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Effect of Low Doses of Red Wine and Pure Resveratrol on Circulating Endothelial Progenitor Cells

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Circulating endothelial progenitor cells (EPCs) play a significant role in neovascularization of ischaemic tissues and in re-endothelization of injured blood vessels. Identification of compounds able to enhance EPC levels and improve their functional activity, noticeably compromised by risk factors for coronary heart disease, is of clinical interest. This study evaluates the effects of red wine on EPCs. After being isolated from total peripheral blood mononuclear cells, EPC phenotype was confirmed by the presence of double positive cells for DiLDL uptake and lectin binding and by expression of CD34, CD133 and VE-cadherin cell surface markers. Long-term culture in the presence of red wine (1 μl/ml), containing resveratrol (Resv) at physiological concentration (nM), determined a time-dependent amelioration of cell number ($P < 0.05$). The presence of red wine prevented the TNF-α-induced reduction of EPC number ($P < 0.05$) and this effect was accompanied by reduced p38-phosphorylation expression levels ($P < 0.05$) and increased NOx levels ($P < 0.05$) Indeed, pure Resv alone significantly improved the TNF-α reduced EPC number ($P < 0.05$). This evidence indicates novel beneficial effects of red wine and Resv in the positive modulation of EPCs levels.

Key words: antioxidants, endothelial progenitor cells, red wine, resveratrol.

Abbreviations: CHD, coronary heart disease; DilLDL, 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanina-labelled acetylated LDL; EC, endothelial cells; EPCs, Endothelial progenitor cells; LDL, low density lipoprotein; LDLR−/−, hypercholesterolaemic LDL receptor-deficient; MAP, kinase, mitogen activating protein kinase; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; PBMCs, peripheral blood mononuclear cells; PE, phycoerythrin; TNF-α, tumour necrosis factor-alpha; RW, red wine; Resv, resveratrol; VE-cad, VE-cadherin; VEGF, vascular endothelial growth factor.

Circulating endothelial progenitor cells (EPCs) isolated from peripheral blood mononuclear cells (PBMCs), characterized by the surface markers CD34, CD133 and vascular endothelial growth factor (VEGF) receptor 2 (*1*, *2*), participate in post-natal growth of new blood vessels. Recently, clonal studies provided functional criteria to dissect the origin and *in vitro* and *in vivo* functional characteristics of true EPCs as well as the counterparts of monocyte-like EPCs (*3*). This population of progenitor cells circulates in the blood and homes to the site of injured endothelium or extravascular tissue (*4*, *5*).

Maintenance of endothelial integrity, function and post-natal neovascularization are considerably influenced by the number and functional activities of circulating EPCs (*6–8*). Correlative studies suggested that number and functional activities of EPCs inversely correlate with cardiovascular risk factors among apparently healthy people and in patients with coronary heart disease (CHD) (*9*, *10*). Moreover, EPCs display a dysregulated proliferation and adhesion to tumour necrosis-alpha (TNF-α) in subject with diabetes (*11*). Indeed, the decrease of VEGF and nitric oxide (NO) with age may play a synergistic role in mobilization, migration, proliferation and survival of EPCs (*12–14*). Statin (*15*), oestrogen (*16*), PPAR-γ antagonists (*17*, *18*) and physical exercise (*12*) counteract the reduction of EPC number and functional activity.

The abundant quantities of polyphenolic compounds contained in red wine (RW), not necessarily present in other alcoholic beverages, promote its cardioprotective effects (*19*, *20*). RW polyphenols contribute to the prevention of endothelial dysfunction (*21–24*), inhibition of oxidized low-density lipoprotein (LDL) (*25*), and at low doses show proangiogenic effect on post-ischaemic neovascularization *in vivo* (*26*). Beneficial effect of RW consumption on ischaemia-induced neovascularization in hypercolesterolaemic ApoE-deficient mice were recently shown to be associated with an increase in bone marrow haematopoietic progenitor cell (HPC) number (*27*).

Although individuals always need to be cautioned against the dangers of heavy alcohol drinking, epidemiological studies are consistent with the fact that regular,

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moderate consumption of RW is linked to a reduced risk of CHD and to lower overall mortality (*28–30*).

We have previously shown that intervention with polyphenols contained in pomegranate juice and antioxidants/L-arginine reverses shear-stress-related redox gene activation and increases endothelial nitric oxide synthase (eNOS) expression both in cultured endothelial cells (EC) and hypercholesterolaemic LDL receptor-deficient ($LDLR^{-/-}$) mice (*31*, *32*). More recently, we demonstrated that moderate treatment with RW significantly attenuated the activation of redox-sensitive genes (ELK-1 and *p*-JUN) and increased eNOS expression (which was decreased by perturbed shear stress) *in vitro* in EC and *in vivo* in atherosclerosis-prone areas of $LDLR^{-/-}$ mice (*33*). Moreover, RW supplementation for 28 days significantly increased upregulation of aortic eNOS and SIRT1 expression induced by physical training in C57BL/6J mice (*33*).

Multiple beneficial events responsible for the cardioprotective effect of polyphenols and other components of RW (*29*) could include protection against factors that influence the number of EPC circulating in the peripheral blood. To date, mechanism(s) responsible for the effect of RW on circulating EPC number remains to be determined. Here, we investigated the *in vitro* effect of RW on human circulating EPCs to complement our *in vivo* observations (*33*) and to elucidate potential mechanism underlying RW effect.

METHODS

Determination of Polyphenolic Content and In Vitro Antioxidant Capacity of Wine—Fresh RW (Aglianico, Feudi di San Gregorio) was used for this study. Although the most common type of isomerization is *cis*- to *trans*, special care was taken in order to avoid exposure to light and thermal heating of the wine samples and standards. The wine samples were filtered before chromatographic analysis. Total phenols were determined using the Folin–Ciocalteu's phenol reagent (Sigma) (*34*). The results were expressed in gallic acid equivalent (GAE), a naturally occurring polyphenol. Antioxidant capacity was measured according to the FRAP (Ferric Reducing Ability of Plasma) method (*35*) as previously described (*33*). The results were expressed as FRA (mmol/l) (*35*).

Determination of Resveratrol (Resv) Content—The quantification of Resv (*trans*-resveratrol) was performed as previously described (*33*, *36*) with minor modifications. Briefly, stock solution of *trans*-resveratrol, from 0.1 to 1.0 mg/l, (99% GC) (Sigma) was prepared in a 12% alcoholic (aqueous) solution. Each standard and wine sample was analysed by HPLC (Agilent chromatograph) with UV detection, equipped with a C18 from Phenomenex (250 × 4.6 mm, with 5 μm particles) and pre-column with the same stationary phase. Mobile phase consisted of a water/acetonitrile/acetic acid mixture (volume ratio 70/29:9/0.1), with a flow rate of 0.8 ml/min. Injection volume was 50 μl. Detection was performed at a 310 nm wavelength. Run time was 8 min. Quantification was based on integration of the peak area, using the external standard method.

Isolation and Culture of Circulating Human EPCs—EPCs were isolated from total human PBMCs as previously described by Casamassimi *et al*. (*37*). Briefly, PBMCs were isolated by density gradient centrifugation (400*g* for 40 min at 4°C) of 15 ml of leucocyte-rich buffy coat of healthy human donor on 20 ml of Histopaque-10771 (1.077 g/ml, Sigma). After centrifugation the interface cells were washed twice with Pipes (1×), centrifuged at 300*g* for 10 min at 4°C and then suspended in 9 ml of H_2O, 3 ml KCl 0.6 M to a final volume of 50 ml of Pipes (1×). After centrifugation at 300*g* for 10 min at 4°C the pellet was suspended in an appropriate volume of Pipes (1×) and cells were counted. Isolated PBMCs were plated on culture dishes (5×10^6 cells/ml medium) coated with human fibronectin and maintained in endothelial basal medium (EBM; Cell Systems) supplemented with 1 μg/ml hydrocortisone, 12 μg/ml bovine brain extract, 50 μg/ml gentamycin, 50 ng/ml amphotericin B, 10 ng/ml epidermal growth factor and 20% FCS (*37*). Cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere for 3 days. After 3 days of culture the non-adherent cells were removed by washing with PBS and adherent cells were used for further analysis.

DiLDL/Lectin Staining—Total PBMCs (5×10^6 cells/ml medium) were grown on microscope fibronectin coated glasses in 24-multiwell plates for 3 days. The 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labelled acetylated LDL (DiLDL)/Lectin staining was performed as described (*37*). Briefly, after 3 days of culture, EPCs were incubated with 2 μg/ml of DiLDL (Biomedical Technologies Inc.) for 3 h at 37°C (*37*). Cells were fixed in 4% paraformaldehyde and counterstained with 50 μg/ml FITC-labelled lectin from *Ulex europaeus* (Sigma) for 1 h at 37°C in the dark. Cell counting was performed as recently described by Casamassimi *et al*. (*37*). Briefly, microscope (Leica FW4000) field image was divided in 120 sub-fields and total number of double-positive Dil LDL/Lectin cells was calculated by counting cells in each sub-field. EPC number was expressed as percentage of cells positive for DilLDL/Lectin dual staining (*37*).

Flow Cytometry Analysis (FACS)—FACS was performed on human EPC after 3 days of culture, as described by Casamassimi *et al*. (*37*), by incubating samples with directly conjugated mouse monoclonal antibodies to CD34-phycoerythrin (PE), anti-VE-cadherin-PE (Santa Cruz), conjugated with the corresponding PE-labelled secondary antibody (Sigma) or for 10 min with directly conjugated mouse monoclonal antibodies to CD133-PE (Miltenyi Biotec). A PE isotype-matched antibody was used as negative control. Quantitative fluorescence analysis was performed with a FACS-CANTO instrument (BD Biosciences). Each analysis included 10,000 events.

Human EPC Treatment—EPCs were cultured for 3 days in the presence of different concentrations of RW (from 0.5 up to 120 μl/ml corresponding to 5.85 μg and 441 μg of total polyphenols, respectively). Cell viability was determined by colorimetric assay with XTT (XTT Cell Proliferation Kit II, Roche). To test the effect of RW and Resv (Sigma) on EPCs in the presence of TNF-α (*38*), cells were pre-incubated for 3 h with RW

(1 μl/ml), resveratrol (Resv) (29 nM) or RW + Resv, and then cultured for 3 days in the presence TNF-α. At the end of the treatment, cells were washed twice with PBS and used for cell number counting and western blot analysis. Control EPCs were treated with equal amount of ethanol contained in the wine added to the culture media, *i.e.* 0.012%.

Evaluation of Oxidative NOx Levels—NO was measured as nitrite and nitrate (NOx), which are stable metabolites of NO, by using the Griess reagent according to the manufacture's instructions (Calbiochem) (*39*). Concentration (μM) was calculated based on a standard curve calibration.

Western Blot Analysis—For western blot analysis, ~15 μg of protein extract were separated by 10% SDS-PAGE as described (*31*, *32*). The gel was transblotted onto a nitro-cellulose membrane, blocked with 10% milk powder in Tris-buffered saline (pH 7.4) with 0.1% Tween-20 (TBS-T) overnight and incubated with antibody against total p38 (c-20), (Santa Cruz Biotechnology) and antibody against the phosphorylated form of p38 (Thr180/Tyr182)(3D7) (Cell Signaling). Secondary antibodies were from Santa Cruz Biotechnology. After five washes with TBS-T, the signal was detected with the aid of a chemiluminescence kit (Amersham Pharmacia enhanced chemiluminescence kit) (*31*, *32*). Membranes were normalized with a polyclonal antibody against γ-tubulin protein (GTU-88) (Sigma). Semiquantitative densitometry of western blots was performed by using a Scan LKB (Amersham Pharmacia) (*31*, *32*).

Statistical Analysis—Results are expressed as mean ± SD or SE. The difference among groups was evaluated by a one- or two-factor ANOVA by two independent investigators in a blinded fashion. Statistical significance was accepted at $P < 0.05$.

RESULTS

Wine Analysis—RW showed a high content of Resv (6.69 mg/l) (Fig. 1, panel A) and a strong anti-oxidant capacity (46.1 ± 5.2 nmol/l) (Fig. 1, panel B). The total polyphenols content was 3.68 GAE g/l (Fig. 1, panel B). Neither ethanol (1–100%) nor glucose (5–50%) demonstrated any anti-oxidant capacity in the FRAP assay, indicating that neither of these compounds alone was responsible for wine's anti-oxidant power.

Effect of RW on Human EPC Number and Viability—Total human PBMC cultured for 3 days under standard conditions resulted in an adherent population consisting of 23% ± 4% double-positive cells for DiLDL (red) and lectin (green) (Fig. 2, panel A), as assessed by phase control fluorescent microscope, thus matching the previously described EPC phenotype (*37*). EPC phenotype was confirmed by demonstrating the expression of stem/progenitor cell surface markers CD34 and CD133 and endothelial markers, VE-cadherin (VE-cad) (Fig. 2, panel B). As indicated by the forward and sideward scatter analysis, the two main populations gated, corresponding to the lymphocyte gate (single arrow) and the monocyte gate (double arrow) (Fig. 2, panel B), were both positive for CD34, CD133, VE-cadherin expression. To determine the optimal concentration of

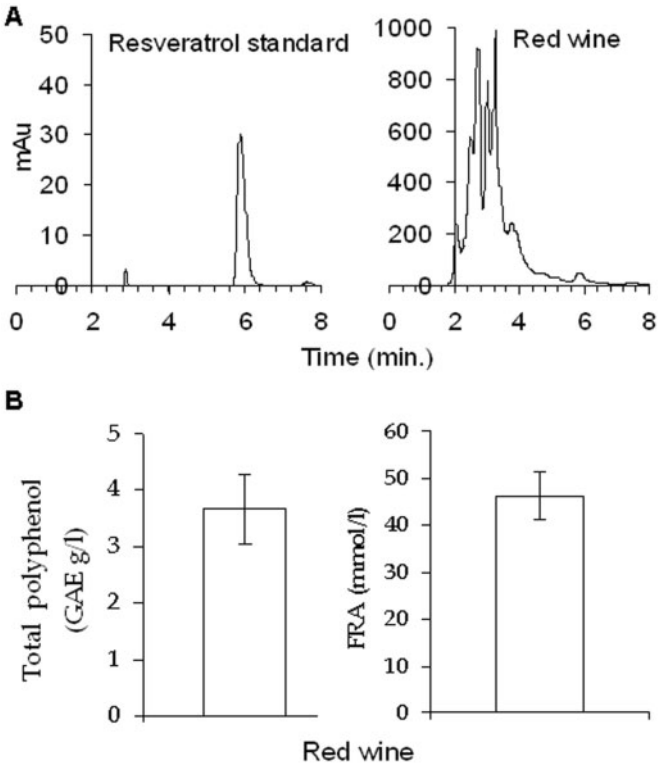

Fig. 1. **Wine analysis.** (A) Chromatogram for Aglianico wine and trans-resveratrol standard solution. (B) Anti-oxidant capacity and total polyphenols content of Aglianico were measured with FRAP assay and Folin–Ciocalteu's phenol reagent method, respectively. Data are the means ± SE and are representative of five determinations in duplicate.

RW (12.5% v/v ethanol), PBMCs were incubated for three days in the presence of different concentration of Aglianico wine (from 0.5 μl/ml up to 120 μl/ml). Dose-dependent EPC viability indicated that no significant effects were observed at a concentration between 1 (*i.e.* 0.012% v/v of ethanol) and 7.5 μl/ml (*i.e.* 0.09% v/v of ethanol) (Fig. 2, panel C). A significant decrease of cell viability was observed at concentrations of 15 μl/ml ($P < 0.05$ versus untreated control cells) up to 120 μl/ml ($P < 0.01$ versus untreated control cells) (*i.e.* from 0.18% to 1.45% v/v of ethanol). The images in the insert of Fig. 2, panel C is representative of cells cultured for 3 days in the presence of RW 1 μl/ml. Therefore, since the concentration of polyphenols in plasma reached *in vivo* after consumption of polyphenols rich foods are in the nM range (*40*), the concentration of 1 μl/ml, corresponding to 3.68 ng/ml of total polyphenols and to 29 nM Resv, was chosen to test the effect of RW on EPC number.

As first approach, a long-term culture of EPCs was performed in the presence of RW (1 μl/ml). During long-term culture, some of the attached cells appeared elongated and became spindle-shaped compared to round cells plated (Fig. 2, insert panel D). Interestingly, results indicated that EPC number was significantly ameliorated in a time-dependent manner in the presence of RW under basal conditions ($P < 0.05$ *vs.* untreated control cells) (Fig. 2, panel D).

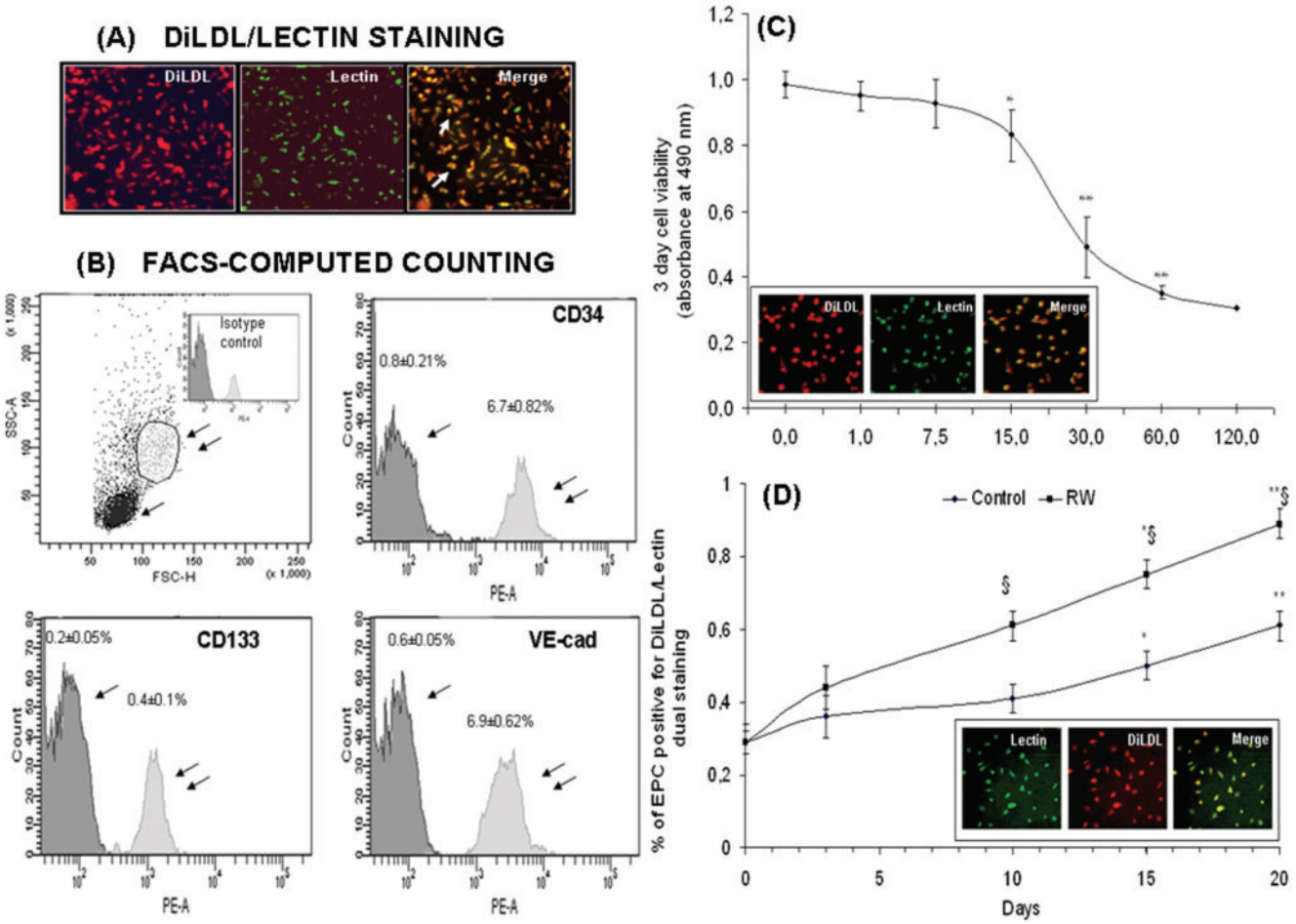

Fig. 2. **Characterization of human EPCs and effect of RW on EPC viability and number.** (A) DiLDL uptake and lectin binding of isolated EPCs were determined by fluorescence microscopy, as described under METHODS section. Overlay image is shown in right panel (arrows indicate double positive cells). (B) Expression of surface markers was performed on cultured PBMCs at 3 days of culture. Controls were corresponding to negative isotope controls. Data are expressed as mean ± SD. Images are representative of three different experiments in duplicate. (C) EPCs were cultured for 3 days in the presence of red wine (from 1 μl/ml up to 120 μl/ml). Cell viability was determined by XTT colorimetric assay. Data are the mean ± SE of three separate experiments in duplicate. (D) EPCs were cultured up to 20 days in the presence or absence of red wine (1 μl/ml). Cells positive for both DiLDL/Lectin dual staining were counted. Control EPCs were treated with equal amount of ethanol contained in the wine. The mean number of cells was expressed as mean ± SE. $^{*}P < 0.05$ versus respective control at day zero. $^{**}P < 0.01$ versus respective control at day zero. $^{\S}P < 0.05$ versus EPCs grown in absence of RW.

Effect of RW on TNF-α Altered Human EPC Number—Treatment of EPCs with TNF-α impairs EPC functional activity and reduces cell number (*38*) Accordingly, when EPCs were incubated with TNF-α (10 ng/ml) for 3 days (day 0–3) the cell number consistently decreased compared to control cells ($P < 0.01$ versus untreated control cells) (Fig. 3, panel A and B). The presence of RW (1 μl/ml) during treatment with TNF-α for 3 days prevented the TNF-α induced reduction of cell number ($P < 0.05$ versus TNF-α). The mechanism underlying the effect of TNF-α on EPC number is mediated by p38 MAP kinase activation (*38*). Accordingly, an increased p38-phosphorylation was observed in EPCs treated with TNF-α for 3 days ($P < 0.05$ versus untreated control cells) (Fig. 3, panel C) and this effect was significantly inhibited by co-incubation with TNF-α and RW ($P < 0.01$ versus TNF-α). Treatments did not influence significantly total p38 expression levels (Fig. 3 panel D). Under the present experimental conditions, NOx levels in the media did not change significantly at Day 1 of treatment (Table 1). NOx levels were consistently reduced in cells treated with TNF-α for 3 days ($P < 0.05$ versus untreated control cells). This effect was completely abolished when RW was added to the culture media for 3 days raising NOx levels near control levels ($P < 0.05$ versus TNF-α) (Table 1).

Effect of Resv on TNF-α Altered Human EPC Number—Incubation of EPCs with pure Resv alone at the same concentration of the Resv contained in the in Aglianico RW (29 nM) significantly prevented TNF-α reduced cell number ($P < 0.05$ versus TNF-α) (Fig. 4). This effect was more significant when EPCs were

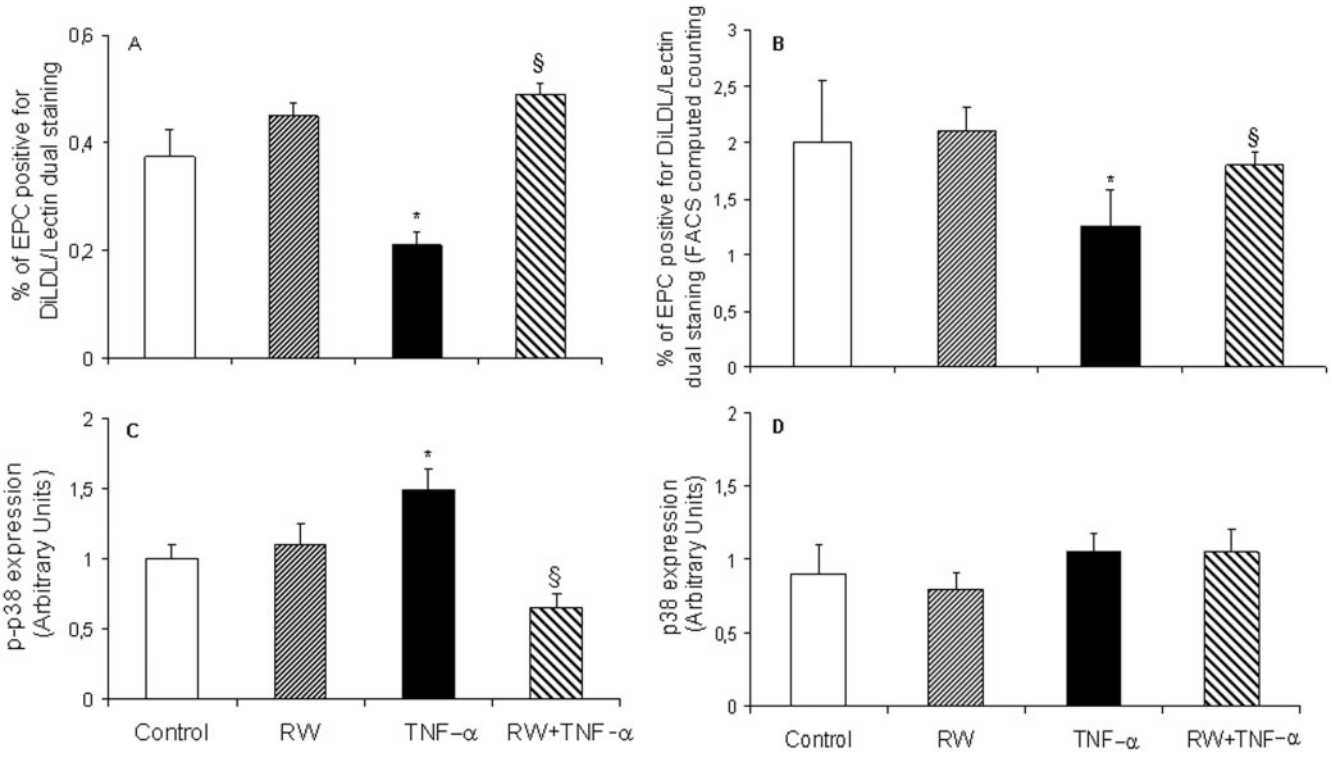

Fig. 3. **Effect of red wine on TNF-α reduced EPC number and p38 phosphorylation.** (A) PBMCs were cultured for 3 days with media alone (control), with red wine (RW, 1 μl/ml), TNF-α (10 ng/ml), or with TNF-α in the presence of red wine (RW + TNF-α). The number of EPCs positive for DiLDL/Lectin dual staining was determined by microscope counting of four randomly selected high-power fields. $^{*}P < 0.01$ versus control; $^{\S}P < 0.01$ versus TNF-α. (B) FACS-computed counting of EPCs positive for DiLDL/Lectin dual staining. $^{*}P < 0.01$ versus control, $^{\S}P < 0.05$ versus TNF-α. (C) Detection of p38 phosphorylation and (D) total p38 in control (lane 1), RW (lane 2), TNF-α (lane 3) or RW+TNF-α (lane 4) samples, $^{*}P < 0.05$ versus control; $^{\S}P < 0.01$ versus TNF-α. The γ-tubulin and total p38 served as loading controls. Control EPCs were treated with equal amount of ethanol contained in the wine (0.012%). Data are mean ± SE ($n = 4$).

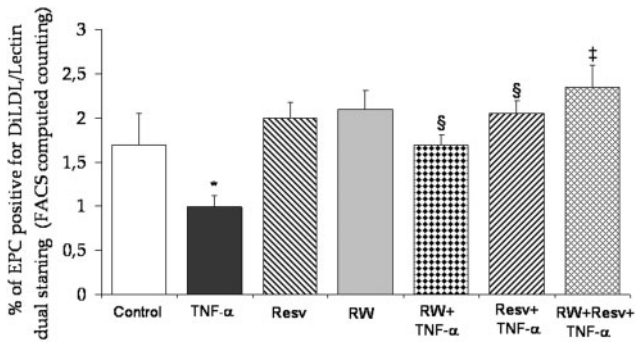

Fig. 4. **Effect of resveratrol on TNF-α reduced EPC number.** PBMCs cultured for 3 days with media alone (control), with resveratrol (Resv, 29 nM), RW (1 μl/ml), TNF-α (10 ng/ml), RW + TNF-α, Resv+TNF-α or RW+Resv+TNF-α. The number of EPCs positive for DiLDL/Lectin dual staining was determined FACS-computed counting. $^{*}P < 0.01$ versus control, $^{\S}P < 0.05$ versus TNF-α, $^{\ddagger}P < 0.01$ versus TNF-α. Control EPCs were treated with equal amount of ethanol contained in the wine (0.012%). Data are mean ± SE ($n = 4$).

incubated with both pure Resv and RW ($P < 0.01$ versus TNF-α). Moreover, the beneficial effect of RW was only slightly potentiated by co-incubation with Resv [Resv + RW + TNF-α (about 1.4-fold versus cells treated with RW and TNF-α)].

Table 1. **NOx levels in the media of in vitro cultured human EPCs.**

	NOx [μM]	
	Day 1	Day 3
Control	18.0 ± 2.71	22.0 ± 1.95
RW	18.0 ± 2.42	19.0 ± 1.72
TNF-α	12.0 ± 1.92	10.5 ± 2.32*
TNF-α + RW	17.0 ± 1.75	24.0 ± 2.68§

PBMCs were cultured for 3 days with media alone (control), with red wine (RW, 1 μl/ml), TNF-α (10 ng/ml) or with TNF-α in the presence of red wine (RW + TNF-α). NOx levels were determined in the culture media by using the Griess reagent according to the manufacture's instructions (Calbiochem). Data are representative of three experiments in duplicate and are expressed as mean ± SD. $^{*}P < 0.01$ versus control; $^{\S}P < 0.01$ versus TNF-α.

DISCUSSION

We report that low doses of RW and Resv, one of the most abundant polyphenols in RW, improve the number of circulating EPCs. We demonstrated that *in vitro* supplementation with RW or Resv at physiological concentration on cultured human EPCs significantly ameliorated cell number in the presence of TNF-α under basal condition during long-term culture. Beneficial effect of RW in cultured human circulating

EPCs was, at least in part mediated by inhibited p38-phosphorylation and enhanced NOx levels. Accumulating data document protective effects of RW in cardiovascular disease by improved NO bioavailability (*19*, *29*). 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 (*41*, *42*). However, recently, Rathel *et al*. (*43*) demonstrated that the biological activity of RWs on eNOS transcription depends neither on grape cultivars nor on growing area in general and that Resv, although it does not account for the complete RW effect, is the most promising polyphenol in RW. Indeed, Resv markedly raised the proliferative, migrative, and adhesive activities of EPCs *in vitro* (*44*) and prevented restenosis by inhibiting intimal hyperplasia of injured artery in experimental rabbit model (*45*).

In our study, pure Resv alone significantly ameliorated the TNF-α reduced EPC number ($P < 0.05$ versus TNF-α alone) but only slightly potentiated the beneficial effect of RW. Multiple components of RW, *i.e*. procyanidins, gallic acid (*20*, *46*), along with Resv are responsible for its beneficial effect and this might conceivably explain the lack of additive beneficial effect when RW was tested in the presence of pure Resv alone. Further studies would be important, in order to determine the possible beneficial effect of Resv as medication.

Moreover, we did not perform a comparative study using white wine in parallel with RW. RW is more antioxidant that white wine in relation to its higher content of polyphenols (*47*, *48*).

Resv at μM concentration (1 μM) has been shown to enhance activities of proliferation, migration and adhesion, as well as to promote endothelial eNOS expression in EPCs, whereas a high concentration (60 μM) inhibited the aforementioned functions and eNOS expression (*44*). However, the physiological concentration of unconjugated Resv reached in plasma after *in vivo* consumption is reported to be about 35 nM and about 2 μM for the biologically active glucuronate and sulphate Resv conjugates (*40*). Indeed, in our study, concentration of pure unconjugated Resv or Resv contained in Aglianico wine was at nM range to mimic *in vitro* appropriate physiological exposure to dietary polyphenols (*40*).

Studies in mice and in patients with coronary artery disease, heart failure and cardiovascular risk factors have demonstrated an improvement of endothelial function during exercise training (*49–52*). The underlying mechanisms may relate to increased circulating number of EPCs *via* a partially NO-dependent, anti-apoptotic effect (*41*, *50*, *51*). Moreover, graduated and moderate exercise training together with metabolic intervention with L-arginine and anti-oxidants have been shown to reduce atherosclerotic lesion formation and to reduce the plaque instability in LDLR$^{-/-}$ mice (*39*, *52*). According to previous findings (*49*), we recently showed that low doses of with RW supplementation, determined a consistent improvement of physical training-induced upregulation of aortic eNOS in C57BL/6J mice (*33*) along with an increased circulating EPC number and VEGF serum levels (data not shown). Low doses of RW supplementation decreased ELK-1 and *p*-JUN activation and increased eNOS expression in atherosclerosis-prone areas of hypercolesterolaemic LDLR$^{-/-}$ mice. In a very recent study, Lefevre *et al*. (*27*) reported that beneficial effect of RW consumption on ischaemia-induced neovascularization in hypercolesterolaemic ApoE-deficient mice were related to an improvement in HPC. However, these findings are limited to the bone marrow HPCs and not to EPC circulating in the peripheral blood (*27*).

Despite polyphenol bioavailability and metabolism should be taken into account when *in vivo* and *in vitro* studies with dietary polyphenols are compared (*40*), in this study we demonstrate that moderate amounts of RW and low concentrations of pure Resv upregulate the number of circulating EPCs during treatment with TNF-α. These data are in line with other studies demonstrating the beneficial effect of natural compounds on EPC number (*44*, *53*). However, definitive large-scale, randomized controlled trials are still necessary to demonstrate efficacy and safety of moderate RW consumption in the prevention of altered EPC number or during therapeutic approaches aimed to improve revascularization of ischaemic tissues via amelioration of EPC levels (*16–19*).

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