Probucol protects circulating endothelial progenitor cells from ambient $PM_{2.5}$ damage via inhibition of reactive oxygen species and inflammatory cytokine production *in vivo*

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Abstract. Bone marrow-derived circulating endothelial progenitor cells (EPCs) contribute to angiogenesis and vascular repair. The number and function of EPCs are significantly decreased following exposure to ambient fine particulate matter of ≤2.5 μm in diameter ($PM_{2.5}$) through reactive oxygen species (ROS) generation and inflammatory cytokine secretion. The anti-oxidant drug probucol reduces ROS and inflammatory cytokine production. The present study was designed to determine the protective effects of probucol on EPCs from $PM_{2.5}$-associated impairment *in vivo* and to explore the potential underlying mechanisms. Male C57BL/6 mice were exposed to ambient air containing $PM_{2.5}$ for one month with or without probucol treatment. Mice that breathed filtered air were used as a control group. Serum and blood cells were collected for analysis. The results indicated that $PM_{2.5}$ exposure induced increases in blood intracellular ROS, serum inflammatory cytokine levels and the blood cell apoptotic rate, while it decreased the number and proliferation rate of circulating EPCs in the mice with $PM_{2.5}$ exposure. These effects were significantly reduced/abrogated by probucol treatment. The present *in vivo* study suggested that probucol protects EPCs from damage through $PM_{2.5}$ exposure by inhibiting ROS generation and inflammatory cytokine production.

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Introduction

Deficiency or injury of the endothelium is closely associated with the initiation of the atherosclerotic process (1). Endothelial progenitor cells (EPCs) are mainly involved in vascular regeneration, angiogenesis and re-endothelialization following vascular damage (2). Thus, it is important to retain the number and function of EPC at normal levels in patients with cardiovascular disease.

Ambient particulate matter (PM) has become a major health threat. Increases in mortality and morbidity due to PM exposure have been reported (3). PM is a mixture of different types of debris, the major components of which are crustal material, metals and bio-aerosols (4). Small PM with a median aerodynamic diameter of ≤2.5 μm ($PM_{2.5}$) is the most harmful type and numerous cardiovascular diseases are associated with exposure to $PM_{2.5}$ (5). $PM_{2.5}$ exposure causes a reduction in heart rate variability, as well as vascular dysfunction, vascular inflammation, an increased coagulation-thrombosis risk and the acceleration of atherosclerosis (5). More importantly, in humans and mice, a decreased number and function of EPC are also associated with $PM_{2.5}$ exposure (6,7). A previous study by our group indicated that exposure of mice to PM of ≤4 μm in size (including $PM_{2.5}$) through intranasal instillation for one month significantly suppressed the number and function of EPCs via increasing blood intracellular reactive oxygen species (ROS) production and inflammatory cytokine generation (8). However, the detailed mechanisms by which the number and function of EPCs decrease after inhalation of atmospheric $PM_{2.5}$ have remained to be elucidated.

A notable reduction in atherosclerosis and restenosis in the coronary artery were observed in subjects treated with probucol (9,10). This potent drug has anti-oxidant and anti-inflammatory effects and preserves endothelial function by

reducing the amount of endogenous nitric oxide (NO) synthase inhibitor, increasing prostacyclin generation, inhibiting the expression of various adhesion molecules and promoting the proliferation of endothelial cells, while preventing the apoptosis of endothelial cells due to oxidative injury (9,10). Of note, cigarette smoking and oxidized high-density lipoprotein (Ox-HDL) induced EPC dysfunction may also be reversed by probucol treatment (11,12). However, the effects of probucol on EPCs exposed to $PM_{2.5}$ remain elusive.

The aim of the present study was to determine whether probucol has any protective effects on EPCs in mice exposed to $PM_{2.5}$. It was observed that the diminished EPC levels in mice under $PM_{2.5}$ exposure were indeed restored to normal levels with probucol treatment.

Materials and methods

Animal model of $PM_{2.5}$ exposure. All animal procedures were performed in accordance with the Guidelines of the Animal Care Committee of the Shandong Provincial Hospital affiliated with Shandong University (Jinan, China). The Animal Care Committee of Shandong University (Jinan, China) approved the experimental protocols. A total of 40 male wild-type C57 BL/6 mice (age, 6-8 weeks; weight, 20-25 g) were purchased from Better Biotechnology Co., Ltd. (Nanjing, China). All mice were housed at the animal facility for 1 week prior to exposure. The center of Jinan city (China), a highly polluted area, was selected for the experiment. The exposure period lasted one month from December 12, 2016 to January 12, 2017. Ambient PM with a diameter equal to 2.5 μm was collected using a high flow rate with an aerosol-into-liquid collector (HRH-PM186; Beijing Huironghe Technology Co., Ltd., Beijing, China). The mean concentration of PM in Jinan measured during the experiment was 135.23±42.12 $\mu g/m^3$. This data was in accordance with previously published data (13). The concentration of PM for mice with exposure and for mice with filtered air (FA; the control mice) was adjusted to 130±65.51 and 2.4±1.1 $\mu g/m^3$, respectively. A total of 10 mice in each group were subjected to PM exposure or used as controls who inhaled FA. All mice were exposed in a chamber system for the experiment as described (14). The high-efficiency particulate air filter (Pall Life Sciences; Pall Corporation, Port Washington, NY, USA) was used for mice with FA exposure. Probucol was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). To evaluate the protective effect of probucol on EPC from $PM_{2.5}$ mice, 5 mg probucol was first dissolved in 100 μl 99% ethanol, then diluted in 99.9 ml PBS to give a final concentration of 50 μg/ml. The final concentration of ethanol was adjusted to 1‰ in PBS. Then, the probucol solution was used to treat mice at a dose of 500 mg/kg/day (10). In a preliminary experiment, probucol at 100, 250, 500 and 600 mg/kg/day was used to treat the mice exposed to $PM_{2.5}$. The maximum anti-oxidant and anti-inflammatory effects of probucol were observed at the dosage of 500 mg/kg/day, while no further increases in the protective effects of probucol were achieved when the dose was 600 mg/kg/day. Probucol was administered by oral gavage for three days prior to $PM_{2.5}$ exposure and for one month during $PM_{2.5}$ exposure. A total of 40 mice were equally divided into four groups: The FA group, 10 mice with FA exposure; the $PM_{2.5}$ group, 10 mice with $PM_{2.5}$ exposure; the Prob+FA group, 10 mice with probucol treatment and FA exposure; the Prob+$PM_{2.5}$ group, 10 mice with probucol treatment and $PM_{2.5}$ exposure.

Measurement of pro-inflammatory factors. Mouse serum was collected after one month of $PM_{2.5}$ exposure. The pro-inflammatory cytokines tumor necrosis factor (TNF)-α (cat. no. 430904), interleukin (IL)-1β (cat. no. 432604) and IL-6 (cat. no. 431304) were measured with an ELISA kit (BioLegend, San Diego, CA, USA) according to the manufacturer's protocols.

Assessment of EPC proliferation, apoptosis and intracellular ROS formation. After $PM_{2.5}$ or FA exposure, murine blood was collected, followed by the elimination of red blood cells (RBCs) with an RBC lysis buffer (cat. no. 420301; BioLegend). The *in vivo* EPC number and proliferation rate were measured at 12 h after i.p. injection of 1 mg bromodeoxyuridine (BrdU). CD34-Alexa Fluor® 700 (cat. no. 560518; BD Biosciences, Franklin Lakes, NJ, USA) and CD133-phycoerythrin (cat. no. 141204; BioLegend) antibodies were used to mark the EPC population. Specifically, 1 μl CD34 and 1 μl CD133 in 100 μl cell staining buffer (420201; BioLegend) were added to $1x10^6$ cells. Then the mixture solution was incubated in ice in a dark room for 30 min. In order to quantify the EPC population, the $CD34^+/CD133^+$ cells were detected in a sample containing at least 50,000 cells. Anti-BrdU fluorescein isothiocyanate (FITC) contained in the BrdU Flow Kit (cat. no. 559619; BD Biosciences) was used to measure the cell proliferation. Blood EPC apoptosis was determined by using the FITC Annexin V Apoptosis Detection kit (cat. no. 556547; BD Biosciences). Early [Annexin V FITC-positive and propidium iodide (PI)-negative cells] and late (Annexin V FITC and PI double-positive cells) apoptotic cells were measured. Total ROS Assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), which contained dichlorofluorescein (DCF)-FITC, was used to measure the intracellular ROS production. Specifically, following the staining of cells with $CD34^+$ and $CD133^+$ antibodies, cells were washed twice with PBS. Then, DCF-FITC was added in the mixture solution for 10 min at 37˚C. The DCF-FITC-labelled cells were washed twice with PBS and then suspended in warm PBS (37˚C) for analysis using flow cytometry. The $CD34^+/CD133^+$ cells with DCF-FITC fluorescence-positive cells were quantitatively evaluated using a BD™ LSR II flow cytometer (BD Biosciences) at the wavelength of 525 nm, as previously described (8).

Statistical analysis. Values are expressed as the mean ± the standard deviation. PRISM version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the statistical analyses. The unpaired Student's t-test (two-sided) was used for comparison between two groups. One-way analysis of variance followed by a post-hoc conservative Tukey's test were used for comparison between three or more groups to minimize type-I errors as appropriate. A two-tailed $P<0.05$ was considered to indicate a statistically significant difference.

Results

$PM_{2.5}$ treatment reduces circulating EPCs in association with increased apoptosis and decreased proliferation. After exposure to $PM_{2.5}$ for one month, murine blood cells were collected

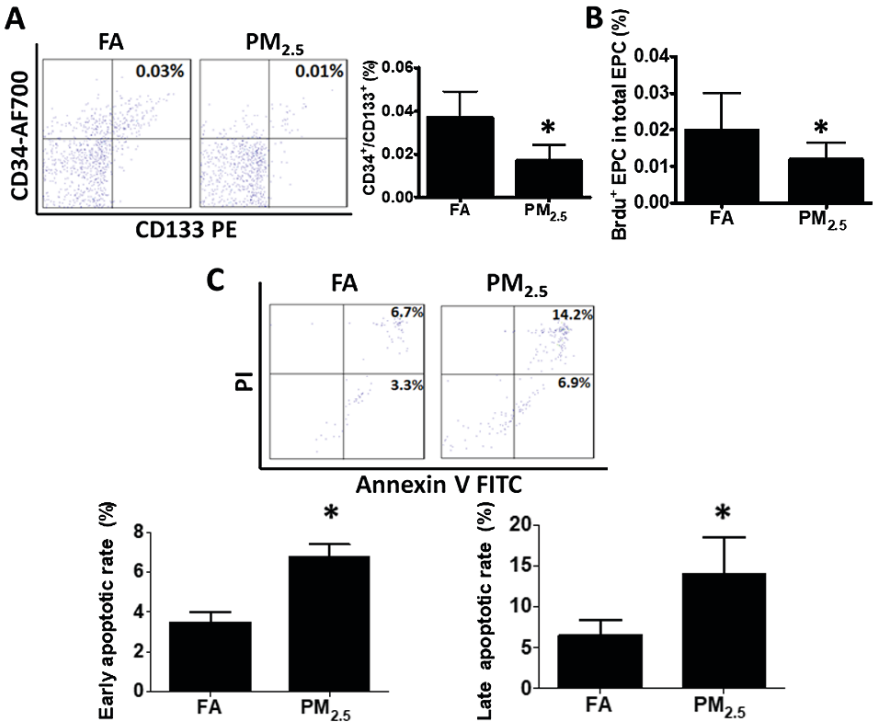

Figure 1. Exposure to $PM_{2.5}$ decreases circulating EPC levels in mice by suppression of EPC proliferation and induction of EPC apoptosis. (A) Following $PM_{2.5}$ or FA exposure for 1 month, the blood cells from C57BL/6 mice were collected and stained with CD34-AF700 and CD133-PE antibody for quantification of EPCs ($CD34^+/CD133^+$). The amount of EPCs was significantly decreased in C57BL/6 mice subjected to $PM_{2.5}$ exposure compared with that in the FA control. (B) After $PM_{2.5}$ exposure, mice were injected intraperitonally with 1 mg BrdU. After 12 h, cells were obtained, permeabilized and incubated with anti-BrdU FITC after staining with CD34-AF700 and CD133-PE antibody. The proliferation rate of murine EPCs was significantly declined with $PM_{2.5}$ exposure compared with that in the FA group. (C) Blood cell apoptosis was measured by staining with annexin V and PI. The early and late apoptotic rate of murine blood cells was significantly increased with $PM_{2.5}$ exposure as compared with that in the FA group. Values are expressed as the mean ± standard deviation (n=10). *P<0.05 vs. the FA group. EPCs, bone marrow-derived circulating endothelial progenitor cells; FA, filtered air; $PM_{2.5}$, ambient fine particulate matter of ≤2.5 μm in diameter; PE, phycoerythrin; PI, propidium iodide; FITC, fluorescein isothiocyanate; BrdU, bromodeoxyuridine; AF700, Alexa Fluor® 700.

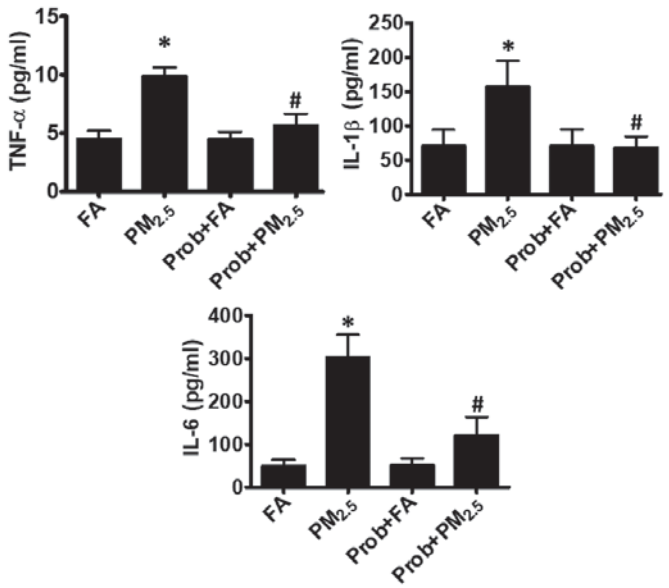

Figure 2. Prob treatment inhibits increases in inflammatory cytokines following $PM_{2.5}$ exposure. Elevation of the inflammatory cytokines, TNF-α, IL-1β and IL-6, induced by $PM_{2.5}$ was effectively prevented by Prob. Groups: FA, C57BL/6 mice kept in ambient FA; $PM_{2.5}$, C57BL/6 mice with $PM_{2.5}$ exposure; Prob+FA, C57BL/6 mice with Prob treatment kept in FA; Prob+$PM_{2.5}$, C57BL/6 mice with Prob treatment and $PM_{2.5}$ exposure. Values are expressed as the mean ± standard deviation (n=10). *P<0.05, $PM_{2.5}$ vs. FA or Prob+FA or Prob+$PM_{2.5}$; #P<0.05, FA vs. Prob+FA or $PM_{2.5}$ vs. Prob+$PM_{2.5}$. IL, interleukin; TNF, tumor necrosis factor; FA, filtered air; $PM_{2.5}$, ambient fine particulate matter of ≤2.5 μm in diameter; Prob, probucol.

for EPC analysis. The results indicated that $PM_{2.5}$ significantly decreased the $CD34^+/CD133^+$ cell population (0.017±0.007%) compared with that in the control (0.037±0.012%; Fig. 1A). To identify the possible reasons for the decrease in the EPC population, the EPC proliferation and apoptotic rate were assessed. As presented in Fig. 1B, the EPC proliferation rate was significantly decreased compared with that in the control group (0.012±0.004% vs. 0.026±0.005%). Furthermore, the early apoptotic rate (6.74±0.67%) and the late apoptotic rate (14.04±4.38%) of EPCs were substantially elevated compared with those in the control group (3.48±0.51 and 6.58±1.77%, respectively; Fig. 1C).

$PM_{2.5}$ increases serum inflammatory factors. The serum levels of inflammatory factors, including TNF-α, IL-1β and IL-6, are known to be closely associated with an increased blood cell apoptosis and decreased circulating EPC proliferation (8,15). In the present study, the serum levels of TNF-α, IL-1β and IL-6 were measured after $PM_{2.5}$ exposure. As presented in Fig. 2, the TNF-α and IL-1β levels were increased to up to 2-fold of those in the control group, while IL-6 was 6-fold of that in the control group.

$PM_{2.5}$ increases intracellular ROS levels in blood EPCs. It has been previously reported that intracellular ROS may cause an elevation of blood cell apoptosis and a decline of EPC proliferation (8,15). After exposure to $PM_{2.5}$ for one

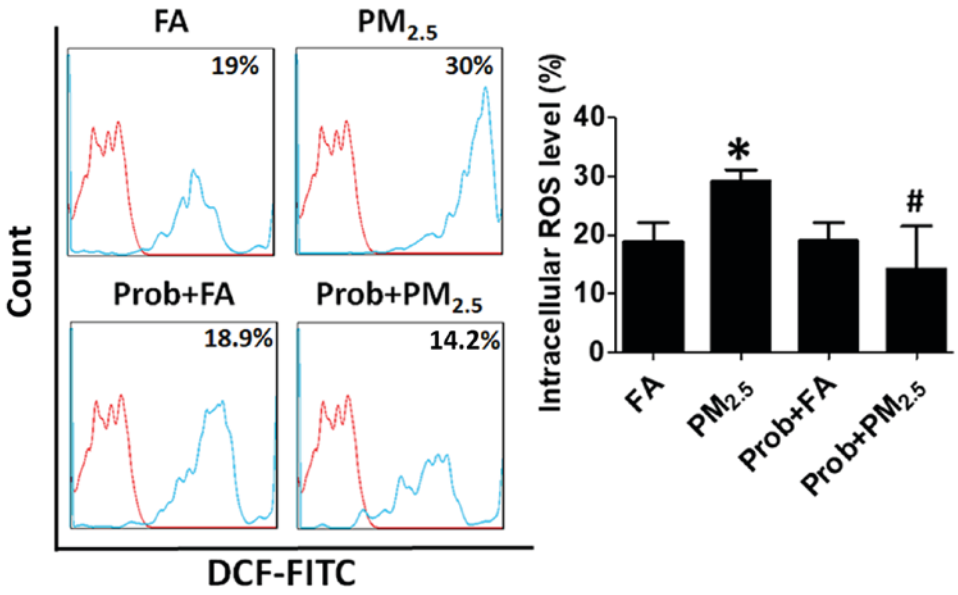

Figure 3. Prob treatment decreases ROS production induced by $PM_{2.5}$ exposure. Increased ROS production in the blood EPCs of mice exposed to $PM_{2.5}$ was effectively blocked by Prob treatment. Blood EPCs without DCF staining was used as baseline in the histograph. Groups: FA, C57BL/6 mice kept in ambient FA; $PM_{2.5}$, C57BL/6 mice with $PM_{2.5}$ exposure; Prob+FA, C57BL/6 mice with Prob treatment kept in FA; Prob+$PM_{2.5}$, C57BL/6 mice with Prob treatment and $PM_{2.5}$ exposure. Values are expressed as the mean ± standard deviation (n=10). *P<0.05, $PM_{2.5}$ vs. FA or Prob+FA or Prob+$PM_{2.5}$; $^{\#}$P<0.05, FA vs. Prob+FA or $PM_{2.5}$ vs. Prob+$PM_{2.5}$. FA, filtered air; $PM_{2.5}$, ambient fine particulate matter of ≤2.5 μm in diameter; Prob, probucol; ROS, reactive oxygen species; DCF-FITC, dichlorofluorescein-fluoresceinisothiocyanate.

month, the ROS levels in the blood EPCs were assessed. As presented in Fig. 3, the blood intracellular ROS levels were significantly increased in the mice with $PM_{2.5}$ exposure compared to those in the control group (29.14±2% vs. 18.96±3.14%).

Probucol treatment attenuates the detrimental effects of $PM_{2.5}$ on EPCs. To evaluate the protective effect of probucol against the reduction of circulating EPCs due to $PM_{2.5}$ exposure, mice were pre-treated with probucol prior $PM_{2.5}$ exposure for 3 days and treatment was continued for 1 month with $PM_{2.5}$ exposure. The intracellular ROS production was completely blocked by probucol treatment (Fig. 3). In the probucol treatment group, the $PM_{2.5}$-associated elevated serum inflammatory factors (TNF-α, IL-1β and IL-6) were also reduced to the normal level of the control group (Fig. 2).

To determine whether probucol treatment of mice exposed to $PM_{2.5}$ is able to restore the circulating EPC proliferation and blood EPCs apoptotic rate to the normal level, the EPC proliferation and blood cell apoptotic rate were measured after probucol treatment. As presented in Fig. 4B, the EPC proliferation rate was recovered with probucol treatment, while the early and late apoptotic rate of blood cells were also restored to the normal level in the mice with probucol treatment (Fig. 4C).

Finally, it was assessed whether probucol was able to prevent the reduction in EPCs after $PM_{2.5}$ exposure. As presented in Fig. 4A, the decrease in $CD34^{+}/CD133^{+}$ cells (0.017±0.007%) associated with $PM_{2.5}$ exposure was inhibited by probucol treatment, resulting in near normal levels (0.029±0.002%).

These data indicated that probucol effectively inhibited the effects of $PM_{2.5}$ on murine circulating EPCs via the inhibiton of blood cell apoptosis and increase in EPCs proliferation through the blocking of intracellular ROS production and inflammatory cytokine secretion (Fig. 5).

Discussion

In the present study, it was demonstrated that exposure to $PM_{2.5}$ induced apoptosis of blood cells and suppressed EPC proliferation to reduce the number of EPCs via increasing the blood intracellular ROS levels and serum inflammatory cytokine levels. However, probucol prevented the blood intracellular ROS generation, increases in serum inflammatory cytokines and EPC apoptosis. The proliferation rate and the percentage of circulating EPCs were also recovered with probucol treatment.

It has been widely reported that $PM_{2.5}$ exposure is associated with various systemic diseases, including cardiovascular (5), neuronal (16) and hepatic disease (17), as well as diabetes (18). The mechanisms mainly involve $PM_{2.5}$ triggering systemic oxidative stress and inflammation (19). Of note, the increases in ROS production and the levels of inflammatory factors following PM exposure are associated with endothelial injury as well as a decreased number of EPCs (20,21). The results of the present study also suggested that intracellular ROS production as well as serum inflammatory factors were increased after $PM_{2.5}$ exposure. These contributed to the decrease in the number of circulating EPCs, which is associated with an increased risk of cardiovascular disease.

It is well documented that after endothelial injury, EPCs contribute to angiogenesis and vascular regeneration, as well as maintaining a normal endothelial function (2). Of note, increases in cardiovascular disease are associated

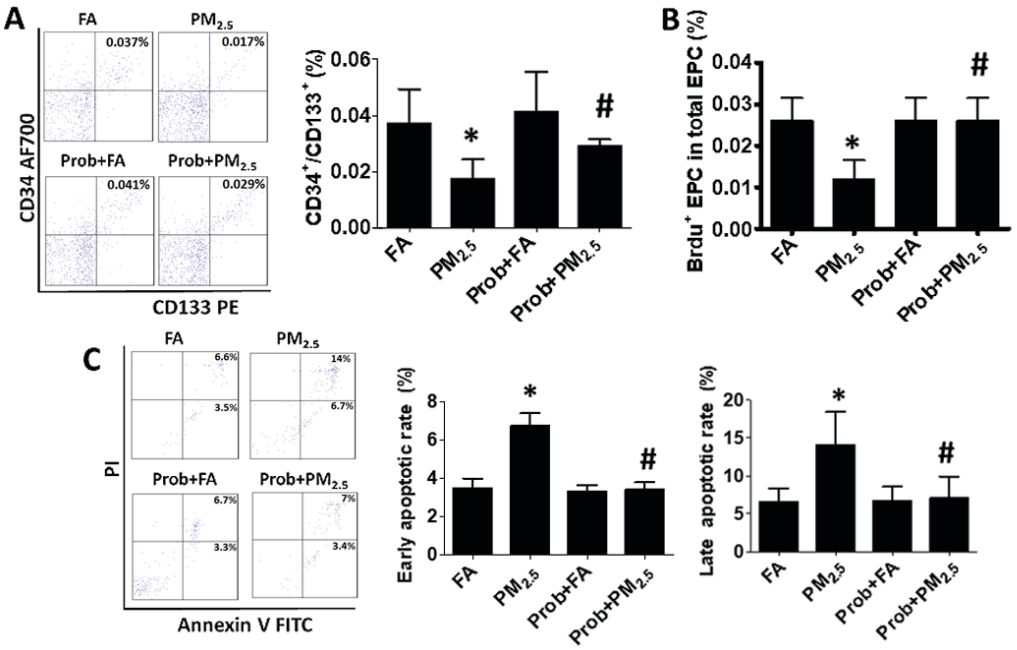

Figure 4. Prob treatment recovers the EPC population in mice exposed to $PM_{2.5}$ by restoring EPC proliferation and preventing EPC apoptosis. (A and B) The decreased (A) EPC population and (B) EPC proliferation due to $PM_{2.5}$ exposure were effectively recovered with Prob treatment. (C) The early and late apoptotic rate were restored to the normal level with Prob treatment. Groups: FA, C57BL/6 mice kept in ambient FA; $PM_{2.5}$, C57BL/6 mice with $PM_{2.5}$ exposure; Prob+FA, C57BL/6 mice with Prob treatment kept in FA; Prob+$PM_{2.5}$, C57BL/6 mice with Prob treatment and $PM_{2.5}$ exposure. Values are expressed as the mean ± standard deviation (n=10). *P<0.05, $PM_{2.5}$ vs. FA or Prob+FA or Prob+$PM_{2.5}$; #P<0.05, FA vs. Prob+FA or $PM_{2.5}$ vs. Prob+$PM_{2.5}$. EPCs, bone marrow-derived circulating endothelial progenitor cells; FA, filtered air; $PM_{2.5}$, ambient fine particulate matter of ≤2.5 μm in diameter; PE, phycoerythrin; PI, propidium iodide; FITC, fluorescein isothiocyanate; BrdU, bromodeoxyuridine; AF700, Alexa Fluor® 700; Prob, probucol.

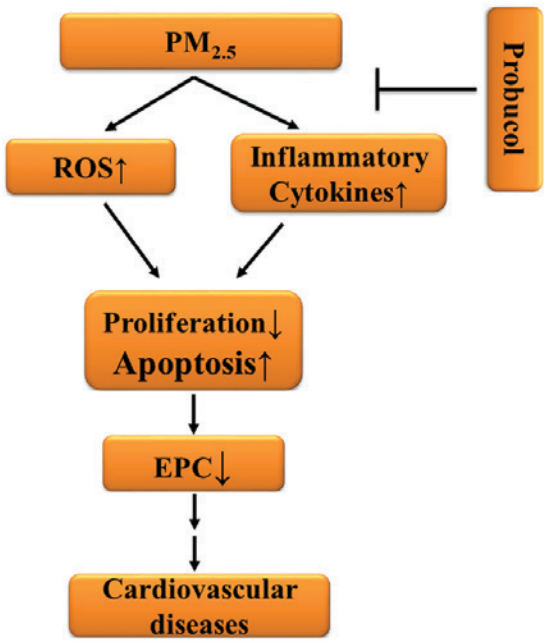

Figure 5. Schematic illustrating the possible mechanisms for the decreased number of circulating EPCs in the mice subjected to $PM_{2.5}$ exposure. Blood intracellular ROS production and inflammatory cytokine levels were elevated following $PM_{2.5}$ exposure. The proliferation rate of the EPCs was notably decreased, while the apoptotic rate of blood EPCs was significantly increased. As a result, the number of circulating EPCs was extensively decreased, which is associated with the development of cardiovascular diseases. Prob efficiently inhibited the $PM_{2.5}$-induced ROS generation, increase in inflammatory cytokines and apoptotic rate of blood cells, and decrease in EPC proliferation. The number of circulating EPCs was significantly increased with Prob treatment after $PM_{2.5}$ exposure. ROS, reactive oxygen species; EPC, endothelial progenitor cell; ↑, increase; ↓, decrease; Prob, probucol; $PM_{2.5}$, ambient fine particulate matter of ≤2.5 μm in diameter.

with a decreased number and function of EPCs after PM exposure (5). It has been reported that after $PM_{2.5}$ or nickel exposure, the number of murine bone marrow and circulating EPCs ($CD34^+/CD31^+/CD45^+/CD133^+$) was decreased (21), and the function of EPCs ($CD34^+$/vascular endothelial growth factor receptor-$2^+/CD11b^-$), including tube formation and chemotaxis, were also significantly suppressed (6). A study on a Chinese cohort also reported that circulating EPCs [$CD34^+$/kinase insert domain receptor $(KDR)^+$, $CD34^+/KDR^+/CD45^-$ or $CD34^+/KDR^+/CD133^+$] were notably reduced following $PM_{2.5}$ exposure (22). Furthermore, in accordance with a previously published study (8), the present results indicated that PM exposure significantly suppressed the circulating EPC population in mice through promoting apoptosis of EPCs ($CD34^+/CD133^+$) in association with an elevated ROS generation as well as serum TNF-α and IL-1β levels *in vivo*. Furthermore, the present study confirmed that inhalation of ambient $PM_{2.5}$ suppressed the proliferation of EPCs and promoted their apoptosis via increasing the levels of ROS in blood cells and of TNF-α, IL-1β and IL-6 in the serum. However, certain studies reported that circulating EPCs increased following short-term $PM_{2.5\text{-}10}$ exposure (7,23). The mechanisms were described to mainly involve sympathetic nervous system activation and decreased mobilization of bone marrow EPCs into the circulation through a systemic reaction to an acute 'endothelial injury' following PM exposure.

Reagents and methods for the protection of EPCs have been reported in numerous studies and included the use of microRNA, triterine, granulocyte-macrophage colony-stimulating factor, urinary trypsin and inhibition of

CD40 (24-27). Of note, probucol, as a cholesterol modulator, significantly inhibits the initiation and progression of atherosclerosis. The mechanisms mainly include the suppression of ROS formation (10), promotion of endothelial recovery, inhibition of monocyte activation and adhesion (28), attenuation of vascular smooth muscle cell (VSMC) growth and migration (29), an influence on VSMC and macrophage proliferation and apoptosis, as well as a decrease of cytokine secretion by macrophages (30,31). Of note, the cigarette smoke-induced impairment and ischemia-triggered neovascularization was rescued by probucol through its protective effects on EPCs (11). The deleterious effects of Ox-HDL on EPCs were also reversed by probucol (12). In addition, probucol prevents ROS-induced inactivation of endothelium-derived NO, decreases endogenous NO synthase inhibitor formation and increases the level and function of NO to further benefit EPCs (9,10,32). In the present study, after the treatment of mice with probucol at 500 mg/kg/day for one month, it was observed the population and the proliferation of circulating EPCs were effectively restored, EPC apoptosis was inhibited, ROS formation blocked and serum inflammatory cytokines were reduced in the mice with $PM_{2.5}$ exposure.

Although the benefits of probucol on EPCs have been reported in numerous studies, the detailed mechanisms of its protective effects on EPCs following $PM_{2.5}$ exposure have remained elusive. Further questions, including what specific type of ROS is generated following $PM_{2.5}$ exposure, whether any other mechanisms are involved in the protection of EPCs by probucol, and whether ROS and inflammatory cytokines that are induced by $PM_{2.5}$ exposure are the two major factors that impair EPCs remain to be addressed in further studies. Future projects, including an experiment to discriminate between different types of ROS generated in EPCs and the application of vitamin C after $PM_{2.5}$ exposure, are currently in planning.

In conclusion, the present study indicated that probucol effectively prevented the effects of $PM_{2.5}$ on murine circulating EPCs via inhibition of blood cell apoptosis and recovery of EPC proliferation through blocking of blood intracellular ROS generation and inflammatory cytokine secretion. Thus, probucol may be an effective medicine for the prevention and treatment of $PM_{2.5}$-induced cardiovascular diseases.

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Availability of data and materials

All data generated or analyzed during the current study are included in this published article.

Authors' contributions

YC, LC, QS and ZL designed the experiments, and YC, KH, LY, HX, PZ and HS performed them. ZS, HL, LC and HB collected and analyzed the data. YC wrote the manuscript.

Ethical approval and consent to participate

The Animal Care Committee of Shandong University (Jinan, China) approved the experimental protocols.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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