and migration [14,15], and at low doses show pro-angiogenic effect on post-ischemic neovascularization in vivo [16]. Moreover, a recent study showed the beneficial effects of red wine on circulating EPC number and functional activity [17].

The activity of red wine has largely been reported and proved, but it has been suggested that ethyl alcohol fails to show any protective effect in this regard, and that, the antioxidant activity of red wine does not arise from its alcohol content, but rather from its phenol content [18].

Alcohol consumption increases the incidence of cardiomyopathies, hypertension, and ictus. Moreover, alcohol appears to accelerate the deterioration process of antioxidants. Recent studies showed that the amount of red wine necessary to provide the organism with a level of phenolic compounds adequate to obtain a significant antioxidant effect is too high to avoid the deleterious alcohol consequences [19].

For all these reasons, grape berries have recently been revaluated for their role as polyphenolic compounds source. Such compounds are receiving growing attention as materials for potential application, as functional food additives or ingredients, or as products from the pharmaceutical industry.

During the wine-making processes, indeed, a lot of by-products are produced, representing around 25% of the grape dry matter. In fact, starting from 100 kg of grape, 25 kg of pomaces is produced. Their composition is 50% skin, 25% clusters, and 25% seeds. Grape skin, which represents about 5–10% of the total dry weight of the grape berry, generally contains the highest concentration of tannins. Quercetin and kaempferol glucosides and glucuronides, gallic acid and its glucosides, caftaric and coutaric acid complete the phenolic composition of grape skins [20]. The seeds are the highest in phenol content (5–8% phenol by weight). Tannins are the main compounds present in the seeds as well as in the skin. Significant amounts of other compounds, namely, quercetin-3-glucuronide, followed by catechin, caftaric acid, and astilbin have also been detected in grape seeds. Procyanidin B1 and B2 have been found as well, along with minor quantities of epigallocatechin and gallic acid [21].

The health effects of polyphenols are largely dependent on both their respective intake and bioavailability, which can vary greatly. Bioavailability appears to differ deeply among phenols, the differences being primarily due to their structure, including degree of glycosylation, molecular size, and level of conjugation with other polyphenols. Consequently, the most common compounds in human diet are not necessarily the most active within the body, and they are not necessarily those leading to the highest concentration of active metabolites in target tissue [22]. Metabolism of polyphenols takes place through a general common pathway, with differences depending on the characteristics of the compounds. In general, just the aglycones and some glucosides can be absorbed in the small intestine, whereas esters, the most part of glycosides and polymers, must be hydrolyzed by intestinal enzymes or the colonic microflora before they can be absorbed [23].

These oral bioavailability problems might be addressed by resorting to intestinal absorption promoters. Low molecular weight enhancers, such as bile salts, surfactants, fatty acids, glycerides, and chelators, have been studied and applied for decades now. More recent reports have appeared in the literature evaluating the efficacy, mechanisms of action, structure–activity relationships, and safety of polymeric absorption enhancers [24]. Unlike the low molecular weight enhancers, the physicochemical characteristics of which favor their own absorption, high molecular weight polymers are generally not absorbed, and this minimizes the risk of systemic toxicity. Another important advantage of macromolecular enhancers over small molecular weight compounds resides in the fact that most of the former have mucoadhesive properties, which often act in synergism with the permeability-enhancing ones by facilitating and prolonging contact of the polymer molecule with the epithelium surface.

Over the past few years, the synthesis, characterization, and bioactivity of polymeric epithelial permeability enhancers derived from chitosan (Ch) have been described [25–27]. Ch is obtained by alkaline deacetylation of the natural polysaccharide chitin, which is the polymer of β-(1-4)-linked 2-acetamido-2-deoxy-D-glucopiranose and is obtained from the shells of marine crustaceans such as crabs and shrimps. Ch is commercially available in different molecular weights and deacetylation degrees. Ch derivatives having the structure of quaternary ammonium-chitosan conjugates (N^+-Ch) have been synthesized [26]. The conjugates are characterized by short pendant chains, made of n adjacent diethyl-dimethylene-ammonium groups, substituted onto the primary amino group of the Ch repeating units. The ability of N^+-Ch to promote the penetration of hydrophobic as well as hydrophilic drug models across different excised epithelia, namely, porcine buccal mucosa [25], rabbit cornea [27], rat jejunum [26], has been evidenced. Ch derivatives of the N^+-Ch family have proved apt to promote either the transcellular or the paracellular transepithelial penetration route. Permeation data across excised rat intestine have substantiated the hypothesis that such structural parameters of conjugates as degree of substitution by pendant chains (DS) and number of quaternary ammonium groups in each chain (n) determine the conjugate ability to promote the one rather than the other penetration route [26]. As in the cases of protonated or quaternized Chs, the activity of which as epithelial permeability enhancers is now well documented (see, e.g., [24]), an electrostatic interaction with negatively charged sites in the mucus, on the cell membranes, or in the tight junctions joining epithelial cells is supposed to be at the basis of the bioactivity of N^+-Ch. The more significant representatives of these Ch derivatives are characterized by DS values of 40% and 60% [26].

Based on the consideration that the red wine contributes to preventing oxidative stress and consequent loss of viability and functional activity of EPC, the present study first aimed at ascertaining whether the antioxidant products, essentially polyphenols, extracted from red grape skin and seeds can exert the same beneficial effects on glucose-stressed EPC. This event would allow taking advantage of the benefits granted by the wine, while preventing the noxious effects of alcohol. The ultimate purpose of this work has been to test the ability of a quaternary ammonium-chitosan conjugate with DS = 60%, n = 1.7 (N^+(60)-Ch) to enhance polyphenol permeation across excised rat intestine, in the perspective of designing novel pharmaceutical systems for the oral administration of the biologically active principles contained in red grape skin and seeds.

2. Materials and methods

2.1. Preparation of grape skin, seeds, and red wine

Fresh Sangiovese red wine (RW) and Sangiovese red grape skin (S) and seeds (GS) (collected at vintage), from Montecucco DOC

area (Tuscany, Italy), were used for this study. Phenols were extracted according to Di Stefano and Cravero method [28] with some modifications. Immediately after sampling, both S and GS of 10 berries were freed from the attached mesocarp and quickly immersed in 25 ml of an extraction solution buffer [28]. After 4 h in the extraction buffer, S and GS were homogenized and centrifuged 10 min at 3000 g, and the supernatant was extracted from S and GS used for analysis of total antioxidant capacity, essentially ascribed to polyphenols. This was determined using the Folin–Ciocalteu reagent (Sigma), as previously described [29], by measuring the absorbance (Perkin Elmer, Lambda 25) of an aliquot of supernatant at 750 nm.

The concentration of antioxidants was expressed as [μg catechin]/[L of supernatant] with reference to a (+)-catechin standard calibration curve. Afterward, the S or GS supernatant was lyophilized (VirTis adVantage, thermal treatment step: −35 °C for 360 min; drying cycle steps: −35 °C for 240 min; −25 °C for 360 min; +10 °C for 300 min; +25 °C for 120 min) to obtain dry S extract (SE) or GS extract (GSE).

For cell treatment or intestinal absorption studies, SE and GSE were dissolved in appropriate buffers.

2.2. Isolation and cultivation of human EPC

Human EPC were isolated from total peripheral blood mononuclear cells (PBMC) after 3 days of culture in a selective medium, as previously described [30]. Briefly, PBMC from healthy male donors (age <40) were isolated by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway; density = 1.077 g/ml) at 400 g for 30 min at room temperature according to the manufacturer's protocol. Isolated PBMC (1×10^6 cells/cm^2) were plated on 6-well plates (Greiner Bio One, Frickenhausen, Germany), 96-well plates (Greiner Bio One), or 8-well chamber slides (BD Biosciences, San Jose, CA) coated with human fibronectin and maintained in endothelial basal medium (EBM-2, Cambrex, Walkersville, MD) supplemented with EGM-2-MV-Single Quots containing human endothelial growth factor, hydrocortisone, insulin-like growth factor, fibroblast growth factor, vascular endothelial grow factor (VEGF), antibiotics, and 5% fetal bovine serum (FBS, Cambrex). Cells were cultured at 37 °C with 5% CO_2 in a humidified atmosphere. After 3 days of culture, non-adherent cells were removed by washing with PBS, and adherent cells were exposed to the different experimental conditions.

2.3. EPC characterization

To evaluate surface marker phenotype, cultured EPC were detached by a pre-treatment with PBS for 20 min, followed by incubation with Trypsin–EDTA (Cambrex) and labeled (5×10^5 cells/sample) for 20 min at room temperature at manufactured-recommended concentrations with the following monoclonal antibodies: anti-KDR-PE (R&D Systems, Minneapolis, MN), anti-VE-cadherin (Santa Cruz, Heidelberg, Germany), anti-CD31-FITC (Beckman Coulter, Marseille, France), anti-vWF (Santa Cruz), and anti-CD14-PE (BD Biosciences). For the analysis of VE-cadherin and vWF, the cells were further incubated with a FITC-conjugated goat anti-mouse IgG1 antibody (Santa Cruz). Appropriate isotype was used as negative control. Cells were washed and analyzed on a FACS-Calibur Instrument (Becton–Dickinson), acquiring 1×10^4 events.

The 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine-labeled acetylated LDL (DiI-ac-LDL)/Lectin staining was performed incubating EPC with 2 mg/ml of DiI-ac-LDL (Invitrogen) for 3 h at 37 °C. Cells were fixed in 4% paraformaldehyde and counterstained with 50 mg/ml FITC-labeled lectin from *Ulex europaeus* (Sigma) for 1 h at 37 °C in dark conditions. Cell counting was performed using a fluorescence microscope, and the total number of double-positive DiI-ac-LDL/Lectin cells was calculated by counting cells in each sub-field. EPC number was expressed as percentage of cells positive for DiI-ac-LDL/Lectin dual staining.

EPC, after 3 days of culture under standard conditions, resulted in an adherent population consisting of double-positive cells for DiI-Ac-LDL (red) and lectin (UEA-1-FITC) (green), as established by fluorescent microscope analysis (Fig. 1A). EPC phenotype was confirmed by the expression of endothelial cell surface markers CD31 (20 ± 14%), vWF (39 ± 15%), KDR (66 ± 13%), Ve-Cad (35 ± 13%), and monocyte marker CD14 (64 ± 8%) by flow cytometry analysis (Fig. 1B).

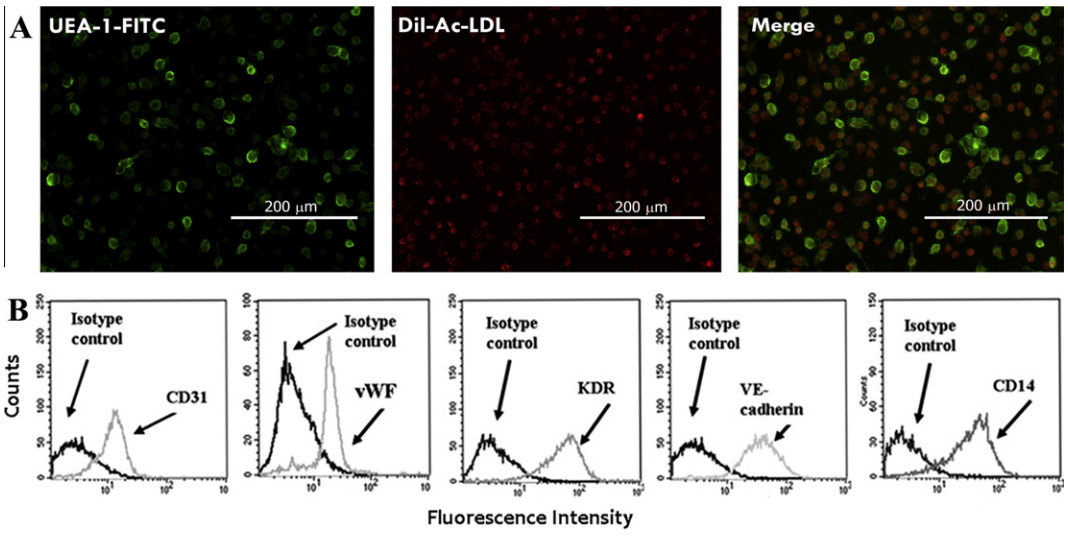

Fig. 1. EPC characterization. (A) EPC phenotype characterization by double staining with lectin UEA-1-FITC (green) binding and DiI-Ac-LDL (red) uptake. Micrographs showing EPC double positive for DiI-Ac-LDL and lectin staining (merge) are representative of three different experiments in duplicate. (B) EPC phenotype characterization by flow cytometry. Representative flow cytometric analysis ($n = 8$, means ± SD) of markers of EPC cultured for 3 days on fibronectin. CD31, vWF, KDR, VE-cadherin, and CD14 staining histograms are shown, compared with their isotype control. Fluorescence intensity, proportional to the surface abundance of the antigen detected by the fluorescent-labeled monoclonal antibody, is reported on the abscissa, while cell number is reported on the ordinate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.4. EPC treatment

After 3 days of culture, adherent EPC were pre-incubated for 2 h with RW, SE, or GSE diluted in a serum-free medium to different concentrations of catechin equivalents (5 μg/ml, 50 μg/ml, 150 μg/ml).

EPC were then cultured for 3 days in the presence of high D-glucose levels (HG, 25 mM), to simulate hyperglycemia conditions. At the end of the treatment, cells were washed twice with PBS and analyzed for viability and functional activities.

2.5. EPC viability

Cell viability was tested by WST-1 assay, based on the cleavage of tetrazolium salt (WST-1, 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate, Roche Applied Science, Mannheim, Germany) by mitochondrial dehydrogenases in viable cells. Briefly, after treatment, EPC were incubated with tetrazolium salt (10 μl/well) for 4 h at 37 °C, 5% CO_2. Then, the formazan dye formed was quantified by measuring the optical density at 450 nm (reference wavelength: 650 nm), by the use of a multiplate reader (Titertek). The absorbance directly correlated to the number of metabolically active cells.

2.6. EPC migration assay

The migratory function of EPC was evaluated by a modified Boyden chamber (Transwell, Coster) assay [9]. In brief, after treatment, EPC were detached with trypsin/EDTA, and then 4×10^4 EPC were placed in the upper chamber of 24-well Transwell plates with polycarbonate membrane (5 μm pores) with serum-free endothelial growth medium; VEGF (50 ng/ml) in medium was placed in the lower chamber. After incubation for 24 h, the membrane was briefly washed with PBS and fixed with 2% paraformaldehyde. The upper side of membrane was gently wiped with a cotton ball. The membrane was then stained using hematoxylin solution and removed. The magnitude of migration of EPC was evaluated by counting the migrated cells in six random high-power (100×) microscope fields.

2.7. EPC adhesion assay

After treatment, human EPC were washed with PBS and gently detached with 1 mmol/l EDTA in PBS. After centrifugation and resuspension in the medium with 5% FBS, identical cell numbers were re-plated onto fibronectin-coated culture dishes and incubated for 30 min at 37 °C. Adherent cells were counted manually in five random microscopic fields (200×), by independent-blinded investigators [31].

2.8. ROS production

ROS production was evaluated by ROS-sensitive fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichloro-di-hydro-fluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen). Briefly, after treatment, EPC were washed with PBS and incubated with CM-H_2DCFDA (10 μM/well) for 30 min at room temperature in the dark. Then cells were washed twice with fresh pre-warmed PBS, and ROS production was detected in EPC by measuring the increase in fluorescence by microplate reader. Fluorescence was measured by excitation at 488 nm and emission at 510 nm.

2.9. Stability testing

A discoloration of the GSE solutions was observed 3 h after their preparation, which was ascribed to an oxidation reaction [32]. This was inhibited by adding 0.163 mg/ml N-acetylcystein (NAC). NAC (0.163 mg/ml) was also added to the standards for the spectrophotometric calibration curve for determination of total antioxidant capacity in NAC-stabilized solutions. These were stable for at least 4 h, as determined by titration.

2.10. Permeation experiments

For permeation studies, the intestinal mucosa was excised from non-fasting male Wistar rats weighing 250–300 g. After sacrificing the rats, the first 20 cm of jejunum was immediately removed. The excised intestine was cut into strips of 1.5 cm, rinsed free of luminal contents, and mounted in Ussing-type chambers (0.78 cm^2 exposed surface area) without stripping off the underlying muscle layer. One milliliter of phosphate buffer pH 6.8, 0.13 M, made isotonic by the addition of sodium chloride (PBS 6.8), was added to the apical side and 3 ml of a phosphate buffer solution pH 7.4, 0.13 M, isotonic (PB 7.4), was added to the basolateral side (acceptor medium). In order to ensure oxygenation and agitation, a mixture of 95% O_2 and 5% CO_2 was bubbled through each compartment. The Ussing chambers were then placed in a water bath at 37 °C. After a 20-min equilibration period, the medium in each apical compartment was replaced by 1 ml of pre-thermostated GSE solution, 2 mg/ml in PBS 6.8 (corresponding to 2 g/l of catechin). The apical to basolateral transport of GSE components was investigated in the absence (control) or presence of 1% (w/v) of the permeability enhancer in the donor.

A quaternary ammonium-chitosan conjugate with a degree of substitution by pendant chains of 60% and an n value of 1.7 (N^+(60)-Ch) were used as the permeability enhancer [26]. N^+(60)-Ch was synthesized from shrimp shell Ch, minimum 90% deacetylated (Chitoclear FG90, Primex, Drammen, Norway). An average viscometric molecular weight of 590 kDa was determined for the commercial Ch. Its deacetylation degree, determined by IR and NMR, was 90% and 82%, respectively.

RW, diluted by PBS 6.8 to a concentration of antioxidants corresponding to 2 mg/ml catechin, was used as a further control. At 30-min interval over 240 min, 20 μl of samples were withdrawn from the acceptor compartments and replaced by the same volume of fresh medium.

The amount of permeated antioxidants was determined using the Folin–Ciocalteu micro-method, previously described. It must be pointed out that although no NAC was added to the donor solution, no discoloration of this solution was observed at the end of the permeation experiments.

2.11. Measurement of GSE components–polymer interaction

The apparatus consisted in five identical cells. Each cell consisted of two Teflon blocks, each having a cylindrical cavity (9.6 ml capacity; 1 cm depth). The blocks had a threaded hole entering the cavity, used for the introduction of the solutions and the removal of sample for analysis. For use, each cell was assembled by pressing together the two blocks in the stainless steel holder. A dialysis membrane, which had been pre-soaked in distilled water for at least 12 h (Spectra/Por, molecular weight cut-off, 3500 Da, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA), was fitted in each cell to separate the cavity into two compartments. The two parts of each cell were then firmly tightened by means of the cell holder, thus securing the membrane in place. At time $t = 0$, 8 ml of a titrated aqueous GSE solution (5 mg/ml) containing 2.5% of N^+(60)-Ch and 0.163 mg/ml of NAC was introduced in the reference compartment. The test compartment was filled with 8 ml of N^+(60)-Ch-free GSE solution was stabilized with 0.163 mg/ml NAC. A different initial GSE concentration was used for the test compartment of each cell, spanning a range to

presumably include the unknown equilibrium concentration with the solution contained in the reference compartment. After stoppering, agitation of cells was simultaneously initiated in a shaker water bath at 37 °C. In each cell, the GSE components diffused across the membrane along their concentration gradients, tending to equilibrate between compartments, while the polymer was impermeable. After 3 h, sufficient for a positive or negative concentration change in each test compartment to be detectable, such a change was determined and plotted vs. the corresponding initial concentration. As expected [33,34], a straight line with negative slope was obtained ($r^2 = 0.957$; $n = 5$), the abscissa intersection of which gave the equilibrium concentration with the N^+(60)-Ch-containing reference solution. Under the above experimental conditions, an equilibrium concentration lower than the GSE concentration in the reference compartment was considered as a sign and a measure of permeant–polymer interactions. The fraction of non-interacting permeant, f_F, was expressed by the following equation:

$$f_F = C_{equilibrium}/C_{total} \quad (1)$$

where C_{total} is the GSE concentration in the reference compartment.

2.12. Permeation data treatment

Permeation data were treated as previously described [27,35]. Briefly, for each permeation run, a value of the apparent permeability coefficient, P^*_{app}, of permeant across the excised rat intestinal mucosa was calculated from the following equation:

$$P^*_{app} = dM/dt * 1/AC_0f_F \quad (2)$$

where $(dM/dt)(1/A)$, the permeation flux, is the slope of the linear portion of the cumulative amount permeated per unit surface area vs. time plot, C_0 is the initial concentration of the permeant dissolved in the donor solution, and f_F is the permeant fraction free from polymer binding, expressed by Eq. (1). For each plot, the linear regression analysis was extended to the set of data points that gave the best fit, as judged from the r^2 value. This, in all of the cases investigated, was greater than 0.9 ($n = 6$). Also the lag time, L^*, which is the time axis intercept of the regression line, was calculated for each plot. The single P^*_{app} and L^* values were averaged to calculate the mean apparent permeability, P_{app}, and lag time, L ($n = 6$). The mean cumulative amount permeated per unit area in any given time was calculated to plot each permeation profile and to determine T_{4h}, i.e., the cumulative transport over the whole time of experiment. The significance of the difference between two P_{app}, or L, or T_{4h} values was assessed by the Student's t-test ($P < 0.05$). The P_{app} increase produced by N^+(60)-Ch was measured by the enhancement ratio (ER), defined as the ratio between the P_{app} values obtained in the presence and in the absence of enhancer.

2.13. Statistical analysis

Data represent means ± SD of at least three independent experiments. The difference among groups was evaluated by a one- or two-factor ANOVA by two independent investigators in a blinded fashion. Post hoc analysis was performed by Student–Newman–Keuls testing. Statistical significance was accepted at $P < 0.05$ or less.

3. Results

3.1. Effect of RW, SE, or GSE on EPC viability

Cell viability was evaluated by WST-1 colorimetric assay. As shown in Fig. 2, incubation of cells with constant HG levels for 3 days decreases significant early EPC viability as compared to 5 mM glucose levels (Control) ($P < 0.05$ vs. control). Pre-incubation with RW, SE, or GSE prevents the negative effect of HG exposure ($P < 0.01$ vs. HG) in a dose-dependent manner. Moreover, results indicate that RW, SE, or GSE alone does not affect cell viability significantly. Interestingly, the SE or GSE effect is comparable with the effect of fresh RW. Since an antioxidant concentration

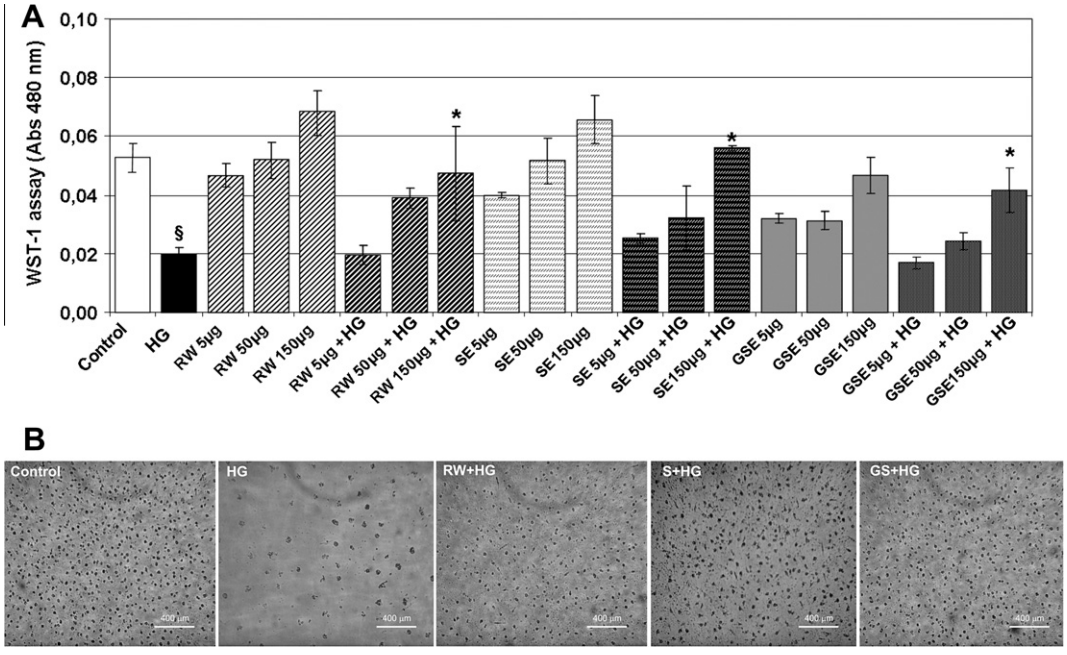

Fig. 2. Effects of SE, GSE, and RW on EPC viability. (A) Viable cells are evidenced by the presence of formazan dye in cell cytoplasm quantified by measuring the optical density at 480 nm. Pre-incubation of EPC with SE, GSE, and RW for 2 h dose-dependently improve cells viability. In particular, SE, GSE, or RW pre-treatment prevents the negative effect induced by 3 days of high glucose exposure (HG, 25 mM), ($n = 6$, means ± SD, $^{§}P < 0.05$ vs. Control and $^{*}P < 0.01$ vs. HG). (B) Representative image of EPC treated with 150 µg of SE, GSE, and RW in the presence of HG levels.

corresponding to 150 μg/ml catechin did not affect cell viability and protected cells from hyperglycemic conditions, this concentration was chosen to test the effect of RW, SE, or GSE on EPC function.

3.2. Effect of red grape components or wine on EPC migration and adhesiveness

As shown in Fig. 3A, the migration activity of EPC was significantly reduced by HG levels (25 mM) compared to untreated cells ($P < 0.01$ vs. control). Pre-treatment for 2 h with RW, SE, or GSE (150 μg/ml catechin equivalents) prevents the negative effect of glucose ($P < 0.01$ vs. HG). RW, SE, or GSE alone does not alter EPC migration. Interestingly, the effect of either SE or GSE is comparable with the RW effect. Fig. 3B shows the effect of pre-treatment with fresh RW or grape components on EPC adherence to fibronectin under HG exposition. We assessed the adhesion of EPC, because adhesion to the extracellular matrix is believed to be important during new blood vessel growth [36].

Results show a significant reduction in EPC adhesion capacity to fibronectin ($P < 0.01$ vs. control). Two hours of pre-treatment with RW, SE, or GSE prevents the effect of glucose significantly ($P < 0.01$ vs. HG). Moreover, the effect of either SE or GSE is comparable with the RW effect.

3.3. ROS production

Fig. 4 shows ROS accumulation in EPC as determined by CM-H2DCFDA. Exposure to 25 mM of HG resulted in an increase in the intracellular ROS levels ($P < 0.01$ vs. control). No significant effect was found in osmotic control experiments (data not shown). Interestingly, pre-treatment with fresh RW or GSE, but not SE, significantly prevented intracellular ROS formation induced by hyperglycemia ($P < 0.01$ vs. HG).

3.4. Measurement of GSE components–polymer interaction

Fig. 5 shows the plot of the variation of GSE concentration in test compartment after 3 h as a function of initial concentration in such a compartment. The abscissa intersection (1.549 mg/ml) represents the theoretical GSE concentration in the test compartment at thermodynamic equilibrium with the polymer containing 5 mg/ml of GSE solution in the reference compartment; hence, it equals the concentration of GSE components in this solution free from polymer binding. It corresponds to an f_F value of 0.31.

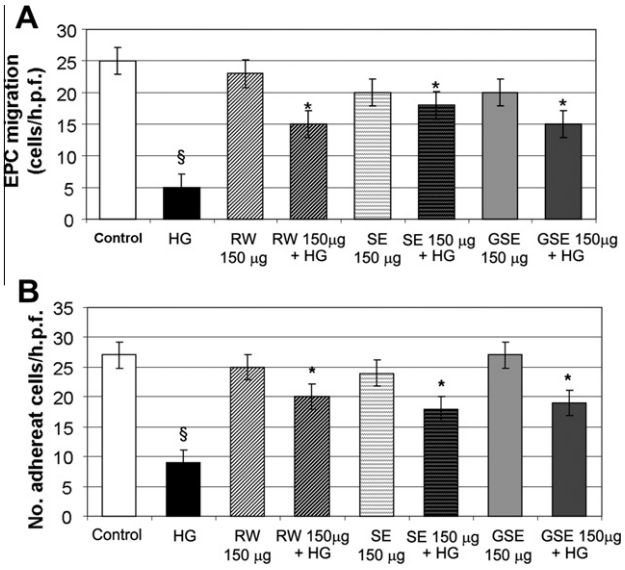

Fig. 3. Effects of SE, GSE, and RW on (A) EPC migration and (B) adhesiveness. Pre-incubation of EPC with 150 μg/ml of antioxidants in SE, GSE, and RW for 2 h prevent the negative effect induced by 3 days of high glucose exposure (HG, 25 mM), both on EPC migration (A) and fibronectin adhesion (B). ($n = 3$, means ± SD, $^{§}P < 0.01$ vs. Control and $^{*}P < 0.01$ vs. HG).

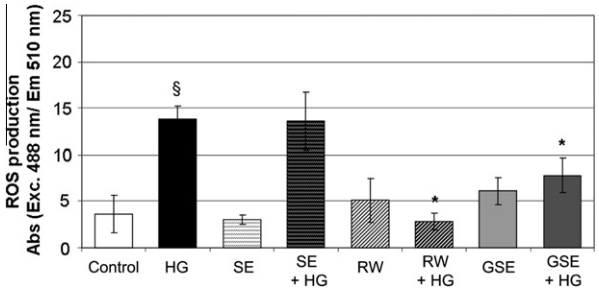

Fig. 4. Effects of SE, GSE, and RW on ROS production in EPC. Incubation of EPC with 150 μg/ml of GSE and RW for 2 h induced a significant reduction in intracellular ROS generation compared with cell exposed to high glucose levels (HG), ($n = 6$, means ± SD, $^{§}P < 0.01$ vs. control and $^{*}P < 0.01$ vs. HG).

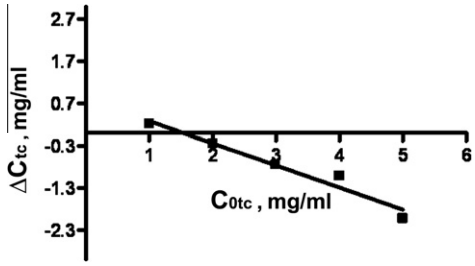

Fig. 5. Variation of concentration in test compartment (ΔC_{tc}) vs. initial concentration in test compartment (C_{0tc}). $C_{equilibrium}$ ($\Delta C_{tc} = 0$) = 1.549 mg/ml, $r^2 = 0.957$.

3.5. Permeation experiments

Each flux value in Table 1 measures steady-state permeation, subsequent to a transient period of variable permeation rate, which is quantified by the corresponding L value. With RW control, L is positive, indicating a lag time, i.e., a period of increasing rate before steady state, whereas with GSE alone (control) or in the presence of N^+(60)-Ch, L is negative, indicating an initial burst of permeation followed by a decreasing rate until steady state. The lag time is accounted for by the time necessary for the permeant to enter the barrier and attain a constant concentration gradient across it. To explain the burst effect, the presence of a fraction of particularly permeable, lower molecular weight polyphenols in GSE is admitted. These could permeate at higher rate during the transient period.

The steady-state flux values listed in Table 1 are not significantly different from one another; nevertheless, the P_{app} value calculated from Eq. (2) for the case of presence of N^+(60)-Ch is remarkably higher than that for the control, as quantified by the relevant ER value. For the calculation, the f_F value determined by the procedure described in the above section was used. This value is validated by the GSE/polymer w/w ratio being the same as in the permeation experiments. On the other hand, the P_{app} for the RW control is practically equal to that for the control. The differences in the T_{4h} values in Table 1 depend on whether the constant-rate permeation was preceded by a burst effect or a lag time.

Table 1
Data on drug permeation across excised rat jejunal epithelium from PBS 6.8 containing 2 mg/ml of GSE and 1% w/v of enhancer (N^+(60)-Ch). Means ± SD of at least 6 runs.

Donor	Flux (μg cm^{-2} h^{-1})	f_F[a]	L[b] (h)	P_{app}[c] 10^7 (cm s^{-1})	ER[d]	T_{4h}[e] (μg cm^{-2})
Control	2.93 ± 0.36	–	−1.30 ± 0.75	4.06 ± 0.50	–	16.21 ± 0.21
RW control	3.06 ± 1.18	–	0.98 ± 0.29*	4.25 ± 1.63	–	9.39 ± 4.39*
Enhancer	3.24 ± 0.49	0.31	−1.11 ± 0.28	14.51 ± 2.19*	3.4	16.79 ± 4.52

The data marked by * is significantly different from the Control ($P < 0.05$).
[a] Fraction of free permeant.
[b] Lag time.
[c] Apparent permeability.
[d] Enhancement ratio.
[e] Cumulative transport over the whole time of experiment (4 h).

4. Discussion

Maintenance of endothelial integrity, function, and post-natal neovascularization is considerably influenced by the number and functional activities of circulating EPC [9,37]. Correlative studies suggested that number and functional activities of EPC inversely correlate with cardiovascular risk factors among apparently healthy people and in patients with CHD [9,38].

Epidemiological studies indicate that regular intake of moderate amounts of beverages rich in polyphenols, particularly those of RW, is associated with a reduced risk of coronary disease [39,40]. Lefevre et al. [41] reported that beneficial effect of RW consumption on ischemia-induced neovascularization in hypercholesterolemic ApoE-deficient mice was related to an improvement in hematopoietic progenitor cells (HPC). However, these findings are limited to the bone marrow HPC and do not apply to EPC circulating in the peripheral blood [41]. Recently, the molecular mechanism of EPC migration in young, healthy individuals who consumed red wine was demonstrated [42].

Not all wines, however, seem to be equally effective. Differences in the extent of beneficial effects of wines have been related to the impact of grape cultivars, growing area, and the vinification/fermentation process on the total polyphenol composition [43,44]. Moreover, individuals always need to be cautioned against the dangers of heavy alcohol drinking.

In this study, we evaluated the effects of polyphenols extracted from grape skin and seeds, from Tuscany area (Italy), on EPC viability and functional activity compared to those of red wine, with the purpose of overcoming the alcohol-linked problems. Numerous studies show the protective effect of grape seed extract on endothelial injury by up-regulating eNOS and NO expression, by activating p-AKT or modulating AGEs/RAGE/NF-kappaB pathway in endothelial cells, or their effect in Type 2 diabetic subjects [45–48]. In our study, we analyzed the effects of grape seed and skin extracts on circulating EPC in the presence of high glucose level.

Contrary to other cardiovascular risk factors, high glucose concentrations have been shown to lower EPC migration and proliferation [49]. Therefore, in this study, we used glucose-stressed EPC to perturb EPC functional activity, in order to highlight the effects of the extracts from grape skin and seeds on the preservation of EPC migration, adhesiveness, and viability, compared to those of red wine. Moreover, we studied the protective effects on ROS production.

We observed that different concentrations of total polyphenols in SE, GSE, or RW did not alter EPC viability; in fact, antioxidant products, essentially polyphenols, corresponding to 150 μg/ml catechin, in SE, GSE, or RW, significantly ameliorated the HG-reduced EPC viability ($P < 0.01$ vs. HG). Moreover, we observed no differences among the RW, SE, and GSE effects. Most probably, this is due to multiple components of RW, namely, procyanidins and gallic acid [50], which are responsible for the RW beneficial effect. Conceivably, this might also explain the same beneficial effects observed when SE and GSE were tested (Fig. 2).

Since 150 μg/ml of active principles was effective on cell viability preservation of glucose-stressed EPC, we used this concentration to study the effects on EPC migration and adhesion. Again, we observed that SE and GSE increased migration and adhesiveness of glucose-stressed EPC, and their effects were similar to those of RW (Fig. 3A and B).

ROS play a key role in glucose-induced apoptosis of mature vascular endothelial cells, a phenomenon that can be reversed by antioxidants [51]. Therefore, we have assessed ROS generation induced by HG (25 mM). Interestingly, the results showed that ROS accumulation, as indicated by fluorescent CM-H2DCFDA, is lower in EPC pre-treated with GSE, but not with SE. This may be due to the higher concentration of tannins and phenols in the former [20].

The results of this study are in accord with previously published data reporting that RW enhances EPC activities [52,53]. The major findings of this study are that treatment of cultured glucose-stressed EPC with SE or GSE normalize the EPC viability, migration, and adhesiveness to the same extent as RW and that GSE, but not SE, acts as well as RW to reduce ROS production. From these results, we conclude that the beneficial effects of grape components on EPC are similar to those exerted by RW and that these effects are mediated by activation of the signaling pathway previously studied [42].

The second goal of this work was to obviate the insufficient intestinal absorption of polyphenols contained in grape derivatives. Since the total content of active principles in GSE was more effective than that of SE, we used the former to perform the intestinal permeation experiments. The excised rat jejunum was chosen among the known intestinal epithelium models for the permeability enhancement studies, because its tight junctions are similar in tightness and number to those of the human jejunum [54], and because the Ch derivatives under study are expected to act by modulating intercellular tight junctions [27].

Altogether, the data in Table 1 point to N^+(60)-Ch as an effective permeability enhancer of the GSE components across the excised rat intestine. This chitosan derivative has been previously shown to be non-toxic to epithelial cells at the concentration used in the present permeation experiments [35,55].

The solid GSE and N^+(60)-Ch products, obtained as described in Section 2, may be thought to be co-formulated into a conventional gastro-protected tablet formulation containing the biologically effective amounts of the active principles and intestinal absorption enhancer. The above is a realistic hypothesis, which would warrant a pre-formulation work.

5. Conclusions

The hypotheses at the basis of the present study, i.e., that the natural antioxidant products, essentially polyphenols, extracted from Italian red grape skin and seeds can exert the same beneficial effects on EPC as RW, and that, the quaternary ammonium-chitosan conjugate, N^+(60)-Ch, has the ability to enhance the permeation of such products across excised rat intestine, have been

confirmed. These results show the potential advantages of an oral dosage formulation comprising the above dry extracts as the active principles and the Ch derivative as the absorption enhancer. Such a formulation would allow taking advantage of the benefits granted by the wine while preventing the noxious effects of alcohol. As a future prospect, the present study could lead to the development of novel strategies to prevent the complications of ischemic vascular diseases.

References

[1] S. Yusuf, S. Reddy, S. Ounpuu, S. Anand, Global burden of cardiovascular diseases: part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization, Circulation 104 (2001) 2746–2753.
[2] U. Landmesser, B. Hornig, H. Drexler, Endothelial function: a critical determinant in atherosclerosis? Circulation 109 (2004) 27–33.
[3] R. Di Stefano, F. Felice, A. Balbarini, Angiogenesis as risk factor for plaque vulnerability, Curr. Pharm. Des. 15 (2009) 1095–1106.
[4] S. Dimmeler, A. Aicher, M. Vasa, C. Mildner-Rihm, K. Adler, M. Tiemann, H. Rutten, S. Fichtlscherer, H. Martin, A.M. Zeiher, HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway, J. Clin. Invest. 108 (2001) 391–397.
[5] T. Asahara, T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, J.M. Isner, Isolation of putative progenitor endothelial cells for angiogenesis, Science 275 (1997) 964–967.
[6] M. Vasa, S. Fichtlscherer, K. Adler, A. Aicher, H. Martin, A.M. Zeiher, S. Dimmeler, Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease, Circulation 103 (2001) 2885–2890.
[7] S. Kässmeyer, J. Plendl, P. Custodis, M. Bahramsoltani, New insights in vascular development: vasculogenesis and endothelial progenitor cells, Anat. Histol. Embryol. 38 (2009) 1–11.
[8] T. Povsic, PJ. Goldschmidt-Clermont, Endothelial progenitor cells: markers of vascular reparative capacity, Ther. Adv. Cardiovasc. Dis. 2 (2008) 199–213.
[9] M. Vasa, S. Fichtlscherer, A. Aicher, K. Adler, C. Urbich, H. Martin, A.M. Zeiher, S. Dimmeler, Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease, Circ. Res. 89 (2001) E1–7.
[10] O.M. Tepper, R.D. Galiano, J.M. Capla, C. Kalka, P.J. Gagne, G.R. Jacobowitz, J.P. Levine, G.C. Gurtner, Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures, Circulation 106 (2002) 2781–2786.
[11] S. Renaud, M. de Lorgeril, Wine, alcohol, platelets, and the French paradox for coronary heart disease, Lancet 339 (1992) 1523–1526.
[12] T. Wallerath, D. Poleo, H. Li, U. Forstermann, Red wine increases the expression of human endothelial nitric oxide synthase: a mechanism that may contribute to its beneficial cardiovascular effects, J. Am. Coll. Cardiol. 41 (2003) 471–478.
[13] B. Fuhrman, A. Lavy, M. Aviram, Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation, Am. J. Clin. Nutr. 61 (1995) 549–554.
[14] K. Iijima, M. Yoshizumi, M. Hashimoto, S. Kim, M. Eto, J. Ako, Y.Q. Liang, N. Sudoh, K. Hosoda, K. Nakahara, K. Toba, Y. Ouchi, Red wine polyphenols inhibit proliferation of vascular smooth muscle cells and downregulate expression of cyclin A gene, Circulation 101 (2000) 805–811.
[15] K. Iijima, M. Yoshizumi, M. Hashimoto, M. Akishita, K. Kozaki, J. Ako, T. Watanabe, Y. Ohike, B. Son, J. Yu, K. Nakahara, Y. Ouchi, Red wine polyphenols inhibit vascular smooth muscle cell migration through two distinct signaling pathways, Circulation 105 (2002) 2404–2410.
[16] J. Lekakis, L.S. Rallidis, I. Andreadou, G. Vamvakou, G. Kazantzoglou, P. Magiatis, A.L. Skaltsounis, D.T. Kremastinos, Polyphenolic compounds from red grapes acutely improve endothelial function in patients with coronary heart disease, Eur. J. Cardiovasc. Prev. Rehabil. 12 (2005) 596–600.
[17] M.L. Balestrieri, C. Fiorito, E. Crimi, F. Felice, C. Schiano, L. Milone, A. Casamassimi, A. Giovane, V. Grimaldi, V. del Giudice, P.B. Minucci, F.P. Mancini, L. Servillo, F.P. D'Armiento, B. Farzati, C. Napoli, Effect of red wine antioxidants and minor polyphenolic constituents on endothelial progenitor cells after physical training in mice, Int. J. Cardiol. 126 (2008) 295–297.
[18] M. Serafini, G. Maiani, A. Ferro-Luzzi, Alcohol-free red wine enhances plasma antioxidant capacity in humans, J. Nutr. 128 (1998) 1003–1007.
[19] K.K. Rocha, G.A. Souza, F.R. Seiva, G.X. Ebaid, E.L. Novelli, Weekend ethanol consumption and high-sucrose diet: resveratrol effects on energy expenditure, substrate oxidation, lipid profile, oxidative stress and hepatic energy metabolism, Alcohol Alcohol 46 (2011) 10–16.
[20] M. Pinelo, V.F. Laurie, A.L. Waterhouse, A simple method to separate red wine nonpolymeric and polymeric phenols by solid-phase extraction, J. Agric. Food. Chem. 54 (2006) 2839–2844.
[21] J.A. Kennedy, M.A. Matthews, A.L. Waterhouse, Changes in grape seed polyphenols during fruit ripening, Phytochemistry 55 (2000) 77–85.
[22] C. Manach, A. Scalbert, C. Morand, C. Remesy, L. Jimenez, Polyphenols: food sources and bioavailability, Am. J. Clin. Nutr. 79 (2004) 727–747.
[23] A. Scalbert, C. Morand, C. Manach, C. Remesy, Absorption and metabolism of polyphenols in the gut and impact on health, Biomed. Pharmacother. 56 (2002) 276–282.
[24] G. Di Colo, Y. Zambito, C. Zaino, Polymeric enhancers of mucosal epithelia permeability: synthesis, transepithelial penetration-enhancing properties, mechanism of action, safety issues, J. Pharm. Sci. 97 (2008) 1652–1680.
[25] Y. Zambito, G. Uccello-Barretta, C. Zaino, F. Balzano, G. Di Colo, Novel transmucosal absorption enhancers obtained by aminoalkylation of chitosan, Eur. J. Pharm. Sci. 29 (2006) 460–469.
[26] Y. Zambito, C. Zaino, G. Uccello-Barretta, F. Balzano, G. Di Colo, Improved synthesis of quaternary ammonium-chitosan conjugates (N+-Ch) for enhanced intestinal drug permeation, Eur. J. Pharm. Sci. 33 (2008) 343–350.
[27] Y. Zambito, C. Zaino, S. Burchielli, V. Carelli, M.F. Serafini, G. Di Colo, Novel quaternary ammonium-chitosan derivatives for the promotion of intraocular drug absorption, J. Drug Deliv. Sci. Technol. 17 (2007) 19–24.
[28] R. Di Stefano, M.C. Cravero, Metodi per lo studio dei polifenoli dell'uva, Riv. Vitic. Enol. 44 (1991) 37–45.
[29] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventos, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent, Methods Enzymol. 299 (1999) 152–178.
[30] R. Di Stefano, T. Santoni, M.C. Barsotti, C. Armani, B. Chifenti, C. Guida, R. Vanacore, M.T. Locci, M. Mariani, A. Balbarini, M. Mariani, Different growth conditions for peripheral blood endothelial progenitors, Cardiovasc. Radiat. Med. 3 (2002) 172–175.
[31] D.H. Walter, K. Rittig, F.H. Bahlmann, R. Kirchmair, M. Silver, T. Murayama, Statin therapy accelerate reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells, Circulation 105 (2002) 3017–3024.
[32] S. Quideau, D. Deffieux, C. Douat-Casassus, L. Pouységu, Plant polyphenols: chemical properties, biological activities, and synthesis, Angew. Chem. Int. Ed. Engl. 50 (2011) 586–621.
[33] G. Di Colo, Y. Zambito, A study of release mechanisms of different ophthalmic drugs from erodible ocular inserts based on poly(ethylene oxide), Eur. J. Pharm. Biopharm. 54 (2002) 193–199.
[34] F. Bottari, V. Carelli, G. Di Colo, M.R. Firinu, E. Nannipieri, A method for studying drug complexation in semisolid vehicles, Il Farmaco Ed. Pr. 33 (1978) 3–21.
[35] Y. Zambito, S. Fogli, C. Zaino, F. Stefanelli, M.C. Breschi, G. Di Colo, Synthesis, characterization and evaluation of thiolated quaternary ammonium-chitosan conjugates for enhanced intestinal drug permeation, Eur. J. Pharm. Sci. 38 (2009) 112–120.
[36] J. Bischoff, Cell adhesion and angiogenesis, J. Clin. Invest. 100 (1997) S37–S39.
[37] C. Urbich, S. Dimmeler, Endothelial progenitor cells: characterization and role in vascular biology, Circ. Res. 95 (2004) 343–353.
[38] J.M. Hill, G. Zalos, J.P. Halcox, W.H. Schenke, M.A. Waclawiw, A.A. Quyyumi, T. Finkel, Circulating endothelial progenitor cells, vascular function, and cardiovascular risk, N. Engl. J. Med. 348 (2003) 593–600.
[39] E.B. Rimm, P. Williams, K. Fosher, M. Criqui, M.J. Stampfer, Moderate alcohol intake and lower risk of coronary heart disease: meta-analysis of effects on lipids and haemostatic factors, BMJ 319 (1999) 1523–1528.
[40] S.C. Renaud, R. Gueguen, J. Schenker, A. d'Houtaud, Alcohol and mortality in middle-aged men from eastern France, Epidemiology 9 (1998) 184–188.
[41] J. Lefevre, S.E. Michaud, P. Haddad, S. Dussault, C. Menard, J. Groleau, J. Turgeon, A. Rivard, Moderate consumption of red wine (cabernet sauvignon) improves ischemia-induced neovascularization in ApoE-deficient mice: effect on endothelial progenitor cells and nitric oxide, FASEB J. 21 (2007) 3845–3852.
[42] S. Hamed, J. Alshiek, A. Aharon, B. Brenner, A. Roguin, Red wine consumption improves in vitro migration of endothelial progenitor cells in young, healthy individuals, Am. J. Clin. Nutr. 92 (2010) 161–169.
[43] A. Gambuti, D. Strollo, M. Ugliano, L. Lecce, L. Moio, Trans-resveratrol, quercetin, (+)-catechin, and (−)-epicatechin content in south Italian monovarietal wines: relationship with maceration time and marc pressing during winemaking, J. Agric. Food Chem. 52 (2004) 5747–5751.
[44] V. Cheynier, Polyphenols in foods are more complex than often thought, Am. J. Clin. Nutr. 81 (2005) 223S–229S.
[45] Z. Feng, R.B. Wei, Q. Hong, S.Y. Cui, X.M. Chen, Grape seed extract enhances eNOS expression and NO production through regulating calcium-mediated AKT phosphorylation in H_2O_2-treated endothelium, Cell Biol. Int. 34 (2010) 1055–1061.
[46] P. Kar, D. Laight, H.K. Rooprai, K.M. Shaw, M. Cummings, Effects of grape seed extract in type 2 diabetic subjects at high cardiovascular risk: a double blind randomized placebo controlled trial examining metabolic markers, vascular tone, inflammation, oxidative stress and insulin sensitivity, Diabet. Med. 26 (2009) 526–531.
[47] M. Lu, L. Xu, B. Li, W. Zhang, C. Zhang, H. Feng, X. Cui, H. Gao, Protective effects of grape seed proanthocyanidin extracts on cerebral cortex of streptozotocin-induced diabetic rats through modulating AGEs/RAGE/NF-kappaB pathway, J. Nutr. Sci. Vitaminol. (Tokyo) 56 (2010) 87–97.
[48] G. Zhao, H. Gao, J. Qiu, W. Lu, X. Wei, The molecular mechanism of protective effects of grape seed proanthocyanidin extract on reperfusion arrhythmias in rats in vivo, Biol. Pharm. Bull. 33 (2010) 759–767.
[49] Y.H. Chen, S.J. Lin, F.Y. Lin, T.C. Wu, C.R. Tsao, P.H. Huang, P.L. Liu, Y.L. Chen, J.W. Chen, High glucose impairs early and late endothelial progenitor cells by modifying nitric oxide-related but not oxidative stress-mediated mechanisms, Diabetes 56 (2007) 1559–1568.
[50] C.C. Appeldoorn, A. Bonnefoy, B.C. Lutters, K. Daenens, T.J. van Berkel, M.F. Hoylaerts, E.A. Biessen, Gallic acid antagonizes P-selectin-mediated platelet-leukocyte interactions: implications for the French paradox, Circulation 111 (2005) 106–112.

[51] R. Recchioni, F. Marcheselli, F. Moroni, C. Pieri, Apoptosis in human aortic endothelial cells induced by hyperglycemic condition involves mitochondrial depolarization and is prevented by N-acetyl-L-cysteine, Metabolism 51 (2002) 1384–1388.
[52] M.L. Balestrieri, C. Schiano, F. Felice, A. Casamassimi, A. Balestrieri, L. Milone, L. Servillo, C. Napoli, Effect of low doses of red wine and pure resveratrol on circulating endothelial progenitor cells, J. Biochem. 143 (2008) 179–186.
[53] J. Gu, C. Wang, H. Fan, H. Ding, X. Xie, Y. Xu, B. Wang, D. Huang, Effects of resveratrol on endothelial progenitor cells and their contributions to reendothelialization in intima-injured rats, J. Cardiovasc. Pharmacol. 47 (2006) 711–721.
[54] I. Legen, M. Salobir, J. Kerc, Comparison of different intestinal epithelia as models for absorption enhancement studies, Int. J. Pharm. 291 (2005) 183–188.
[55] G. Rassu, E. Gavini, H. Jonassen, Y. Zambito, S. Fogli, M.C. Breschi, P. Giunchedi, New chitosan derivatives for the preparation of rokitamycin loaded microspheres designed for ocular or nasal administration, J. Pharm. Sci. 98 (2009) 4852–4865.