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ORIGINAL PAPER

Senescent endothelial progenitor cells from dogs with pulmonary arterial hypertension: a before–after self-controlled study

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Abstract Previous studies have underlined the importance of endothelial dysfunction and microvascular occlusion in the pathogenesis of pulmonary artery hypertension (PAH). Since the endothelial progenitor cells (EPCs) are involved in maintaining endothelial homeostasis, we observed the change of peripheral EPCs in canines before and after PAH onset. PAH was induced by intra-pulmonary artery injection of dehydromonocrotaline (DHMC) in nine beagles. Before and 48 h and 6 weeks after DHMC injection, 40 ml peripheral blood was obtained from the femoral vein. Circulating EPCs were identified as CD133 + KDR + cells and numerated by fluorescence-activated cell sorter; the EPCs functional capacity was determined by in vitro tubule-forming assay. The senescence of EPCs was determined by β-galactosidase staining. At each time point, 2 ml blood from femoral artery was obtained for arterial oxygen pressure (PaO_2). Forty-eight hours after DHMC injection, treated beagles suffered from hypoxemia; however, both the number and the tubule-forming capacity of EPCs were transiently raised. Six weeks later, PAH was confirmed by obviously high mean pulmonary arterial pressure (20.2 ± 1.64 vs. 11.3 ± 2.0 mmHg, $p < 0.05$) and low PaO_2 (69.30 ± 9.15 vs. 95.94 ± 1.43 mmHg, $p < 0.01$) in beagles after DHMC treatment, and their EPCs exhibited a predominant decrease in either the number (206.1 ± 26.8 vs. 632.8 ± 42.8 cells/ml blood, $p < 0.01$) or the tubule-forming capacity (21.1 ± 2.8 vs. 11.2 ± 2.8 tubules/$\times$200 field, $p < 0.01$). Additionally, senescence-associated β-galactosidase-positive EPCs were significantly increased. Our data suggested that, after the acute stage of DHMC injury to pulmonary vessels, the EPCs from PAH beagles suffered from exhaustion and senescence.

Keywords Endothelial progenitor cells · Pulmonary artery hypertension · Canine · Angiogenesis · Senescence

Abbreviations

DHMC	Dehydromonocrotaline
EPCs	Endothelial progenitor cells
PAH	Pulmonary arterial hypertension
PAP	Pulmonary artery pressures
PaO_2	Arterial oxygen pressure
PE	Phycoerythrobilin
SA-β-gal	Senescence-associated β-galactosidase
VRm	Right ventricular pressure

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Introduction

Pulmonary arterial hypertension (PAH) is a devastating disease characterized by a progressive increase in pulmonary artery pressures and vascular resistance. Its most severe form, idiopathic PAH, with uncertain etiology and pulmonary precapillary lesions [1, 2], leads to right heart failure and death within a median of 2.8 years from diagnosis [3, 4]. Despite significant advances in the drug

therapy of PAH during the last decade, the prognosis remains poor. The vascular contraction, microvascular occlusive remodeling, inflammation and thrombosis are all speculated to contribute [5–9]. Evidence from animals and human lung specimens suggested that endothelial dysfunction may play a central role in the development of PAH [10]. The injured endothelium will induce the increase of vasoconstrictors (thromboxane A_2 and endothelin-1) and decrease of vasodilators (nitric oxide and prostacyclin), so contribute to the vascular tension and remodeling.

A variety of evidence suggests that endothelial progenitor cells (EPCs) constitute one aspect of the endothelium repair process [11, 12]. EPCs are a cell population that have the capacity to circulate, proliferate and differentiate into mature endothelial cells [13, 14]. Laboratory evidence suggests that these precursors participate in postnatal neovascularization and reendothelialization [11, 12, 15–17]. EPC dysfunction may contribute to an insufficient regeneration of the endothelium, which may lead to endothelial dysfunction. On the other hand, the repair of damaged endothelium may exploit the presumed finite EPCs pool, so finally results in EPCs depletion in return.

On the basis of the above consideration, we hypothesize that the establishment of PAH may lead to the impairment of circulating EPCs, which has previously been suggested to be associated with endothelial dysfunction and promotes the disease progression. To test this hypothesis, the numbers and activity of EPCs from peripheral blood of dogs were determined before and at several different time points after dehydromonocrotaline (DHMC)-induced PAH. In addition, EPC senescence, which is essential for cellular proliferation and function, was also observed.

Methods

Animal models of PAH

To indue PAH in dogs, nine healthy adult male beagle dogs, 8–10 months old and weighing 9–10 kg, were anesthetized with pentobarbital (30 mg/kg body weight, intraperitoneal). Dehydromonocrotaline was prepared as previously described [18] and was dissolved in dimethylformamide (0.1 ml/kg) just before injection. Then a 5-F Swan-Ganz catheter (Cordis, Miami, FL) was advanced through the right jugular vein into the right atrium, the right ventricle and the pulmonary artery. Hemodynamic measurements were performed in recumbent position. Continuous hemodynamic monitoring included heart rate, pulmonary artery pressures (PAP), pulmonary capillary wedge pressure (PCWP), right ventricular pressure (VRm) and transcutaneous oxygen saturation. After baseline hemodynamic measurements were obtained, DHMC (3 mg/kg) was administered via the main pulmonary artery. Six weeks later, hemodynamic changes were evaluated according to the procedure as described above. All hemodynamic measurements and drug injections were done under sterile conditions. Nine dogs were treated with DHMC. Just before and 48 h and 6 weeks after the injection of DHMC, 20 ml vein blood and 2 ml artery blood were obtained from these dogs' femoral vessels and anticoagulated by heparin for EPCs culture and assessment or blood gas analysis. Animal care, housing, feeding and surgery were performed in accordance with the international guidelines and relevant national laws.

Flow cytometry analysis

For determining absolute counts of circulating EPCs per ml of peripheral blood, TruCOUNT^{TM} tubes (Becton Dickinson, Franklin Lakes, NJ) containing a certain amount of microbeads were used in flow cytometry analysis. According to Szmitko et al. [13, 14, 19], circulating EPCs were identified as CD133 +/KDR + cells. Briefly, 50 μl of well-mixed anticoagulated whole blood was incubated with PE-labeled mouse anti-dog KDR antibody (Becton Dickinson) and APC-labeled mouse anti-human CD133 antibody (Miltenyi Biotec, Krohne, Germany) in manufacturer-recommended concentrations just above the stainless steel retainer of the tube for 30 min at 4°C. Fluorescent isotype-matched antibodies (Becton Dickinson) were used as controls. After incubating the suspension with 450 μl FACS lysing solution (Becton Dickinson) for 15 min in the dark at room temperature, the samples were analyzed on a FACSCalibur Instrument (Becton Dickinson). Surface markers CD133 and KDR were determined on peripheral blood cells. The absolute counts of positive cells per ml blood were calculated using the following equation:

$$\frac{\text{count of events in region containing positive cells} \times \text{count of beads per test}^{*}}{\text{count of events in absolute count bead region} \times \text{test volume}} = \text{absolute count of cells}$$

*This value is remarked on the TruCOUNT™ tubes' foil pouch (ranges from 12,310 to 10,740).

Isolation, culture and characterization of circulating EPCs

EPCs were isolated, cultured and characterized according to previously described techniques [20]. Briefly, mononuclear cells (MNCs) were isolated from peripheral blood of dogs by Ficoll density gradient centrifugation and cultured on fibronectin (Biosciences, Franklin Lakes, NJ)-coated dishes in EC basal medium. The medium was supplemented with EBM-2 SingleQuotes (Clonetics, East Rutherford, NJ) containing 5% fetal bovine serum (FBS), human vascular endothelial growth factor 1 (VEGF-1), human fibroblast growth factor 2 (FGF-2), human epidermal growth factor (EGF), insulin-like growth factor I (IGF-I) and ascorbic acid. After 4 days in culture, non-adherent cells were removed by washing with phosphate-buffered saline (PBS), new medium was supplied, and the culture was maintained for a further 3 days. Then the adherent cells were incubated with DiLDL (Invitrogen, Carlsbad, CA) and stained with FITC-labeled Ulex europaeus agglutinin (UEA-1; Sigma, Bremen, Germany). The differentiating EPCs were identified as DiLDL/FITC-UEA-1 double-positive cells. Two or three independent investigators evaluated the number of EPCs per well by counting 15 randomly selected high-power fields (×200) with an inverted fluorescent microscope.

In vitro tubule-forming assay

In vitro tubule-forming assay was performed with the In Vitro Angiogenesis Assay Kit (Chemicon). The protocol was according to the manufacturer's instructions. Briefly, ECMatrix™ solution was thawed on ice overnight, then mixed with 10 × ECMatrix™ diluent and placed in a 96-well tissue culture plate at 37°C for 1 h to allow the matrix solution to solidify. Isolated EPCs were detached using 1 mmol/l EDTA in PBS (pH 7.4), harvested by centrifugation, resuspended in 500 μl liquid cell culture medium-M199 supplemented with 10 ng/ml VEGF and then counted and replated (10,000 cells per well) on top of the solidified matrix solution. Cells were incubated at 37°C for 24 h. Tubule formation was inspected under an inverted light microscope at ×200 magnification. Tubule formation was defined as a structure exhibiting a length more than four times its width [16]. Five independent fields were assessed for each well, and three wells were assessed for each group. The average number of tubules/×200 field was determined.

Senescence-associated β-galactosidase activity assay

Senescence-associated β-galactosidase (SA-β-gal) activity was measured with the β-Galactosidase Staining Kit (BioVision). The protocol was conducted according to the manufacturer's instructions. Briefly, after 7 days of culture, EPCs were washed in PBS, fixed for 10–15 min at room temperature with 0.5 ml of fixative solution, washed and incubated overnight at 37°C with the staining solution mix. Cells were observed under a microscope for development of blue color (×200 total magnification) [21].

Histological analysis

The fixed lungs were paraffin embedded *en face* and then sectioned. The tissues were stained with Masson triple stain, by which the vascular smooth muscle is stained pink, the fibril tissues green and the nucleus brown as stained by hematoxylin. Then the vessels in the lung tissues were observed under a light microscope (×40 magnification). We analyzed the medial wall thickness of the pulmonary arterioles in the middle region of the right lung (20 muscular arteries/dog, ranging in external diameter from 75 to 150 μm). The medial wall thickness was expressed as follows: %wall thickness = [(medial thickness in long axis + medial thickness in short axis)/(external diameter in long axis + external diameter in short axis)] × 100. Representative sections were photographed.

Statistical analysis

All data are presented as mean ± SD. Differences of means between baseline and pulmonary hypertension status were assessed by paired *t* test. Differences of means between baseline and 48 h and 6 weeks after DHMC injection were assessed by one-way ANOVA followed by Tukey test for post-hoc multiple comparisons. Pearson's test was performed for correlation analysis. Values of $p < 0.05$ were considered significant. All statistical analyses were performed with SPSS 13.0.

Results

Hemodynamics, arterial oxygen pressure and histology

Six weeks after DHMC administration, right heart catheterization showed significant pulmonary hypertension in hemodynamics in treated dogs (mean PAP 20.2 ± 1.64 vs. 11.3 ± 2.0 mmHg, $p < 0.05$; RVm 14.1 ± 3.2 vs. 23.9 ± 3.9 mmHg, $p < 0.05$; Fig. 1a, b). During the 6 weeks, treated dogs suffered from continuous hypoxia, which was

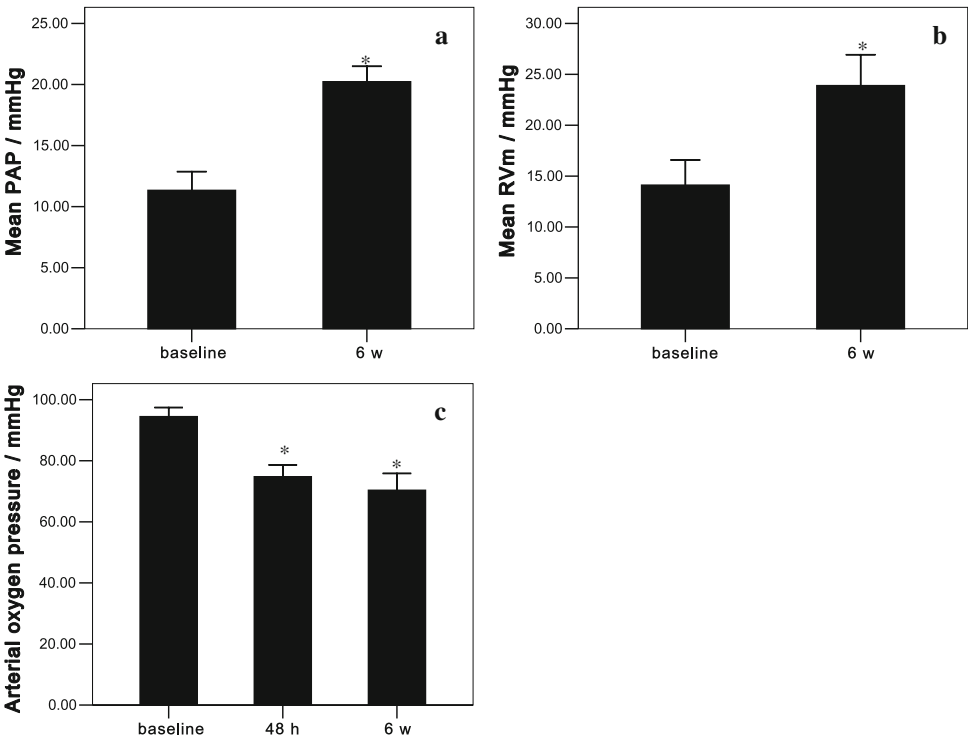

Fig. 1 The quantitative comparison of mean PAP (**a**) and RVm (**b**) before and after DHMC administration. **c** The DHMC-treated dogs suffered from continuous hypoxemia. However, there was no significant difference in the PaO_2 between 48 h and 6 weeks after DHMC. Data are mean ± SD. *$p < 0.05$ versus baseline. $N = 9$

indicated by significantly low PaO_2 (74.8 ± 5.0 mmHg for 48 h after DHMC vs. 95.94 ± 1.43 mmHg for baseline, $p < 0.01$; 70.3 ± 7.2 mmHg for 6 weeks after DHMC vs. 95.94 ± 1.43 mmHg for baseline, $p < 0.01$; Fig 1c).

Furthermore, the PAH was confirmed by histological analysis on the lung tissues from DHMC-treated dogs as well as adult male body weight-matched healthy ones. As shown in Fig. 2, the inter-acinar pulmonary arterioles (diameters ≤100 μm) of treated dogs suffered from muscular remodeling 6 weeks after DHMC, and the medial hypertrophy led to increased medial wall thickness (21.75 ± 1.2 vs. 37.4 ± 1.4%, $p < 0.05$; Fig. 2c).

EPCs levels

The number of circulating EPCs in peripheral blood was assayed via fluorescence-activated cell sorter analysis (Fig. 3a, b). A rapid and impressive increase was observed in circulating EPCs 48 h after DHMC injection (778.0 ± 93.3 vs. 632.8 ± 42.8 cells/ml blood, $p < 0.01$; Fig. 3c). However, the circulating EPCs were significantly reduced 6 weeks later, when PAH had been well established (206.1 ± 26.8 vs. 632.8 ± 42.8 cells/ml blood, $p < 0.01$; Fig. 3c). In addition, EPCs were expanded from the peripheral blood in vitro and identified by DiLDL uptake and lectin staining. EPC numbers raised 48 h after DHMC (57.7 ± 10.4 vs. 40.5 ± 9.6 EPCs/×200 field, $p = 0.03$; Fig. 3d) and decreased 6 weeks later (22.1 ± 9.4 vs. 40.5 ± 9.6 EPCs/×200 field, $p = 0.02$; Fig. 3d) when compared with the baseline level in vitro cultivation.

EPCs vasculogenesis in vitro

In vitro tubule-forming assay was used to investigate the ability of EPCs to participate in neovascularization. We analyzed the vasculogenesis capacity of isolated EPCs from nine beagles before and 48 h and 6 weeks after the treatment of DHMC. Forty-eight hours after DHMC injection, beagles' EPCs formed more quantitative tubules than that of baseline (26.6 ± 5.3 vs. 21.1 ± 2.8 tubules/×200 field, $p < 0.05$; Fig. 4b). But 6 weeks later, the tubules formed by beagles' EPCs were much more sparse (11.2 ± 2.8 tubules/×200 field) compared with that at previous time points.

EPCs senescence

Six weeks after DHMC, PAH was associated with an increase in SA-β-gal-positive EPCs, (42.1 ± 5.6% for 6 weeks vs. 28.9 ± 4.7% for baseline, $p < 0.01$, Fig. 5). However, an inhibitory effect on EPCs senescence has been observed at 48 h after DHMC injection (21.1 ± 3.8% for 48 h vs. 28.9 ± 4.7% for baseline, $p < 0.01$, Fig. 5), suggesting that during the early stage of pulmonary arterial endothelial injury, circulating EPCs are activated. Pearson's analysis revealed that the percentage of senescent

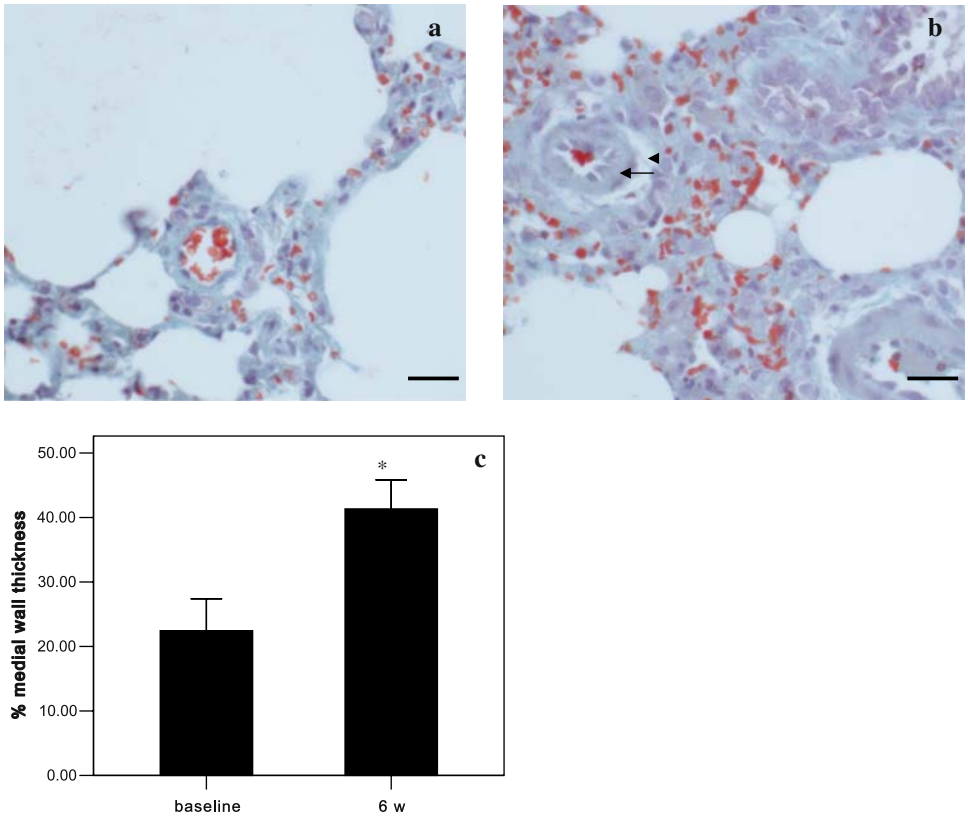

Fig. 2 Representative histological photomicrographs of inter-acinar pulmonary arterioles (diameter ≤100 μm) from 10-month-old male beagles (20 arterioles/dog were assessed). **a** A small interalveolar artery from a normal beagle's lung. The lumen of the arteries is surrounded by thin vessel wall without evident medium and adventicium. **b** A muscularized interalveolar artery in a treated beagle's lung. Medial (*arrow*) hypertrophy and adventitial (*arrowhead*) fibrosis were observed. **c** Quantitative analysis of pulmonary arteriole medial wall thickness before and 6 weeks after DHMC. Data are mean ± SD. $*p < 0.05$ versus baseline. (*Scale bars* 50 μm)

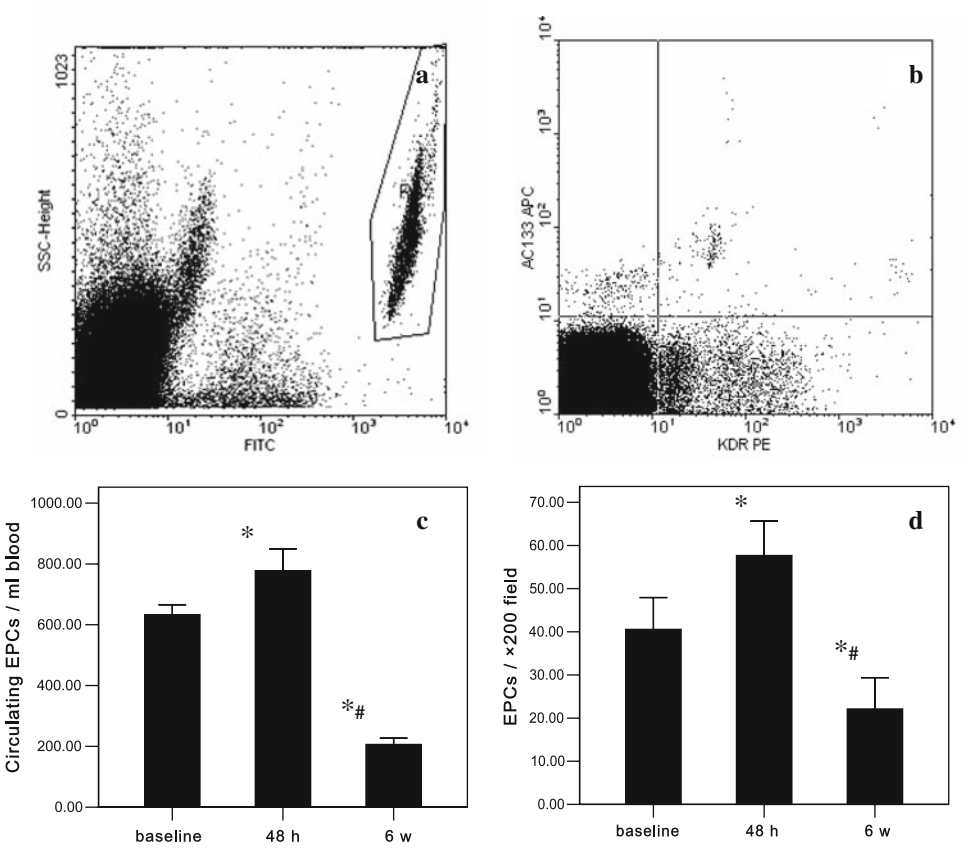

Fig. 3 CD133/KDR-positive cells were detected in peripheral blood and identified as circulating EPCs by flow cytometry using counting beads tubes. **a** Gated and counted beads from a fluorescein isothiocyanate (FITC) versus side-scattering corner (SSC) dot plot. Quadrants were set on the basis of isotype-identical controls. **b** Dual-positive CD133/KDR cells were identified and numerated in the upper right quadrant of the scatter plot from blood of dogs. The representative dot plots are represented. The absolute counts of positive cells per ml blood were calculated using the equation described in "Methods". **c** Comparison of circulating EPCs number before and after PAH. **d** Differentiated EPCs were significantly reduced in PAH but transiently raised at the early stage after DHMC. Data are mean ± SD. $*p < 0.05$ versus baseline, $^{\#}p < 0.05$ versus 48 h

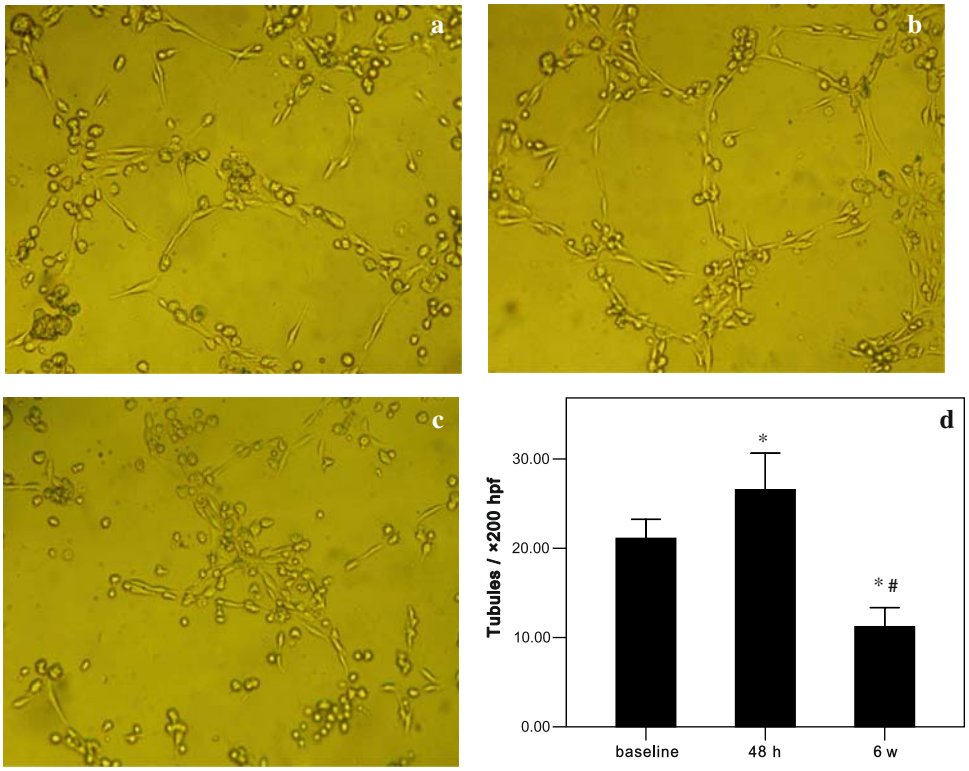

Fig. 4 **a–c** Twenty-four hours after being replated on the top of the solidified matrix, EPCs from beagles formed tubules, which were defined as a structure exhibiting a length four times more than its width. Representative micrographs of tubule formation of EPCs derived from beagles at baseline, 48 h and 6 weeks after DHMC treatment, respectively, are shown. **b** Quantitative comparison of the numbers of the tubules per ×200 field among baseline, 48 h and 6 weeks after DHMC. Data are mean ± SD. $^{*}p < 0.05$ versus baseline, $^{\#}p < 0.05$ versus 48 h

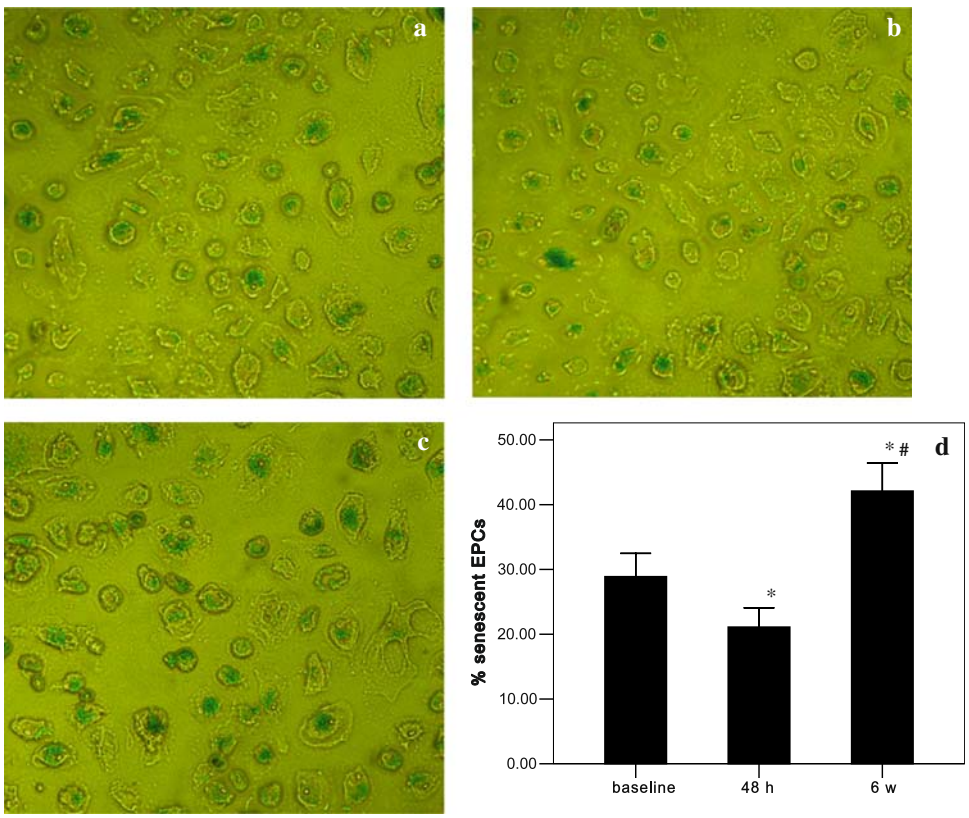

Fig. 5 **a–c** Representative micrographs of senescence-associated β-galactosidase (SA-β-gal)-positive cells (senescent cells) in EPCs derived from beagles at baseline, 48 h and 6 weeks after DHMC treatment, respectively, are shown. **d** The number of *blue* cells was counted manually from a total of 200 cells. Data are mean ± SD. $^{*}p < 0.05$ versus baseline, $^{\#}p < 0.05$ versus 48 h

EPCs was negatively correlated with either circulating EPC number ($r = 0.699$, $p = 0.036$) or EPCs vasculogenesis capacity ($r = 0.744$, $p = 0.022$).

Discussion

To the best of our knowledge, this is the first before-after self-controlled study on the number and functional capacity of circulating EPCs from PAH. We used an induced-PAH beagle model by intra-pulmonary arterial DHMC injection and confirmed the PAH establishment by catheterization, blood gas analysis and histological examination. We found that 6 weeks after DHMC administration, when PAH has been well established, circulating EPCs from beagles exhibited reduction, and their in vitro vasculogenesis activity was impaired. However, at the early acute stage of pulmonary artery injury (48 h after DHMC administration), the EPC number and functional capacity were raised, which suggested the increased mobilization from bone marrow. More importantly, we found that all these variations were contradictory to cellular senescence.

A canine PAH model achieved by a single injection of artificially synthesized DHMC is among the most commonly used animal models of experimental PAH. It develops not only moderate PAH in hemodynamics, but also pulmonary vascular histological changes similar to those of human idiopathic PAH, such as medial hypertrophy and adventitial thickening [18, 22, 23]. Meanwhile, by employing the large-sized animal model, it is possible to perform catheterization repeatedly to monitor the change of hemodynamics. For reference, the mPAP in normal dogs was 13.4 ± 5.4 mmHg [24] and increased to 22.5 ± 8.1 mmHg 6 weeks after DHMC treatment [25]. Our data were consistent with them. Additionally, in the present study, the blood gas analysis after DHMC showed continuous hypoxemia, and the histology examination on lung tissue revealed muscularization and occlusion remodeling of pulmonary arterioles, which are both common in human PAH. It suggests that we induced PAH by this approach successfully.

Regardless of PAH etiology, endothelial dysfunction underlies PAH [10]. Lopes and his colleagues [26–28] found that the plasma levels of von Willebrand factor, a marker of endothelial perturbation, were exceedingly high in idiopathic PAH patients, and the short-term survival of PAH is related to the severity of endothelial cell dysfunction. Endothelial dysfunction could result in an imbalance between vasodilators and vasoconstrictors, which has a key role in the progression of PAH [11]. EPCs play an important role in endothelial repair [10–15]. So the reduction and functional impairment of EPCs may have critical sense in PAH.

The mechanisms mediating the depletion of circulating EPCs observed in this study remain to be clarified. Some may question that whether it was caused by the direct toxicity of DHMC to the bone marrow. In fact, due to rapid hydrolysis in aqueous environment, the half-time of DHMC in the bloodstream is less than 14 s [29]. Thus, the effects of DHMC are mainly limited to the pulmonary vasculature. Moreover, our data showed that as early as 48 h after DHMC injection, the blood PaO_2 had declined, but the number of circulating EPCs and their tubule-forming capacity were elevated, which indicated the increased mobilization of EPCs from bone marrow in response to the acute DHMC-induced injury to pulmonary vessels and short-term hypoxia. However, in the chronic or subacute stage, the continuous endothelial damage and subsequent repair will eventually deplete the presumed finite precursors pool. Fadini et al. [30] have also reported the reduction of circulating EPCs in patients with chronic lung disease and long-lasting hypoxia. So it is reasonable to consider the decrease of circulating EPCs as a consequence of long-term exhaustion and hypoxia.

In addition, we found that 6 weeks after DHMC, the EPCs decrease and functional impairment were correlated with cellular senescence. Cellular senescence is characterized by cell-cycle arrest and determined by the length of the telomere. Along with cellular division, the telomere is shortening gradually. When its length reduces to some certain extent, the cells stop dividing and become senescent. Studies have demonstrated that EPC senescence was associated with EPC numbers and activity [31]. So it helps explain the EPC decrease and functional impairment in that when exposed to long-term stimulation, the bone marrow-derived EPCs may undergo frequent cellular division and then finally become senescent. The senescent EPCs may have reduced adherence to fibronectin as well as reduced response to VEGF, which may result in reduced vasculogenesis capacity together. Furthermore, given the protective roles the VEGF plays for the endothelium, the reduced response of senescent EPCs to VEGF may help interpret the paradox of elevated serum levels of VEGF in idiopathic PAH patients [32]. Referring to the impaired response of EPCs to VEGF, the downregulation of VEGF receptor on EPCs surface, defects in the downstream of signaling pathway and/or dysregulated integrin synthesis may be involved, and this needs further study [33, 34].

Recently, autologous EPC transplantation has been reported to benefit idiopathic PAH patients modestly in hemodynamics and labor capacity [35]. Although genetically transduced EPCs have been shown to be more efficient in preventing or reversing PAH by animal studies before [16, 17], the intense safety concern will limit its clinic application. Meanwhile, on account of potential immune rejection, heterologous EPC transplantation will

also be considered less acceptable. So the autologous manner ought to be the most practical and feasible for EPC therapy, and the patients' EPC number and functional capacity will become a major bottleneck for improving the therapeutic efficacy. Given the close link between senescence and EPC function, reducing EPC senescence through the in vitro expansion and manipulation procedures before delivery may help improve the therapy outcome [31].

Taken together, this before–after controlled study demonstrates that after the acute stage of DHMC injury to pulmonary vessels, the EPCs from PAH beagles suffered from exhaustion and senescence. Our data give potential insight into the pathophysiological mechanisms of EPCs depletion and indicate the necessity of an intervention procedure before delivery to reduce the cellular senescence.

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