

Original Article

Correlation between congenital heart disease complicated with pulmonary artery hypertension and circulating endothelial cells as well as endothelin-1

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Abstract: Objective: To investigate changes in the level of circulating endothelial cells (CECs) and endothelin-1 (ET-1) in peripheral venous blood of the patients with congenital heart disease (CHD) complicated with pulmonary artery hypertension (PAH), and research on their effects in the onset and progress of CHD complicated with PAH. Methods: A case-control study including 30 cases of healthy controls, 15 cases of left-to-right shunt CHD without PAH, 26 cases of CHD complicated with mild PAH, and 17 cases of CHD complicated with moderate-severe PAH was performed. We used flow cytometry to measure the percentage of CECs accounting for nucleated cells in whole blood, and enzyme linked immunosorbent assay (ELISA) to measure the level of ET-1 in serum. The differences of above-mentioned biomarkers between different groups were compared. Results: (1) The level of CECs and ET-1 in the group of moderate-severe PAH was significantly higher than those in the group of mild PAH and the group of CHD without PAH. Significant difference was also observed between the level of CECs and ET-1 in the group of mild PAH and those in the group of CHD without PAH and the control group. Meanwhile, the level of CECs and ET-1 in the group of large shunt was significantly higher than those in the group few shunt and few-medium shunt. (2) Strong positive correlations were observed between pulmonary artery systolic pressure and percentage of CECs as well as ET-1 production. Mean pulmonary artery pressure also positively correlated with percentage of CECs as well as ET-1 production. (3) Arterial partial pressure of oxygen as well as arterial oxygen saturation negatively correlated with the level of CECs, whereas the volume of left-to-right shunt positively correlated with the level of ET-1. (4) The level of CECs and ET-1 were positively correlated as well in CHD patients. Conclusions: CHD complicated with PAH is associated with increased CEC counts and ET-1 production. This study suggests that CECs and ET-1 could be used as clinical biomarkers to define medical strategies for control of PAH.

Keywords: Congenital heart disease, pulmonary artery hypertension, circulating endothelial cells, endothelin-1

Introduction

Congenital heart disease (CHD) is the most common congenital cardiovascular anomaly. Commonly encountered CHD with left-to-right shunt in the clinic include atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus (PDA) and so on. Pulmonary arterial hypertension (PAH) is a complex and rapidly progressive disorder [1]. Though PAH is a common complication of left-to-right shunts, the pathogenesis of CHD complicated with PAH is not yet fully elucidated.

It is commonly believed that the structural, functional and metabolic change of endothelial

cell (ECs) is an important pathological feature of the pulmonary vessels of patients with PAH. ECs dysfunction has been identified to play a decisive role in mediating the structural changes in the pulmonary vasculature and in pathogenesis of PAH [2]. Circulating endothelial cells (CECs), a novel marker of endothelial damage, are vascular ECs detected in the peripheral blood of body under physiological and pathological conditions [3]. These cells are characterized by an expression of specific markers and proteins, such as vascular cell adhesion molecule-1, E-selectin, and P-selectin, which may herald increased coagulability, proliferation, and vasoconstriction. The enumeration of CEC

may provide useful information in the monitoring of endothelial injury and reflect the degree of severity of the insults toward the endothelium.

Endothelin-1 (ET-1), a 21-amino acid peptide originally isolated from the supernatants of cultured porcine aortic endothelial cells, is considered to be one of the most potent and long-acting vasoconstrictors known [4]. Although produced mainly by ECs, ET-1 is also produced by other cell types, including vascular smooth muscle cell, fibroblasts and inflammatory cells [5, 6]. Several studies have indicated that ET-1 could cause vasoconstriction and vascular remodeling [7].

In this study, we hypothesized that CHD complicated with PAH was correlated with CECs as well as ET-1. Thus, we investigated the level of ET-1 and CECs to explore their effects in CHD complicated with PAH and explored their correlation with patients' haemodynamic characteristics. The aim of this study was to look for noninvasive and reliable biomarkers for clinical assessment of pulmonary artery pressure, and provide a theoretical basis for clinical diagnosis and therapy of CHD patients with PAH.

Patients and methods

Study population

The present study was approved by the ethics committee at Affiliated Hospital of Nantong University (Nantong, China), and signed informed consent was obtained from all patients and control subjects. Fifty-eight consecutive patients with left-to-right shunt CHD (19 males and 39 females, age range, 1-72 years, median age, 34 ± 19 years) who underwent cardiac catheterization at Department of cardiovascular medicine and surgery, Affiliated Hospital of Nantong University were obtained and enrolled with their parents' consent between February 2014 and February 2015. Thirty cases of healthy people without history of heart disease and other serious diseases and with other normal physiological indexes (10 males and 20 females, age range, 23-77 years, median age, 49 ± 17 years) were studied as control patients. Pulmonary artery pressure of fifty-eight patients under routine cardiac catheterization was measured. They were separated into 3 groups according to their PASP. 15

patients had left-to-right shunt CHD without PAH, 26 had CHD complicated with mild PAH, and 17 still had CHD patients complicated with moderate-severe PAH.

Cases in a specific group were decided on the basis of the task force for the diagnosis and treatment of pulmonary hypertension released by the European Society of Cardiology (ESC) and the European Respiratory Society (ERS) [8]. PAH was defined as mean pulmonary arterial pressure (PAPm) ≥ 25 mmHg at rest as assessed by right heart catheterization. PAPm of 15 patients had left-to-right shunt CHD without PAH was ≤ 25 mmHg, 26 had CHD complicated with mild PAH 26~35 mmHg, and 17 still had CHD patients complicated with moderate-severe PAH ≥ 36 mmHg.

Judgement of the pulmonary left-to-right shunt volume (Q_s/Q_t) and criteria for grouping: shunt volume was calculated using the pulmonary to systemic flow ratio (Q_p/Q_s). $1 < Q_p/Q_s < 1.5$ was defined as few shunt, $1.5 \leq Q_p/Q_s < 2$ medium shunt, $Q_p/Q_s \geq 2$ large shunt.

Exclusion criteria for case and control groups: Complicated by acute infection and inflammation; Complicated by coronary heart disease, cardiomyopathy, high blood pressure and other heart diseases; Complicated by respiratory system disease, liver disease, kidney disease, diabetes, cerebrovascular disease, tumor, autoimmune disease, Down syndrome; Complicated by PAH caused by other factors; Has a major surgery recently or a history of trauma.

Flow cytometric quantification of CECs

4 ml venous blood of all objects with an empty stomach was collected in the morning. Half of the venous blood was put in disposable normal serum tube and centrifuged in a low-speed centrifuge for 10 min after coagulation. Supernatant was aspirate and saved standby at -80°C . Remaining 2 ml venous blood was put in EDTA-anticoagulant tube for flow cytometric quantification of CECs.

CECs in peripheral venous blood of the patients with CHD were quantified by means of flow cytometry. Briefly, blood sample was incubated with FITC-labeled anti-human CD3 monoclonal antibody and PE-labeled anti-human-CD31 monoclonal antibody purchased from BioLegend (BioLegend, San Diego, USA). 10000 cells were collected from each sample to sepa-

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Table 1. Clinical and haemodynamic data of different PAH groups

Parameter	Without PAH	Mild PAH	Moderate-severe PAH	Control
Gender (female)	15 (8)	26 (17)	17 (14)	30 (10)
Age (year)	17±14	38±19	43±18	49±17
Height (cm)	139.0±32.1	156.4±23.0	154.2±12.3	155.9±16.3
Weight (kg)	42.4±22.0	58.7±16.9	55.8±16.1	57.4±15.8
Hb concentration (g/L)	126.20±16.93	135.08±16.74	133.24±17.68	-
PaO ₂ (mmHg)	224.46±169.02	117.43±62.60	103.13±48.87	-
SaO ₂ (%)	96.48±7.42	97.48±1.65	97.01±2.94	-
Arterial PH	7.40±0.05	7.41±0.05	7.42±0.04	-
PASP (mmHg)	28.17±1.94	41.27±5.20	65.70±18.01	-
mPAP (mmHg)	17.40±3.58	24.40±4.86	39.50±13.57	-

rate CD31 and CD3 from nucleated cells in whole blood based on the forward scatter (FSC). The number of positive peripheral blood cells was counted within the endotheliocyte gate was determined with non-immune monoclonal antibodies as negative controls to set appropriate regions, which possess the same isotype and the same fluorochrome as the immune monoclonal antibodies provided by the same manufacturer.

Measurement of ET-1

Endothelin-1 secretion was measured in supernatants collected from venous blood in serum-free medium by using an enzyme-linked immunosorbent assay (ELISA) kit (HCB, Ontario, Canada) as recommended by the manufacturer's directions.

Statistical analysis

Data were analyzed using SPSS version 16.0 (WSPSS Ltd., Surrey, UK) and graphs were generated using GraphPad Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). All measurement data fitted normal distribution are presented as mean ± SD. Comparisons between groups were assessed using the unpaired *t* test analysis. Correlation between two variables was analyzed using Spearman's correlation coefficient. Statistical significance was assumed at *P*<0.05.

Results

Clinical data of the study population

The patients' and controls' clinical and haemodynamic characteristics are summarized in **Table 1**.

Percentage of CECs in different groups

The level of CECs was presented as the percentage of CD31 and CD3 positive peripheral blood cells accounting for nucleated cells in whole blood quantified by flow cytometry. Results revealed that percentage of CECs was in the group of moderate-severe PAH was significantly higher than those in the group of mild PAH and the group of CHD without PAH (**Figure 1A**, *P*<0.05). Significant difference was also observed between the level of CECs in the group of mild PAH and those in the group of CHD without PAH and the control group (*P*<0.05), but percentage of CECs in the group of CHD without PAH was not different from those in the control group.

On the other hand, the level of CECs in the group of large shunt was significantly higher than those in the group of few shunt and few-medium shunt but not different from those in the group of medium shunt (**Figure 1B, 1C**, *P*<0.05). Moreover, there was also no difference between the percentage of CECs in the group of few shunt and those in the control group.

Linear-regression analysis of parameters and percentage of CECs

Correlation between the parameters measured under routine cardiac catheterization and percentage of CECs in CHD patients was further studied. In the present study we found a strong positive correlations between pulmonary artery systolic pressure as well as mean pulmonary artery pressure and the level of CECs (*r*=0.8365, *P*<0.0001) (*r*=0.7448, *P*<0.0001) (**Figure 2A, 2B**), whereas the level of CECs negatively cor-

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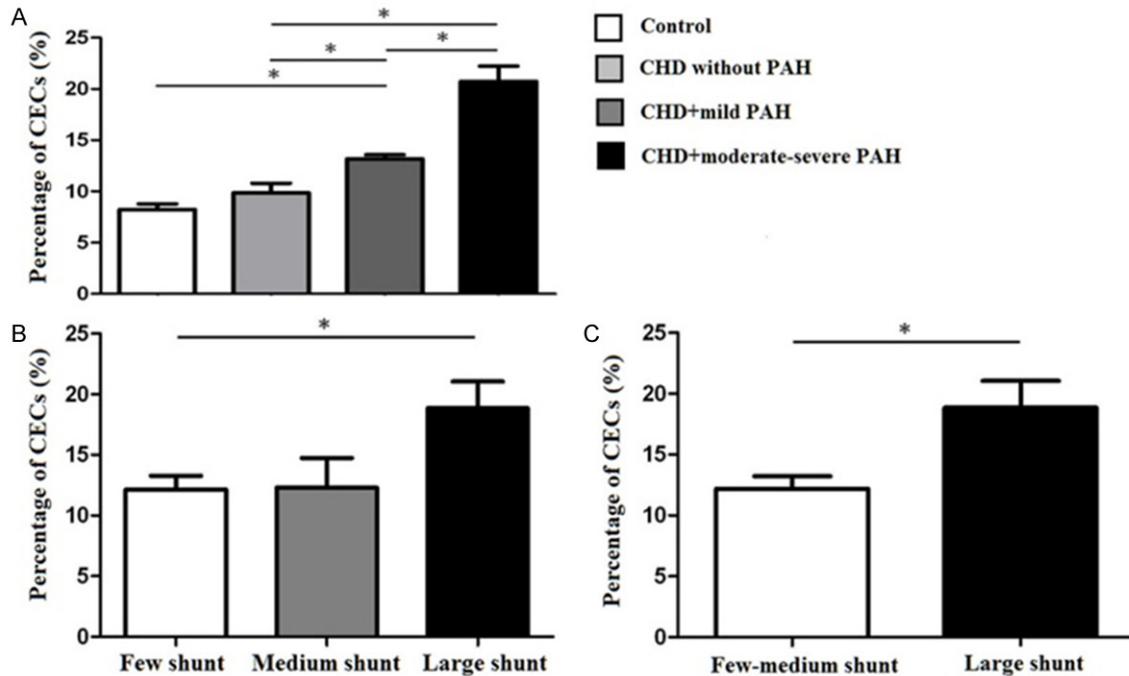


Figure 1. The level of CECs in peripheral venous blood of CHD patients. Percentage of CECs was determined by the percentage of CD31 and CD3 positive peripheral blood cells accounting for nucleated cells in whole blood quantified by flow cytometry. The level of CECs measured in the group of left-to-right shunt CHD without PAH, the group of CHD complicated with mild PAH, and the group of CHD complicated with moderate-severe PAH (A). Percentage of CECs quantified in the group of few shunt, medium shunt, large shunt, and few-medium shunt (B, C), * $P < 0.05$.

related with arterial partial pressure of oxygen as well as arterial oxygen saturation ($r = -0.3406$, $P < 0.05$) ($r = -0.4900$, $P < 0.01$) (**Figure 2C, 2D**).

ET-1 production between different groups

ET-1 has been considered as a potent vasoconstrictive peptide that shows a widespread tissue distribution and many actions. To further explore the fluctuation ET-1 secretion in CHD with or without PAH and control patients, we used the ELISA kit to quantify its production in supernatants from venous blood. Data present demonstrated that ET-1 production in the group of moderate-severe PAH was significantly higher than those in the group of mild PAH and the group of CHD without PAH (**Figure 3A**, $P < 0.05$). Meanwhile, secretion of ET-1 in the group of mild PAH was also significantly higher than those in the group of CHD without PAH and the control group ($P < 0.05$). However, no significant difference between ET-1 production in the group of CHD without PAH and those in the control group was observed.

Interestingly, the change of ET-1 secretion and CECs level in venous blood with various shunt volume is in the same tendency. Briefly, ET-1 production in the group of large shunt was significantly higher than those in the group of few shunt and few-medium shunt but not different from those in the group of medium shunt (**Figure 3B, 3C**, $P < 0.05$). Similarly, no significant difference between the percentage of CECs in the group of few shunt and those in the control group was presented.

Linear-regression analysis of parameters and ET-1 production

To extend above data, we determined correlations between the parameters measured under routine cardiac catheterization and ET-1 production in CHD patients. As illustrated in **Figure 4A** and **4B**, ET-1 production displayed significant positive correlations with pulmonary artery systolic pressure as well as mean pulmonary artery pressure ($r = 0.8563$, $P < 0.0001$) ($r = 0.8619$, $P < 0.0001$). Results also demonstrated that the volume of left-to-right shunt positively correlated with the level of ET-1

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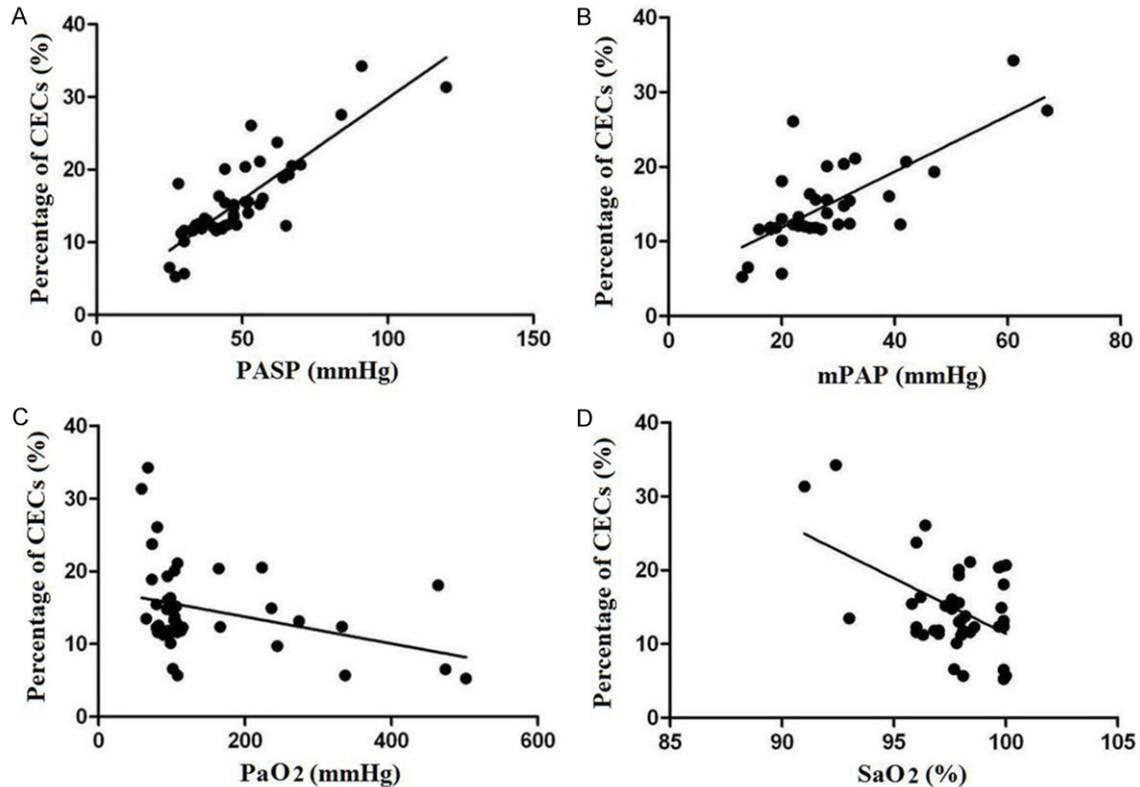


Figure 2. Correlation analysis between parameters and percentage of CECs. Scatter diagram of percentage of CECs and pulmonary artery systolic pressure with regression equation the symbol (A), mean pulmonary artery pressure with regression equation $\hat{y}=4.378+0.3746x$ (B), arterial partial pressure of oxygen with regression equation $\hat{y}=17.45-0.01848x$ (C) as well as arterial oxygen saturation with regression equation $\hat{y}=161.8-1.504x$ (D). \hat{y} : percentage of CECs.

($r=0.4612$, $P<0.05$) (**Figure 4C**). Correlation between CECs and ET-1 in CHD patients was further studied. Results revealed that the level of CECs and ET-1 were positively correlated as well ($r=0.7378$, $P<0.0001$) (**Figure 4D**).

Discussion

Pulmonary arterial hypertension (PAH) is defined as a group of diseases often characterized by a progressive and sustained increase in pulmonary vascular resistance that eventually may lead to right ventricular (RV) failure. In China, CHD is one of the most common causes of PAH. The pathogenesis of CHD complicated with PAH is very sophisticated that has currently been considered associating with elevated levels of pulmonary blood flow and pulmonary vascular resistance. Pulmonary vascular ECs have been thought to play a central role in the pathogenesis of CHD with PAH.

CECs is the one and only noninvasive, direct and specific biomarker of vascular damage,

remodeling, and dysfunction in lung biopsy at present [9]. Cardiovascular disease, endothelial injury, as well as exposure to stress and lack of oxygen could induce significantly accelerated vascular ECs apoptosis. When a large number of exfoliated vascular ECs flowed into blood circulation, the amount of CECs increased significantly. In this study, we found that percentage of CECs was in the group of moderate-severe PAH was significantly higher than those in the group of mild PAH and the group of CHD without PAH (**Figure 1A**). Moreover, the level of CECs was strongly positive correlated with pulmonary artery systolic pressure as well as mean pulmonary artery pressure and (**Figure 2A, 2B**). These results indicated that the elevation of pulmonary arterial pressure (PAP) could enhance pulmonary artery wall shear stress, especially circumferential wall tension, and pulsatile stretch, both of which accelerated exfoliation of vascular ECs from the blood vessels wall. Moreover, the elevation of left-to-right shunt volume kept the relationship between

CECs and ET-1 in CHD complicated with PAH

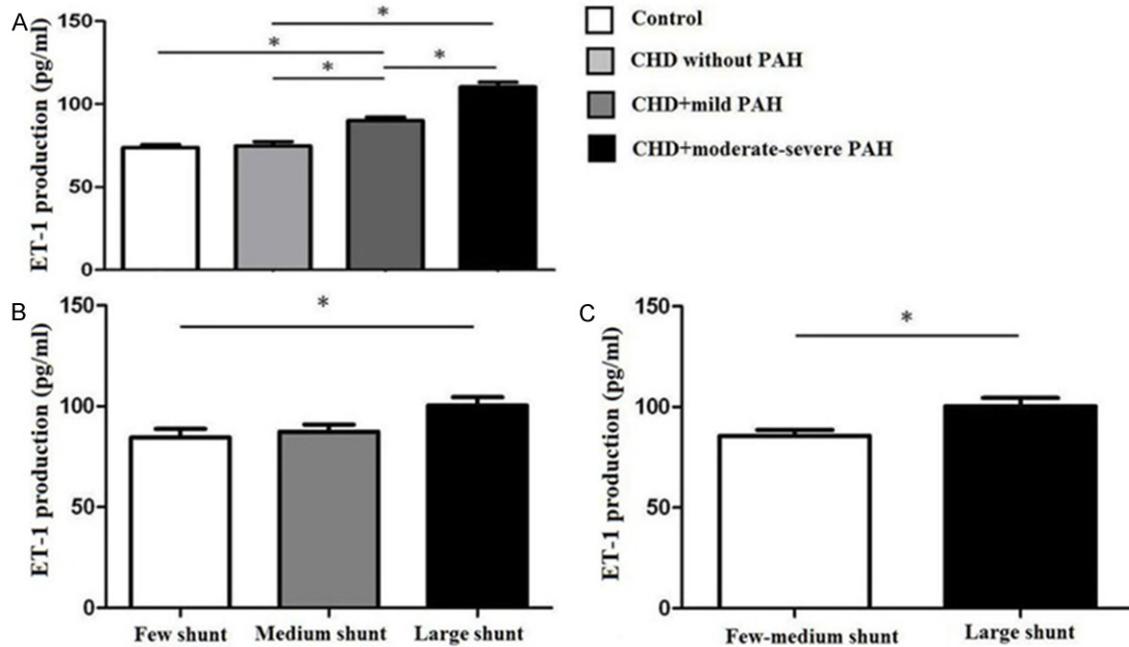


Figure 3. The level of ET-1 in peripheral venous blood of CHD patients. ET-1 production was measured in supernatants collected from venous blood by using an ELISA kit. A. The level of ET-1 secreted in venous blood measured in the group of left-to-right shunt CHD without PAH, the group of CHD complicated with mild PAH, and the group of CHD complicated with moderate-severe PAH. B, C. ET-1 secretion quantified in the group of few shunt, medium shunt, large shunt, and few-medium shunt, * $P < 0.05$.

PAP and the number of CECs. Increase in shunt volume might enlarge pulmonary blood flow in pulmonary circulation, which then markedly increased PAP and the level of vascular ECs shed from the blood vessels wall (namely CECs). Shunt volume was calculated using the pulmonary to systemic flow ratio (Q_p/Q_s). Data in the present study demonstrated that percentage of CECs in the group of large shunt was significantly higher than those in the group of few shunt and few-medium shunt but not different from those in the group of medium shunt (Figure 1B, 1C). Our data support the concept that enhanced shunt volume could increase PAP and percentage of CECs. We further carried out Linear-regression analysis of PaO_2 as well as SaO_2 and percentage of CECs, results showed that the level of CECs negatively correlated with arterial partial pressure of oxygen as well as arterial oxygen saturation, that is, percentage of CECs increased with decrease of PaO_2 and SaO_2 (Figure 2C, 2D). Results displayed that though PaO_2 and SaO_2 were within normal limits in the case groups, there was still a decline of PaO_2 and SaO_2 because of the left-to-right shunt. Meanwhile, percentage of ECs accounting for nucleated cells in whole blood

also increased to varying degrees. Furthermore, the more PaO_2 and SaO_2 decreased, the more percentage of CECs increased. So we speculated that pulmonary vascular spasm and varying degree of pulmonary vascular remodeling could be observed in keeping with increase of PAP, which on one hand resulted in disturbances in gas diffusion between pulmonary microvascular and alveoli, and on the other hand, increased ventilation/blood flow ratio due to insufficient blood flow in parts of the alveoli. It is worth noting that disorders of Oxygenation might lead to different level of decline in PaO_2 and SaO_2 . Subsequently, increase of vascular ECs apoptosis induced by hypoxia contributed to the elevated levels of CECs [10].

Elevated levels of shunt blood flow increases shear stress, and increased shear stress can be postulated as a consequence of pulmonary vasoconstriction or vascular remodeling. Indeed, endothelial dysfunction induced by high fluid shear stress may lead to impaired ECs apoptosis and antiapoptotic signaling from perivascular inflammatory cells and then activated pulmonary ECs apoptosis in left-to-right CHD [11]. Breakdown of the endothelial barrier

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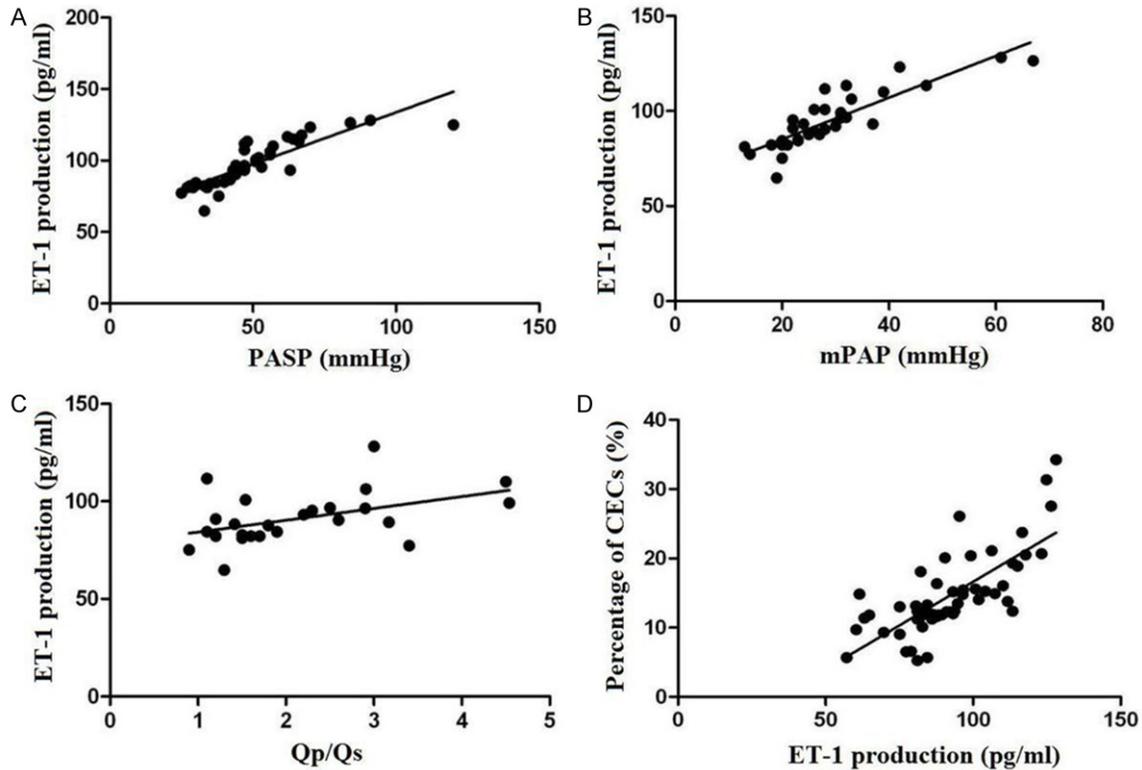


Figure 4. Correlation analysis between parameters and ET-1 production. Scatter diagrams of ET-1 production and pulmonary artery systolic pressure with regression equation $y = 61.09 + 0.7256x$ (A), mean pulmonary artery pressure with regression equation $y = 63.19 + 1.096x$ (B), the volume of left-to-right shunt with regression equation $y = 78.21 + 6.063x$ (C) as well as the level of CECs with regression equation $y = -8.616 + 0.2525x$ (D). y : ET-1 production in (A-C). y : percentage of CECs.

function resulting from pulmonary ECs apoptosis could give rise to direct contact of vascular smooth muscle with VEGF, which was followed by pulmonary artery remodeling [12].

As to the reasons for the increased number of vascular ECs in blood circulation, the opinion that high fluid shear stress leads to vascular endothelial dysfunction was widely accepted. However, some other scholars believed that vascular endothelial growth factor (VEGF) markedly increased in vascular ECs could promote release of endothelial progenitor cells (EPCs) from bone marrow to blood circulation. It is noteworthy that CECs may be derived from the endothelial progenitor cells obtained from the mobilization of bone marrow by growth factors such as VEGF. And EPCs have thought to be a sub-population of CECs and enhance CECs counts [13].

ET-1 is considered a highly potent endogenous vasoconstrictor that may contribute to vascular

and tissue remodeling and reflects functional status of vascular ECs. Effects of ET-1 on extracellular matrix production [14], fibroblast proliferation [15], angiogenesis and inflammation [16] may contribute to the pathogenesis of PAH.

In the present study, when comparing the level of ET-1 in different groups, we found that ET-1 production in the group of moderate-severe PAH was significantly higher than those in the group of mild PAH and the group of CHD without PAH. Meanwhile, secretion of ET-1 in the group of mild PAH was also significantly higher than those in the group of CHD without PAH and the control group (**Figure 3A**). This affirmed that ET-1 caused greater vasoconstriction in hypertensive pulmonary arteries which could result in elevated levels of PAP. Results also demonstrated that both pulmonary artery systolic pressure and mean pulmonary artery pressure were positively correlated with ET-1 production (**Figure 4A, 4B**). The common view is emerging

that ET-1 production actively secreted by pulmonary ECs increases evidently thanks to pulmonary arterial dilation caused by blood shunting in left to right CHD. To confirm this opinion, relationships between left-to-right shunt volume and ET-1 production were studied. As shown in **Figure 3B** and **3C**, ET-1 production in the group of large shunt was significantly higher than that in the group of few shunt and few-medium shunt. Linear-regression analysis of Qp/Qs and ET-1 production further supported the concept that larger shunt volume contributed to obvious pulmonary arterial dilation, and then led to more ET-1 secretion.

Vascular adventitial fibroblasts are able to synthesize and release ET-1 in response to ANG II [17] and TGF- β [18], which contributed to extracellular matrix protein secreted by vascular adventitial fibroblasts in turn, contracted extracellular matrix, and further aggravated PAH. Thus, ET-1 played important biological effects in the pathogenesis of PAH [7].

While it is clear from evidence, the elevated levels of ET-1 secretion and percentage of CECs was observed in CHD patients with PAH, the relationship between them is still not well defined. There is a growing belief that increase in shear stress caused by left to right shunting stimulates vascular ECs to secrete ET-1 and vascular endothelial dysfunction caused by high fluid shear stress induces ECs apoptosis. In order to verify the relationship between CECs and ET-1, we performed linear-regression analysis to explore correlations between the level of CECs and ET-1. As shown in **Figure 4D**, percentage of CECs and ET-1 production were positively correlated. Previous investigations declared that ET-1 enhanced the production of 1,2-diacylglycerol, which endogenously activated protein kinase C (PKC) in ECs. So ET-1 causes pulmonary vasoconstriction via PKC activation, which may further induce vascular ECs apoptosis and increase in CECs in PKC mediated Ca^{2+} sensitization to cause vascular contraction [19]. There are several limitations to acknowledge: Firstly, the number of objects is limited. Therefore, we cannot exclude that there might be an effect of treatment in a larger cohort of patients. Besides, the direct functional study of CECs was not performed. Thus, the contribution of elevated CECs to the development of pulmonary hypertension remains speculative.

What's more, the molecular pathway of ET-1 responsible for impairment in ECs is yet to be determined, especially in this type of human study.

Conclusion

In summary, CECs and ET-1 can be considered indicators of endothelial injury and biomarkers for predicting CHD complicated with PAH, and provide a new clinical thought for diagnosing CHD complicated with PAH.

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Disclosure of conflict of interest

None.

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