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Moderate intake of red wine improves ischemia-induced neovascularization in diabetic mice—Roles of endothelial progenitor cells and nitric oxide

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ABSTRACT

Objective: Circulating endothelial progenitor cells (EPCs) play a significant role in postnatal neovascularization. Patients with diabetes have attenuated EPC functions and impaired angiogenic response after tissue ischemia. We investigated whether moderate red wine consumption can enhance blood flow recovery in response to tissue ischemia by enhancement of EPC functions in diabetic mice.
Methods and results: Starting at 4 weeks after diabetes onset, red wine (4 ml/kg/day) or ethanol were administered to streptozotocin (STZ)-induced (type 1) diabetic mice and KKAy-Ta (type 2) mice. Unilateral hind limb ischemia surgery was conducted after 2 weeks of red wine or ethanol ingestion. Type 1 and type 2 diabetic mice given red wine, but not ethanol, had significantly increased collateral flow about 30% and augmented capillary density in ischemic tissues. These beneficial effects were markedly abolished by an eNOS inhibitor (L-NAME). Flow cytometry analysis showed impaired EPC-like cells (Sca-1^+/Flk-1^+) mobilization after ischemia surgery in diabetic mice, but augmented mobilization in red wine group (baseline vs. 2 days after operation: $0.88 \pm 0.06\%$ vs. $1.73 \pm 0.29\%$, $p = 0.010$). C-kit positive bone marrow cells isolated from diabetic mice given red wine had enhanced adhesion and migration compared to mice given vehicle. By in-vitro studies, incubation with red wine in high-glucose medium significantly reduced H_2O_2 production, and improved high glucose-suppressed EPC functions by nitric oxide-related mechanisms.
Conclusions: Our findings demonstrate that red wine consumption enhances blood flow recovery after tissue ischemia in diabetic mice. These effects may partly derive from enhanced EPC functions by upregulation of eNOS activity.

1. Introduction

Hyperglycemia is a key factor for the development of vascular complications in patients with diabetes [1]. Patients with diabetes or metabolic syndrome are related to endothelial dysfunction and have diminished ability of collateral vessel formation in response to ischemia in the heart and peripheral tissues [2,3]. There is increasing evidence that neovascularization in adults is not solely the result of proliferation of local endothelial cells (angiogenesis), but also involves bone marrow-derived circulating endothelial

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progenitor cells (EPCs) for the processes of vasculogenesis [4]. However, patients with cardiovascular risk factors were shown to have decreased numbers and function of circulating EPCs [5,6]. Our recent study also demonstrated that long-term exposure to high glucose may enhance cellular senescence and decrease cell numbers and functional competencies of EPCs via nitric oxide (NO)-related mechanisms [7]. These findings provide a rationale for potential therapeutic targets for hyperglycemia-related vascular complications in diabetic patients.

Light-to-moderate ethanol intake from any type of beverage has been shown to improve lipoprotein metabolism and lower cardiovascular mortality [8,9]. However, red wine, with its abundant contents of phenolic acids, polyphenols and flavonoids, appears to confer additional healthful benefits [10]. The protective effects of red wine are supported by epidemiological data that show a significantly reduced incidence of coronary artery disease in certain areas of France, in spite of high-fat diets, little exercise and wide-spread cigarette smoking [11]. Previous studies showed that the beneficial effects of red wine are derived from increased endothelium-derived NO, implying that enhanced NO bioavailability may mediate the cardiovascular protection provided by red wine [12]. Although clinical studies have indicated that light-to-moderate consumption of red wine can reduce the incidence of coronary artery disease and decrease cardiovascular events [13], the multi-faceted effects of red wine ingestion on circulating EPCs and diabetes remain unclear. Therefore, we designed this study to test the hypothesis that moderate intake of red wine can improve collateral flow recovery after tissue ischemia in diabetic mice and improve the numbers and functional capacities of EPCs by increasing NO bioavailability.

2. Materials and methods

2.1. Animals

Mice at 6–8 weeks old were purchased from the National Laboratory Animal Center, Taiwan (FVB mice) and the Jackson Laboratory (KKAy-Ta mice, type 2 diabetes model; Bar Harbor, ME, USA). Experimental diabetes was induced in FVB mice by daily intraperitoneal injections of streptozotocin (STZ) in citrate buffer (40 mg/kg) for 5 days for the type 1 diabetic model [14]. Mice were considered diabetic only if they developed glycemia >250 mg/dl and overt glycosuria at 14 days after the first STZ injection. Persistence of diabetes was determined at the end of the study. The KKA^{y}/Ta mice (type 2 diabetes model) produced by transfection of the yellow obese gene (Ay) into KK/Ta mice are obese-diabetic mice showing hyperglycemia, hypertriglyceridemia, hyperinsulinemia and microalbuminuria [15,16]. All mice were kept in microisolator cages on a 12-h day/night cycle. All experimental procedures and protocols involving animals were approved by the institutional animal care committee of National Yang-Ming University (Taipei, Taiwan).

2.2. Mouse ischemic hind limb model

Eight-week-old male STZ-induced diabetic mice and KKAy-Ta mice received vehicle (0.5% carboxymethyl cellulose), ethanol (0.6 ml/kg/day) or red wine (4 ml/kg/day) administered by gavage every day. The red wine used was "Vin De Pays D'OC" (12.5% ethanol), a cabernet sauvignon from France. After 2 weeks of red wine and ethanol ingestion, unilateral hind limb ischemia was induced by excising the right femoral artery as previously described [16]. Briefly, the animals were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). The proximal and distal portions of the femoral artery were ligated. Hind limb blood perfusion was measured with a Laser Doppler perfusion imager system (Moor Instruments Limited, Devon, UK) before and after the surgery and then followed weekly. To avoid the influence of ambient light and temperature, the results were expressed as the ratio of perfusion in the right (ischemic) versus left (non-ischemic) limb.

2.3. Measurement of capillary density in the ischemic limb

At 5 weeks after surgery, mice were sacrificed by intraperitoneal injection of an overdose of pentobarbital. The whole limbs were fixed in methanol overnight. The femora were carefully removed, and the ischemic thigh muscles were embedded in paraffin. Sections (5 μm) were de-paraffinized and incubated with a rat monoclonal antibody against murine CD31 (clone MEC13.1, BD PharMingen, San Diego, CA). Antibody distribution was visualized using the avidin-biotin-complex technique and Vector Red Chromogenic substrate (Vector Laboratories, Burlingame, CA), followed by counterstaining with hematoxylin. Capillaries were identified by positive staining for CD31 and morphology. Ten different fields from each tissue preparation were randomly selected, and visible capillaries were counted. Capillary density was expressed as the number of capillaries per square millimeter.

2.4. Flow cytometry

To investigate the effects of red wine and ethanol ingestion on EPC mobilization in response to tissue ischemia, the fluorescence-activated cell sorting (FACS) Caliber flow cytometer (Becton Dickinson, San Jose, CA, USA) was used to assess EPC mobilization. A volume of 100 μL peripheral blood was incubated with Fluorescein isothiocyanate (FITC) anti-mouse Sca-1 (eBioscience, San Diego, CA, USA), and phycoerythrin (PE) anti-mouse Flk-1 (VEGFR-2, eBioscience) antibodies [17]. Isotype-identical antibodies served as controls (Becton Dickinson, Franklin Lakes, NJ, USA). After incubation for 30 min, cells were lysed (PharmLyse; BD Pharmingen), washed with phosphate-buffered saline (PBS), and fixed in 2% paraformaldehyde before analysis. Each analysis included 100,000 events. Circulating EPCs were considered to be from the mononuclear cell population and were gated with double positive for Sca-1 and Flk-1.

2.5. Bone marrow-derived c-kit^{+} cell isolation

Bone marrow-derived MNCs were obtained from STZ-induced diabetic mice given red wine or vehicle for 2 weeks. Briefly, bone marrow-derived c-kit^{+} cells were isolated using CD117 MicroBeads and MACS (Miltenyi Biotec GmbH) according to the manufacturer's instructions [17]. C-kit^{+} bone marrow cells were >90% positive for CD31^{+}, and these bone marrow-derived progenitor cells were used for cell functional assays.

2.6. Bone marrow transplantation model

Bone marrow transplantation was performed as previously described [17,18]. Recipient wild-type mice at 8 weeks of age were lethally irradiated with a total dose of 9.0 Gy. eGFP transgenic mice (FVB background) that ubiquitously expressed enhanced GFP (Level Biotechnology Inc., Taipei, Taiwan) were used as the donors. After being irradiated, a recipient mouse received unfractionated bone marrow cells (5×10^{6}) from an eGFP mouse by a tail vein injection. Two months after the bone marrow transplantation, the chimeric mice were induced diabetes by daily intravenous injections of STZ as previously described [14]. Then, all mice received an unilateral hind limb ischemic surgery ($n = 6$ in each group). Repopulation by eGFP-positive bone marrow cells was 95%, as measured by flow cytometry. Two weeks after the induction of hind limb ischemic

surgery in the bone marrow-reconstituted and STZ-induced diabetic mice, tissues were harvested for confocal immunofluorescent and histological analysis. Bone marrow-derived EPCs were stained with antibodies directed against eGFP (Chemicon) and CD31 (BD PharMingen). EPC density was estimated by counting $eGFP^+CD31^+$ double-positive cells (yellow color) under high power filed (HPF, ×100) in at least 6 different cross-sections from different animals.

2.7. Human EPC isolation and cultivation

Peripheral blood samples (20 ml) were obtained from healthy young volunteers, and total MNCs were isolated by density gradient centrifugation with Histopaque-1077 (1.077 g/ml, Sigma, St. Louis, MO, USA) [17]. Briefly, MNCs (5×10^6) were plated in 2 ml endothelial growth medium (EGM-2 MV Cambrex, East Rutherford, NJ, USA), with supplements (hydrocortisone, R^3-insulin-like growth factor 1, human endothelial growth factor, vascular endothelial growth factor (VEGF), human fibroblast growth factor, gentamicin, amphotericin B, vitamin C, and 20% fetal bovine serum) on fibronectin-coated 6-well plates. After 4 days of culture, the medium was changed and non-adherent cells were removed; attached early EPCs appeared elongated with spindle shapes. A certain number of early EPCs were allowed to grow into colonies of late EPCs, which emerged 2–4 weeks after the start of the MNC culture. The late EPCs exhibited a cobblestone morphology and monolayer growth pattern typical of mature endothelial cells at confluence [16]. Late EPCs were collected and used for all the assays in this study.

2.8. EPC characterization

The late EPCs were characterized as adherent cells double positive for acetylated low-density lipoprotein uptake and lectin binding by direct fluorescent staining as previously described [7,16]. Briefly, the adherent cells were first incubated with 2.4 μg/ml 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-acetylated low-density lipoprotein (DiI-acLDL; Molecular Probe) for 1 h and then fixed in 2% paraformaldehyde and counterstained with 10 μg/ml FITC-labeled lectin from Ulex europaeus (UEA-1) (Sigma). The late EPC was also characterized by immunofluorescence staining for the expression of CD34, CD133, Von Willebrand factor (vWF), VE-cadherin, platelet/endothelial cell adhesion molecule-1 (PECAM-1) (CD-31), and eNOS (Santa Cruz). The fluorescent images were recorded under a laser scanning confocal microscope. CD45 expression was accessed by fluorescence-activated cell sorting Caliber flow cytometer in late EPCs.

2.9. EPC senescence assay

By in vitro studies, we tested the direct effects of red wine on EPC number, proliferation, senescence and functions in high glucose conditions (25 mM) for 4 days. Red wine or ethanol were administered for 24 h. Cellular aging was determined with a Senescence Cell Staining kit (Sigma). Briefly, after washing with phosphate-buffered saline, late EPCs were fixed for 6 min in 2% formaldehyde and 0.2% glutaraldehyde in PBS, and then incubated for 12 h at 37 °C without CO_2 with fresh X-gal staining solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$; pH 6). After staining, green-stained cells and total cells were counted and the percentage of β-galactosidase-positive cells was calculated [7].

2.10. EPC fibronectin adhesion assay

Late EPCs were washed with PBS and gently detached with 0.5 mM EDTA in PBS [19]. After centrifugation and resuspension in basal medium with 5% fetal bovine serum, identical cells (1×10^4 cell per well) were placed on fibronectin-coated 6-well plates and incubated for 30 min at 37 °C. Gentle washing with PBS for 3 times was performed after 30 min' adhesion, and adherent cells were counted by independent blinded investigators. Phenotyping of endothelial characteristics of adherent cells was done by indirect immunostaining using Dil-acLDL and BS-1 lectin.

2.11. EPC migration assay

The migratory function of EPCs was evaluated by a modified Boyden chamber assay (Transwell, Costar) [7]. Briefly, isolated late EPCs were detached as described above with trypsin/EDTA, and then 4×10^4 late EPCs were placed in the upper chambers of 24-well transwell plates with polycarbonate membranes (8-μm pores) that contained serum-free endothelial growth medium. VEGF (50 ng/ml) was added to medium placed in the lower chambers. After incubation for 24 h, the membrane was washed briefly with PBS and fixed with 4% paraformaldehyde. The upper membrane side was wiped gently with a cotton ball. The membrane was then stained using hematoxylin solution and carefully removed. The extent of migration of late EPCs was evaluated by counting the migrated cells in 6 random high-power (×100) microscopic fields.

2.12. Western blotting analysis

Briefly, EPCs were lysed in buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.5 mM PMSF, 2 μg/ml aprotinin, pepstatin and leupeptin), and the protein lysates were subjected to SDS-PAGE, followed by electroblotting onto a PVDF membrane [20]. Membranes were probed with monoclonal antibodies against phosphorylated endothelial NO synthase (p-eNOS), eNOS (Cell Signaling) and α-tubulin (Sigma). Bands were visualized by chemiluminescence detection reagents. Densitometric analysis used ImageQuant (Promega) software.

2.13. Measurement of reactive oxygen species (ROS) production

The effect of red wine on ROS production in EPCs was determined by a fluorometric assay using 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes) as a probe for the presence of H_2O_2 [20]. Confluent EPCs (10^4 cells/well) in 96-well plates was pretreated with red wine in high glucose medium for 4 days. After the removal of red wine from wells, cells were incubated with 20 μmol/L DCFH-DA for 45 min. The fluorescence intensity (relative fluorescence units) was measured at 485-nm excitation and 530-nm emission using a fluorescence microplate reader.

2.14. Statistical analysis

Results are given as means ± standard errors of the mean (SEM). Statistical analysis was done by unpaired Student's *t* test or analysis of variance, followed by Scheffe's multiple-comparison post hoc test. Data were analyzed using SPSS software (version 14; SPSS, Chicago, IL, USA). A p value of <0.05 was considered statistically significant.

3. Results

3.1. Enhanced collateral blood flow recovery after red wine consumption in diabetic mice

To evaluate any angiogenic effect of red wine and ethanol, we induced tissue ischemia by unilateral hind limb ischemia surgery in wild-type mice and in STZ-induced diabetic mice ($n = 6$ for each

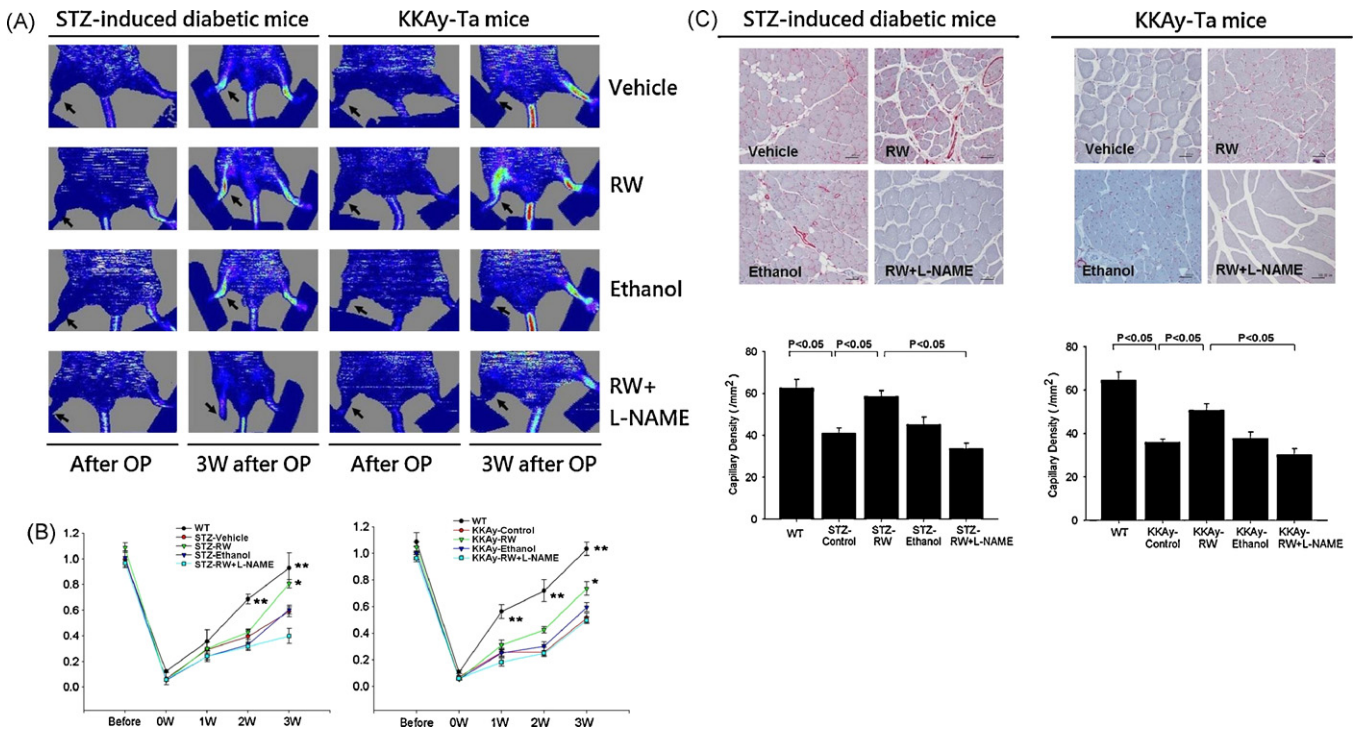

Fig. 1. Effect of red wine on blood flow recovery after hind limb ischemia in wild-type mice, and STZ-induced diabetic and KKAy-Ta mice. (A) Representative results of laser Doppler measurements before operation and 3 weeks after hind limb ischemia surgery in control (vehicle), red wine (RW) and ethanol-drinking mice. Color scale illustrates blood flow variations from minimal (dark blue) to maximal (red) values. Arrows indicate ischemic (right) limb after hind limb ischemia surgery. (B) Doppler perfusion ratios (ischemic/non-ischemic hind limb) over time in the different groups. Administration of L-NAME in drinking water abolished the beneficial effect of RW in diabetic mice. (C) Mice were sacrificed 3 weeks after surgery and capillaries in the ischemic muscles were visualized by anti-CD31 immunostaining. Results are means ± SEM. ($^{*}p < 0.05$ compared with vehicle and ethanol groups; $^{**}p < 0.01$ compared with vehicle and ethanol groups; $n = 6$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

group). As shown in Fig. 1A, the STZ-induced diabetic mice given the vehicle showed delayed blood flow recovery after ischemia surgery compared with that in wild-type mice as determined by laser Doppler imaging, while ingestion of red wine significantly improved blood flow recovery by 30% in STZ-induced diabetic mice. The blood flow of the ischemic limb did not significantly increase in STZ-induced diabetic mice given ethanol compared to the vehicle group (Fig. 1B). However, the benefit of red wine ingestion was significantly abolished after treatment with the endothelial NO synthase (eNOS) inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME, 1 mg/ml in drinking water).

The KKAy-Ta mice given the vehicle showed delayed blood flow recovery after hind limb ischemia surgery compared with that in wild-type mice as determined by laser Doppler imaging, and ingestion of red wine significantly enhanced blood flow recovery by 32%. However, ethanol ingestion had no significant effect, and administration of L-NAME attenuated the benefit of red wine ingestion (Fig. 1B).

Consistent with the measurements by Laser Doppler imaging, anti-CD31 immunostaining revealed that ingestion of red wine significantly increased the number of detectable capillaries in the ischemic muscle, both in STZ-induced diabetes and KKAy-Ta mice (Fig. 1C). However, ethanol ingestion had no significant effect on capillary density after tissue ischemia, and administration of L-NAME abolished the benefit of red wine consumption (Fig. 1C).

3.2. Effects of red wine on EPC mobilization and homing process

To investigate effects of red wine ingestion on EPC mobilization in response to tissue ischemia, levels of Sca-1^{+}/Flk-1^{+} cells in peripheral blood were determined by flow cytometry in STZ-induced diabetic mice ($n = 6$ for each group). The basal number of EPCs did not differ significantly between the groups of wild-type and diabetic mice (Fig. 2A). EPCs Mobilization was enhanced by tissue ischemia in wild-type mice (baseline vs. 2 days after operation: 0.68 ± 0.24% vs. 1.27 ± 0.15%, $p = 0.033$). However, impaired mobilization of EPCs in peripheral blood after hind limb ischemia was noted in STZ-induced diabetic mice (baseline vs. 2 days after operation: 0.67 ± 0.21% vs. 0.92 ± 0.20%, $p = 0.342$). As shown in Fig. 2A, administration of red wine augmented EPCs mobilization after tissue ischemia in STZ-induced diabetic mice (baseline vs. 2 days after operation: 0.88 ± 0.06% vs. 1.73 ± 0.29%, $p = 0.010$), but not in those given ethanol (0.94 ± 0.25 vs. 1.2 ± 0.18, $p = 0.348$). In addition, administration of the eNOS inhibitor significantly diminished the effect of red wine on EPC mobilization.

To evaluate the effect of red wine consumption on bone marrow-derived EPC homing and differentiation to endothelial cells, STZ-induced diabetes was created in FVB mice that received eGFP mouse bone marrow cells ($n = 6$–7 in each group). By immunofluorescence staining, STZ-induced diabetic mice in the red wine group had more $GFP^{+}/CD31^{+}$ double-positive cells in ischemic muscle than those in control group (control vs. red wine: 14 ± 1 vs. 20 ± 2/HPF, $p = 0.019$; Fig. 2B). This suggested that ingestion of red wine promoted EPC mobilization and homing to ischemic tissue.

3.3. Ingestion of red wine improves c-kit positive bone marrow cell adhesion and migration

C-kit positive bone marrow cells were purified from STZ-induced diabetic mice to investigate the effect of red wine ingestion on cell functions. As shown in Fig. 2C, c-kit positive bone marrow cells from STZ-induced diabetic mice given red wine had augmented cellular adhesion (control vs. red wine: 100 ± 11 vs.

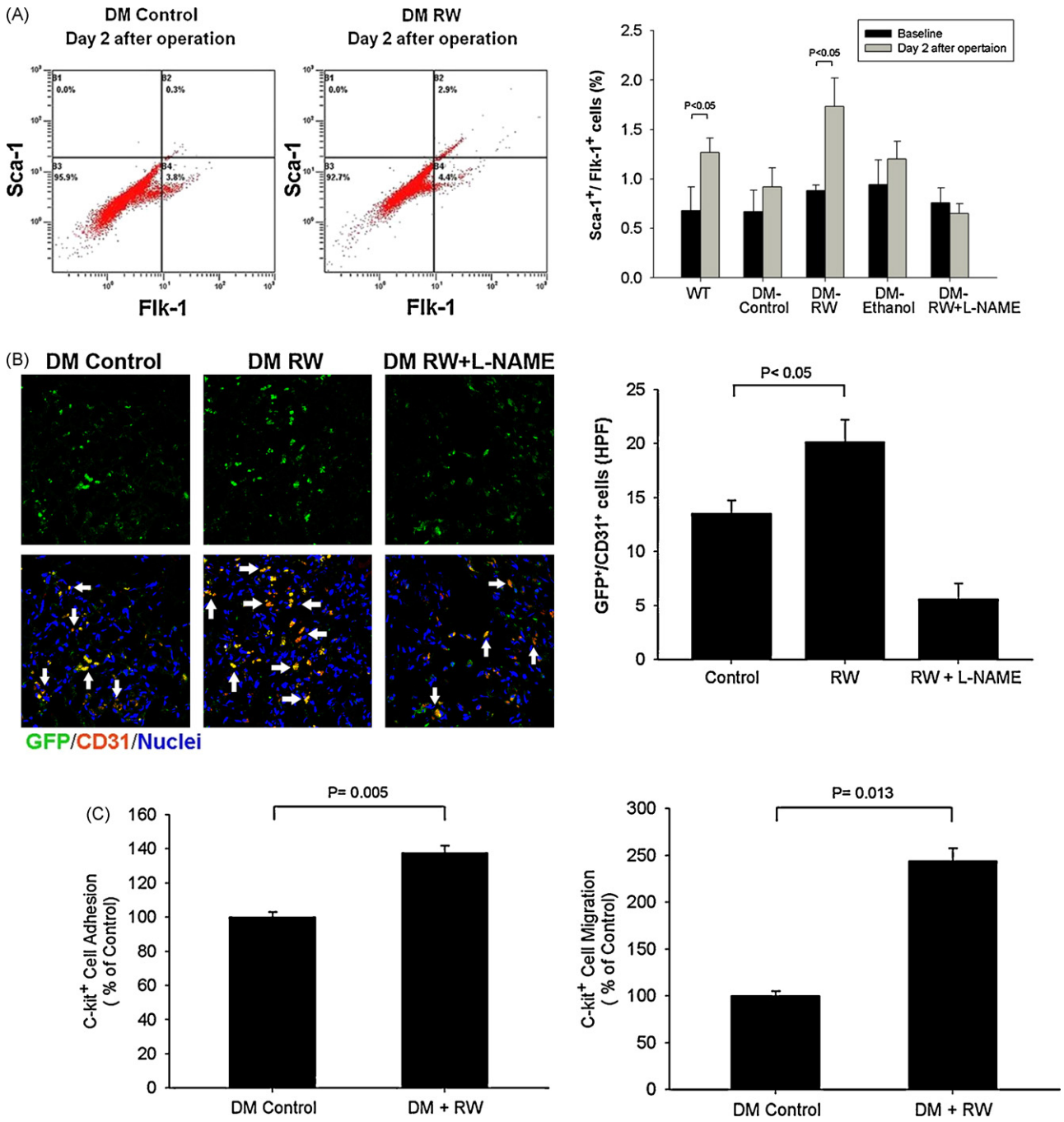

Fig. 2. Effects of red wine on EPC mobilization, tissue homing and c-kit positive bone marrow cell functions in STZ-induced diabetic mice. (A) EPCs (defined as Sca-1$^+$/Flk-1$^+$ cells) mobilization after tissue ischemia was determined by flow cytometry in STZ-induced diabetic mice given the vehicle, red wine (RW) or ethanol. (B) STZ-induced diabetes was created in FVB mice that received eGFP mouse bone marrow cells. By immunofluorescence staining, STZ-induced diabetic mice in RW group had more GFP$^+$/CD31$^+$ double-positive cells in ischemic muscle than those in vehicle group. (C) C-kit positive bone marrow cells from STZ-diabetic mice given the vehicle and RW were tested for cell functions (adhesion and migration) (n = 6–7 for each group).

138 ± 3, $p = 0.012$) and migration capacities (control vs. red wine: 5.6 ± 1.8 vs. 13.8 ± 1.8, $p = 0.007$) compared to cells isolated from STZ-induced diabetic mice given the vehicle.

3.4. Red wine ingestion results in upregulated phosphorylation of eNOS and Akt in ischemic muscle

We further evaluated the effect of red wine ingestion on eNOS activity in ischemic muscle of STZ-induced diabetic mice by Western blotting. Administration of red wine upregulated eNOS phosphorylation at Ser-1177 and Akt phosphorylation at Ser-473 in ischemic muscle, but upregulation was not found in STZ-induced diabetic mice given vehicle or ethanol (Fig. 3). Of note, administration of red wine did not enhance VEGF expression in ischemic muscle, which suggests that red wine-enhanced EPC mobilization was mediated by NO-related mechanisms, but not by VEGF upregulation.

3.5. Characterization of human late EPCs

Late EPCs were isolated from peripheral blood MNCs of healthy young volunteers as previously described [16]. The peripheral

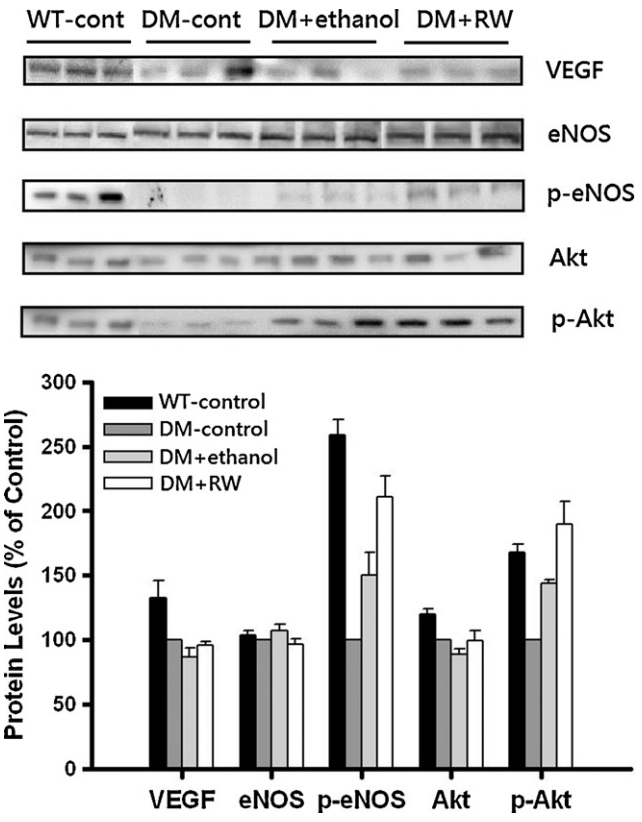

Fig. 3. Red wine enhanced expressions of p-eNOS Ser^{1177} and p-Akt Ser^{473} in ischemic muscle. After red wine (RW) ingestion, STZ-induced diabetic mice had significantly increased expressions of p-eNOS Ser^{1177} and p-Akt Ser^{473} in ischemic tissues compared to those given the vehicle. Administration of red wine did not enhance VEGF expression in ischemic muscle ($n = 3$).

blood MNCs that initially seeded on fibronectin-coated wells were round in shape. After the medium was changed on day 4, attached early EPCs appeared to be elongated with a spindle shape. Late EPCs with a cobblestone-like morphology similar to mature endothelial cells were grown to confluence (Fig. 4A). The late EPCs were characterized as adherent cells positive for acetylated low-density lipoprotein uptake by direct fluorescent staining (Fig. 4B). Low CD45 expression was noted in late EPCs (Fig. 4C). Late EPC characterization was also performed by immunohistochemical staining, and most of the cells expressed hematopoietic stem cell markers and mature endothelial markers, CD34, CD133, vWF, VE-cadherin, PECAM-1 (CD31), and eNOS (Fig. 4D–I), which are considered critical markers of late EPCs.

3.6. Red wine upregulates eNOS phosphorylation and decreases reactive oxidative stress in high glucose conditions

High glucose impairs eNOS activation and reduces NO bioavailability in cultured late EPCs [7]. We therefore investigated the effects of red wine on high glucose-treated EPCs to determine whether red wine could recover impaired eNOS activation in EPCs. After 4 days of incubation in 25 mM high glucose medium, the eNOS phosphorylation at Ser^{1177} was significantly downregulated (Fig. 5A). However, administration of red wine (0.5%) for 24 h following by treatment of EPCs in high-glucose conditions markedly upregulated high glucose-impaired eNOS phosphorylation at Ser^{1177}. In addition, high glucose markedly increased H_2O_2 production determined by the relative DCFH-DA fluorescent intensity, and administration of red wine (0.1–0.75%) significantly suppressed the high glucose-induced ROS index in cultures of late EPCs (Fig. 5B).

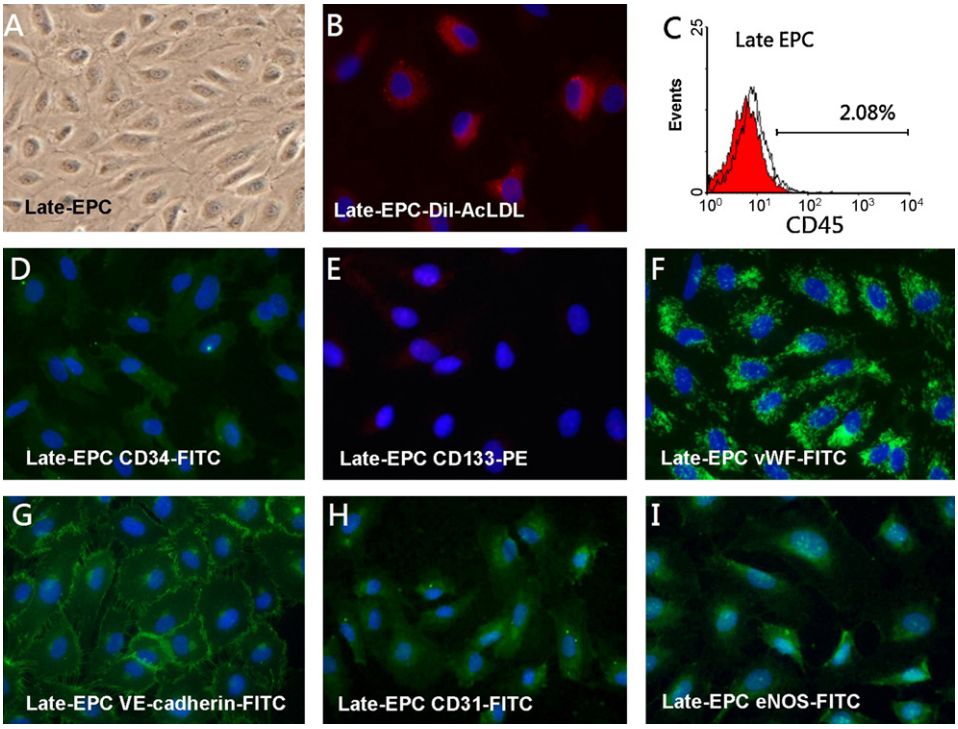

Fig. 4. The late EPCs (A) were characterized as adherent cells double positive for acetylated low-density lipoprotein uptake (B) by direct fluorescent staining. CD45 expression was accessed by fluorescence-activated cell sorting Caliber flow cytometer in late EPCs (C). The late EPC was also characterized by immunofluorescence staining for the expression of CD34 (D), CD133 (E), Von Willebrand factor (vWF) (F), VE-cadherin (G), platelet/endothelial cell adhesion molecule-1 (PECAM-1) (CD-31) (H), and eNOS (I). Cells were counterstained with propidium iodide for nucleus (red).

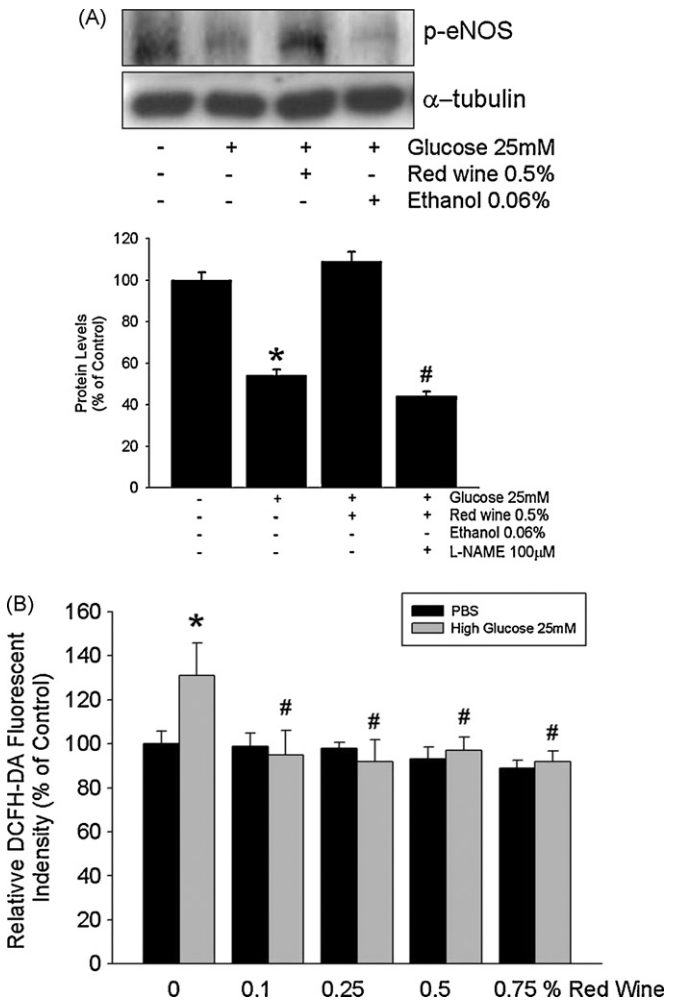

Fig. 5. Red wine upregulated eNOS phosphorylation and decreased reactive oxidative stress in high-glucose conditions. (A) High glucose-suppressed eNOS phosphorylation by Western blotting in cultured late EPCs. After 4 days of incubation, the eNOS phosphorylation at Ser^{1177} was significantly upregulated in high-glucose conditions by treatment of late EPC with red wine (RW). (B) High glucose markedly increased H_2O_2 production determined by the relative DCFH-DA fluorescent intensity, and administration of RW suppressed high glucose-induced reactive oxidative stress (ROS) index in late EPC culture ($n = 4$ for each experiment).

3.7. Red wine improves high glucose-suppressed EPC numbers, proliferation and senescence in high glucose conditions in vitro

After seeding MNCs in wells, incubation of cells with high glucose for 4 days decreased the numbers of differentiated, adherent early EPCs (control vs. high glucose, 100 ± 9 vs. 53 ± 3, $p < 0.001$, Fig. 6A). As compared to the control group, incubation of MNCs with red wine (0.5%) for 24 h following by treatment of EPCs in high glucose medium significantly increased the numbers of EPCs assessed by DiI-acLDL and fluorescein isothiocyanate lectin staining (high glucose vs. high glucose + red wine, 53 ± 3 vs. 69 ± 5, $p = 0.005$, Fig. 6A), but no significant change was found with the ethanol group (high glucose vs. ethanol, $p = 0.270$).

As shown in Fig. 6B, incubation with high glucose culture medium for 4 days markedly reduced EPC proliferation (control vs. high glucose, 100 ± 2 vs. 57 ± 1, $p < 0.001$). However, administration of red wine for 24 h following by treatment of EPCs in high-glucose conditions significantly reversed the reduction in EPC proliferation in response to high glucose (high glucose vs. high glucose + red wine, 57 ± 1 vs. 74 ± 1, $p < 0.001$), but this effect was not observed with ethanol ($p = 0.764$).

Compared with the control group, incubation of late EPCs with high glucose significantly increased the percentage of senescence-associated β-galactosidase-positive EPCs ($100 \pm 2\%$ vs. $141 \pm 6\%$, $p = 0.008$). Administration of red wine for 24 h following by treatment of EPCs in high-glucose conditions significantly attenuated the percentage of senescence-associated β-galactosidase-positive EPCs (high glucose vs. high glucose + red wine, $141 \pm 6\%$ vs. $110 \pm 3\%$, $p = 0.033$) (Fig. 6C), while ethanol treatment had no effect ($p = 0.454$). However, administration of L-NAME abolished the anti-senescence effect of red wine (Fig. 6C).

3.8. Effects of red wine on EPC adhesion, migration and tube formation in high glucose conditions in vitro

As shown in Fig. 7A, incubation of late EPCs with high glucose medium markedly suppressed late EPC adhesion, and administration of red wine (24 h) significantly augmented adhesion in high glucose conditions (high glucose vs. high glucose + red wine, $77 \pm 2\%$ vs. $95 \pm 1\%$, $p = 0.004$). This effect was inhibited by treatment with L-NAME (100 μM) (Fig. 7A). However, there was no effect of ethanol on EPC adhesion in high glucose conditions ($p = 0.171$).

Compared with the control group, incubation of late EPCs with high glucose (4 days) markedly decreased EPC migration (130 ± 8 vs. 54 ± 5/HPF, $p = 0.001$). However, coincubation with red wine for 24 h significantly enhanced high glucose-suppressed late EPC migratory function (high glucose vs. high glucose + red wine, 54 ± 5 vs. 102 ± 2 cells/HPF, $p < 0.001$; Fig. 7B), but this benefit was not observed in the ethanol group ($p = 0.234$). In addition, administration of L-NAME (100 μM) markedly diminished the beneficial effect on EPC migration provided by red wine (Fig. 7B).

After 4 days of culturing, the capacity for tube formation of late EPCs on ECMatrix gel was significantly reduced in the presence of high glucose compared with the control group, whereas incubation with red wine for 24 h following by treatment of EPCs in high-glucose conditions ameliorated this high glucose suppressed-tube formation by EPCs (37 ± 1 vs. 101 ± 3 cells/HPF, $p < 0.001$; Fig. 7C). This effect was not observed by incubation with ethanol. Administration of L-NAME attenuated the effect of red wine on EPC tube formation.

4. Discussion

To the best of our knowledge, this is the first study to show that a favorable effect of moderate intake of red wine on ischemia-induced neovascularization in diabetic mice. We demonstrated that red wine ingestion enhanced blood flow recovery and new vessel formation in response to tissue ischemia in type 1 and type 2 diabetic mice. Intake of red wine upregulated eNOS activity in ischemic tissues, promoted EPC mobilization and improved c-kit positive bone marrow cell functions in STZ-induced diabetic mice. Interestingly, these effects were not seen when the mice were exposed to ethanol only. In addition, incubation with red wine could ameliorate high glucose-induced oxidative stress, attenuate cellular senescence and improve EPC functions, including adhesion, migration and tube formation capacities. Given the evidence mentioned above, our current findings indicated the direct beneficial effects of red wine ingestion on EPCs, which may provide some novel rationales to its potential clinical impact to vascular protection.

The protective effect of red wine is supported by epidemiological data showing a significantly reduced incidence of coronary artery disease in certain areas of France, in spite of high-fat diets, little exercise and wide-spread cigarette smoking. This phenomenon has been termed the “French paradox” [11]. Evidence in support of a more pronounced cardio-protective effect of red wine as compared

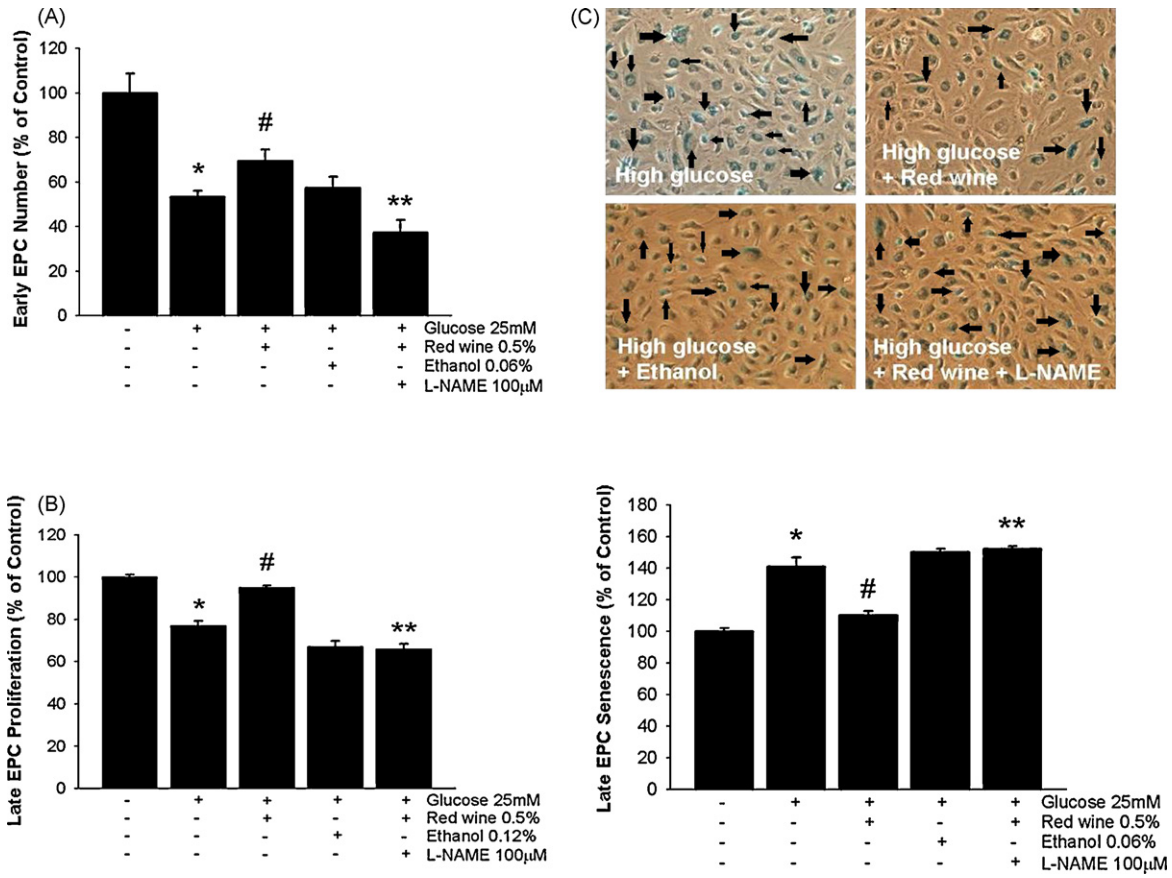

Fig. 6. Effects of Red wine on EPC numbers, proliferation and senescence in vitro. (A) Compared to the control group, incubation of mononuclear cells with high glucose medium suppressed the numbers of differentiated, adherent, early EPCs, as assessed by fluorescein isothiocyanate lectin and DiI-acLDL staining. (B) The effect of red wine on late EPC proliferation was analyzed by MTT assay. (C) To determine the onset of cellular aging, acidic β-galactosidase was used as a biochemical marker for acidification, typical of EPC senescence ($n = 4$ for each experiment).

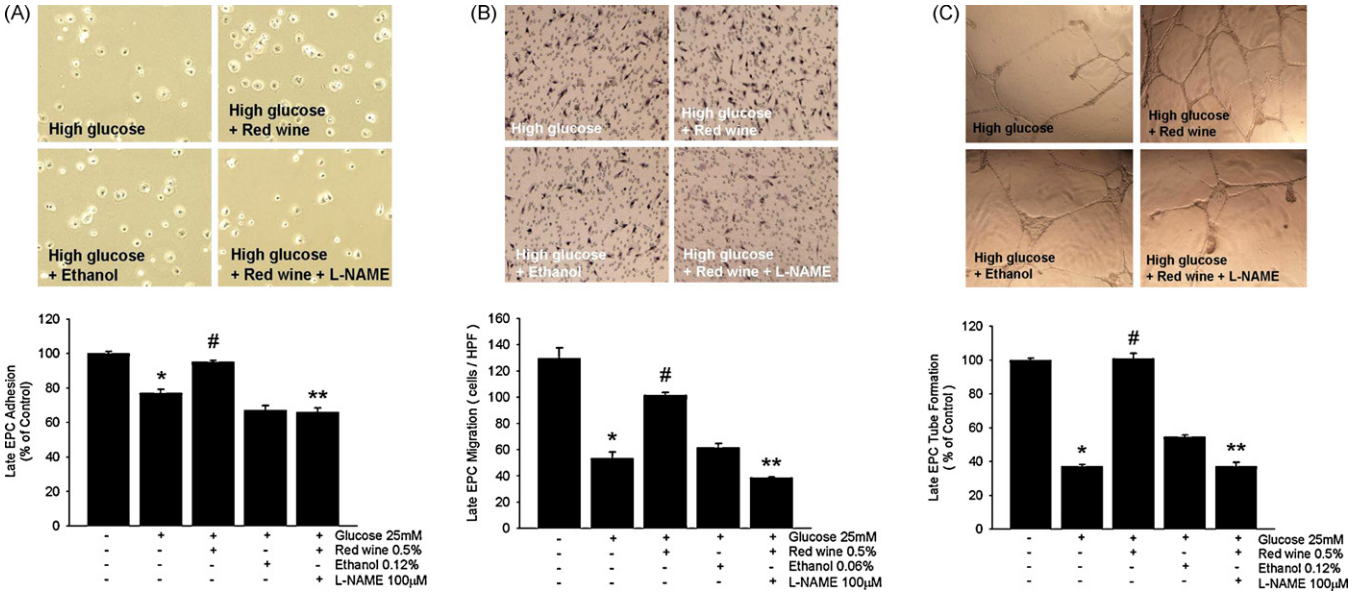

Fig. 7. Effects of red wine on EPC adhesion, migration and tube formation in vitro. (A) Fibronectin adhesion assay was used to evaluate the effect of red wine on EPC adhesion. (B) Boyden chamber assay using VEGF as a chemoattracting factor was used to evaluate the effect of red wine on EPC migration. (C) An in vitro angiogenesis assay for late EPCs used ECMatrix gel. Representative photos for in vitro angiogenesis are shown. Cells were stained with crystal violet, and the averages of the total area of complete tubes formed by cells were compared using computer software ($n = 4$ for each experiment).

with other alcoholic beverages first emerged from the Copenhagen City Heart Study, in which 13,285 men and women were observed for 12 years [21]. The results from this study suggested that patients who drank wine had half the risk of death from coronary heart disease or stroke compared to those who never drank wine. However, those who drank spirits and beer did not experience this advantage [22].

A number of mechanisms have been proposed for the beneficial effect of red wine ingestion, including increased levels of high-density lipoproteins [23], decreased levels of ox-LDL [24], inhibition of platelet aggregation and adhesion [25], improvement of endothelial function [26], and potent anti-oxidant capacity [27]. However, mechanistic studies suggest that many of these beneficial effects of red wine are compatible with the actions of endothelium-derived NO, implying that NO may be a critical mediator of the cardiovascular protection provided by red wine [12].

Improved neovascularization in response to tissue ischemia is an important therapeutic strategy to reduce organ damage. Accumulating evidence suggests that neovascularization in adults is not solely the result of the proliferation of endothelial cells (angiogenesis), but also involves circulating EPCs during vasculogenesis [4]. These circulating EPCs are derived from bone marrow and are mobilized either endogenously, triggered by tissue ischemia, or exogenously by cytokine stimulation. Diabetic patients frequently suffer from micro- or macrovascular abnormalities, including retinopathy, nephropathy, neuropathy and accelerated atherosclerosis. It is evident that decreased bioavailability of NO produced from eNOS plays a crucial role in the development and progression of atherosclerosis. Under various pathological conditions such as type 2 diabetes eNOS may become dysfunctional or its expression may be decreased. Inadequate angiogenic response to ischemia in the myocardium of diabetic patients could result in poor collateral formation and organ damage. Previous studies reported that EPCs are markedly reduced in patients with either type 1 or type 2 diabetes, and the EPCs from diabetic patients showed reduced capacity to induce angiogenesis in vitro [28–31]. These defects in EPC functions may underlie some of the vascular complications associated with diabetes, such as endothelial dysfunction, that predisposes to diffuse atherosclerosis and impaired neovascularization after ischemic events. Our recent work also indicated that the administration of NO donor could reverse the impairments in proliferation and functions of both early and late EPCs induced by long-term exposure to high glucose, suggesting the critical role of NO to reverse hyperglycemia-induced EPC down-regulation [7].

There is increasing evidence showing the interaction of red wine with the endothelial NO system [32]. In animal experiments, red wine or extracts obtained from red wine caused endothelium-dependent, NO-mediated vasorelaxation of rat or rabbit aorta that had been constricted with norepinephrine [33,34]. Wallerath et al reported that red wine upregulated eNOS expression and activity in human endothelial cells by both transcriptional and post-transcriptional (mRNA-stabilizing) mechanisms [12]. Recent evidence suggests that the mobilization and differentiation of EPCs are modified by NO, and that bone marrow-expressed eNOS is essential for the mobilization of stem cells and progenitor cells in vivo [35]. In this study, we first showed that red wine ingestion could enhance collateral blood flow in ischemic tissue in diabetic mice by promoting EPC mobilization and c-kit positive bone marrow cell function through NO-related pathway. By in vitro assays, red wine attenuated high glucose-induced EPC senescence, and promoted high glucose-suppressed EPC functional capacities. Our data are in line with previous studies showing that moderate intake of red wine promoted EPC numbers in wild-type mice subjected to physical exercise and improved ischemia-induced neovascularization in hypercholesterolemic ApoE-deficient mice after hind limb ischemia by increasing the number and functional activities of EPCs [36,37]. Balestrieri and colleagues indicated that red wine and pure resveratrol prevented the TNF-α-induced reduction of EPC number, implying beneficial effects of red wine and resveratrol in the positive modulation of EPCs levels [38]. These findings suggest that the vasoprotective effects may derive from upregulation of eNOS phosphorylation and enhanced NO bioavailability by red wine.

It has also been proposed that the cardiovascular benefits of moderate wine consumption are due, at least in part, to the antioxidant properties of red wine [39]. Oxidative stress is associated with the development of atherosclerotic diseases, and has also been shown to impair endothelial cell growth and angiogenesis [40]. In this study, we demonstrated that in vitro administration of red wine was associated with reduced oxidative stress in high glucose conditions. Therefore, the antioxidant potential of red wine could also contribute to restore EPC number and function in situations of increased oxidative stress such as hyperglycemia.

5. Conclusions

This study first showed that moderate ingestion of red wine augmented blood flow recovery and new vessel formation after tissue ischemia in diabetic mice. These beneficial effects may derive from enhanced EPC functions, mobilization and tissue homing by upregulation of eNOS activity. These novel findings may explain the underlying mechanism responsible for the beneficial effects of red wine consumption on the cardiovascular system.

Conflict of interest

None.

Acknowledgements

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