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Full Paper

Echinacoside prevents hypoxic pulmonary hypertension by regulating the pulmonary artery function

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ABSTRACT

Hypoxic pulmonary hypertension (HPH) is a progressive and irreversible disease that reduces survival. Echinacoside is a phenylethanoid glycoside from Tibetan herbs known for its vasorelaxant effect and for inhibiting the proliferation of rat pulmonary arterial smooth muscle cells. This study aimed to investigate the effect of echinacoside on HPH. Sprague Dawley rats were housed in a hypobaric hypoxia chamber (4500 m) for 28 days to obtain the HPH model. Echinacoside (3.75, 7.5, 15, 30 and 40 mg/kg) was administered by intraperitoneal injection from the 1st to the 28th day. The mean pulmonary artery pressure (mPAP), right ventricular hypertrophy index, hemoglobin, hematocrit, red blood cell concentration and morphological change of pulmonary arteries were evaluated. Vascular perfusion assay was used to assess the pulmonary artery function. Echinacoside reduced mPAP, hemoglobin, hematocrit, right ventricular hypertrophy index and mean wall thickness% of pulmonary arteries in HPH rats. It significantly increased maximum vasoconstriction percentage of pulmonary arteries induced by noradrenaline in a dose-dependent manner. In addition, it improved the responsiveness of pulmonary arteries to acetylcholine and sodium nitroprusside. Therefore, Echinacoside might be an effective treatment against HPH, since it regulated pulmonary artery endothelium and smooth muscle layer function and improved the remodeling of pulmonary artery.

1. Introduction

Hypoxic pulmonary hypertension (HPH) may be due to several causes. Native sea-level inhabitants who move to high altitudes or individuals living at high altitude may permanently develop HPH in the absence of any pre-existing lung disease, which in turn may lead to right ventricular hypertrophy and eventually heart failure.[1–3] HPH is a progressive and irreversible lung disease that reduces survival.[4] It is characterized by a pulmonary artery pressure higher than 30 mmHg, with various factors contributing to the pathogenesis of the disease, leading to an abnormal constriction of pulmonary arteries and inducing proliferation of pulmonary arterial smooth muscle cells (PASMCs).[5] Echinacoside (ECH) (Fig. 1) is a phenylethanoid glycoside from the Tibetan herb *Lagotis brevituba* Maxim and *Cistanche tubulosa*.[6,7] Our previous findings indicated that ECH induces the vasorelaxation of rat pulmonary artery, and also inhibits hypoxia induced-proliferation of rat PASMCs in a concentration-dependent manner.[8,9] Therefore, the main purpose of this study was to evaluate the effect of ECH in a rat HPH model. Our present results indicated that ECH might be a potential agent in the prevention and treatment of HPH. In addition, this study might help the discovery of new drugs to prevent and cure HPH.

2. Materials and methods

2.1. Drugs and reagents

ECH (170307) (HPLC≥98%) was purchased from the Beijing Century Aoke Biotechnology Co. Ltd (Beijing, China), and deposited

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at the School of Pharmacy, Qinghai Nationalities University. ECH was dissolved in dimethylsulfoxide (DMSO), and subsequently diluted with saline. Noradrenaline (NE), acetylcholine (ACh, ≥99%), Sodium nitroprusside dihydrate (SNP, ≥99%) were purchased from Sigma Chemical Co (St Louis, MO, USA); inorganic salts were purchased from Beijing Chemical Reagent Co., Ltd. (Beijing, China).

2.2. Animals

Eight-week-old male Sprague Dawley rats weighing 250–300 g were purchased from the Dilepu bioscience and technology medical co. LTD (Xi'an, China). They were housed in a temperature-controlled environment of 22 ± 2 °C, relative humidity of 45%–55% and fed with a standard laboratory diet and tap water ad libitum during the acclimation period prior to the experiment. All procedures and protocols were approved by the Animal Care and Use Committee of Qinghai Nationalities University.

2.3. Hypoxia-induced pulmonary hypertension rat model

Animals were randomly divided into seven groups: (1) normoxia group (control group); (2) chronic hypoxia group (HPH rat model); (3) ECH (3.75 mg/kg) group; (4) ECH (7.5 mg/kg) group; (5) ECH (15 mg/kg) group; (6) ECH (30 mg/kg) group; (7) ECH (40 mg/kg) group. Except for the rats of the first group, rats in the other groups were housed in a DYC3000 type hypobaric hypoxia chamber (Fenglei, Guizhou, China) in a simulated altitude of 4500 m for 28 days.[10] All animals were maintained in a 12 h light−12 h dark cycle, under a temperature of 22 ± 2 °C, supplied with food ad libitum, and the bedding was changed once every three days. Animals in the (3) to the (7) group were treated with an intraperitoneal injection of ECH, one time daily for 28 days (the first ECH dose was simultaneously administered with the onset of hypoxia). Animals in the (1) and (2) group were treated with normal saline under the same modality used for the ECH groups. The altitude of the hypobaric hypoxia chamber was lowered to 3500 m, and drug administration and bedding change was performed inside the hypobaric hypoxia chamber by the experimenter who entered into the chamber.

2.4. Measurement of pulmonary artery pressure (PAP) and sample preparation

After 28 days of hypoxic exposure, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40~60 mg/kg). A silicone catheter (outer diameter 0.9 mm) was introduced into the right external jugular vein through a venotomy and passed across the tricuspid valve and right ventricle (RV) into the pulmonary artery. The mean pulmonary artery pressure (mPAP) was recorded using the MP100 pressure signal acquisition system (Biopac, California, USA) simultaneously with intubation.[11] After opening the chest, the RV was separated from the heart. Right ventricular hypertrophy index was calculated by the wet weight ratio of RV against left ventricle (LV) plus septum (SP) as follows: RV/(LV + SP).[12] The lungs were rapidly removed, and one was placed into liquid nitrogen for a quick freezing and stored at −80 °C. A blood sample was collected from the abdominal aorta and placed in a heparinized tube. BC5000vet automatic hematocrit analyzer (mindray, Shenzhen, China) was used for the determination of hemoglobin (Hb), hematocrit (Hct), and red blood cell (RBC).

2.5. Morphometric analysis of lung tissues

The other lung was fixed in formalin (10% wt/vol) and cut into 4 μm-thick paraffin sections using the RM2265 paraffin microtome (Leica, Solms, Germany). Sections for morphometric analysis were stained with hematoxylin & eosin (HE) to evaluate pulmonary arterial remodeling.[13] The morphological changes in pulmonary arterioles were observed and photographed under an optical microscope (Olympus Corporation, Shinjuku, Japan). More than 20 consecutive sections of each rat were analyzed. Image-pro Plus 6.0 software was used to measure the external diameter, wall thickness (WT), wall area, lumen area and total wall area of the pulmonary artery on the slice. The ratios of the wall thickness/external diameter (WT%), wall area/total wall area (WA%) and lumen area/total wall area (LA%) were calculated as indices of pulmonary vascular morphology for further statistical analysis.[14]

2.6. Evaluation of rat pulmonary artery function

The intrapulmonary arteries (0.7–1.5 mm in diameter) were dissected to be free of the surrounding connective tissue and adventitia and cut into rings of approximately 5–6 mm in length. The rings were suspended in organ chambers filled with 10 mL KH solution at 37 °C, gassed with 95% O_2–5% CO_2, and isometric tension was measured using a force transducer (JH-2, Space Medico-Engineering Institute, Beijing, China). The KH solution contained (in mM): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4 \cdot 7H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 11.1 glucose (pH 7.4). Vessel rings were allowed to equilibrate for 2 h under a basal tension of 400 mg, and during this time the KH solution was changed every 15 min. Contraction percentage and Relaxation percentage were calculated as follows:

$$\text{Contraction percentage} = \frac{\text{Tension induced by NE}}{\text{Basal tension}} \times 100\%$$

$$\text{Relaxation percentage} = \frac{\text{Tension induced by vasodilator}}{\text{Tension induced by NE}} \times 100\%$$

One μmol/L NE was added into the chamber to pre-contract each pulmonary arterial ring and detect the contractility of rat pulmonary artery.[15] After the NE-induced vasoconstriction reached a

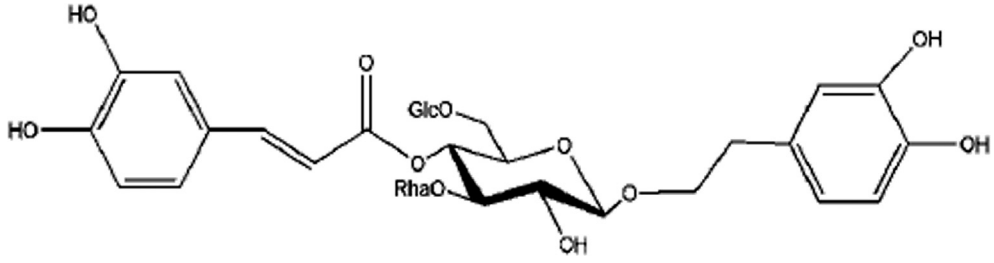


Fig. 1. Chemical structure of echinacoside.

plateau, the relaxant reagents ACh and SNP were added to detect the function of pulmonary artery endothelium and smooth muscle layer, respectively.[16,17] The details of the performed method are listed in a previous study.[9]

2.7. Statistical analysis

Statistical analysis was performed using SPSS 16 software package (IBM, Endicott, NY). Results were expressed as mean ± standard deviation (SD). Multiple-group comparisons were evaluated by one-way ANOVA with Dunnett test, while the unpaired *t*-test was used for the normoxia *vs.* hypoxia comparison. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. ECH reduced mPAP, Hb, Hct and RV/(LV + SP) of HPH rats

The mPAP of normal rats in the normoxia group was 18.18 ± 1.09 mmHg. The results showed that the mPAP of rats rose to 40.83 ± 7.49 mmHg after they were housed in a hypobaric hypoxia chamber of a simulated altitude of 4500 m for 28 days (Fig. 2A), and it was compared with that of the normoxia group, resulting in a significant difference ($P < 0.05$). Besides, RV/(LV + SP), Hb, Hct and RBC in the hypoxia group were all markedly increased to 0.46 ± 0.07, 247.83 ± 12.43 g/L, 65.34 ± 3.07% and 10.14 ± 0.62 10^{12}/L, respectively, compared to 0.25 ± 0.09, 136.69 ± 10.22 g/L, 46.91 ± 3.37% and 7.14 ± 0.65 10^{12}/L in the normoxia group (all $P < 0.05$) (Fig. 2BCDE). These results indicated that the HPH rat model was successfully obtained.

The mPAP was markedly decreased to 32.21 ± 7.32 mmHg, 28.47 ± 8.31 mmHg and 32.64 ± 7.21 mmHg, respectively, in the ECH third dose (15 mg/kg), fourth dose (30 mg/kg) and fifth dose (40 mg/kg) group (compared with that in the hypoxia group, both $P < 0.05$) (Fig. 2A). In addition, ECH (15 mg/kg) reduced Hb and Hct in HPH rats to 216.5 ± 24.08 g/L and 57.66 ± 5.95% (Fig. 2CD), respectively, and ECH (40 mg/kg) reduced Hb and Hct in HPH rats to 219 ± 24.26 g/L and 57.37 ± 5.87% (Fig. 2CD), respectively (compared with the values in the hypoxia group, $P < 0.05$). The RV/(LV + SP) was markedly decreased to 0.36 ± 0.06 in the ECH fifth dose (40 mg/kg) group (compared with that in the hypoxia group, $P < 0.05$) (Fig. 2B).

3.2. ECH improved the remodeling of pulmonary artery

The results of HE-stained lung tissues showed that the pulmonary artery wall in the normoxia group was complete and smooth, with neat and dense cell distribution, thin and uniform vessel wall with no thickening of the muscular layer (Fig. 3A). The pulmonary artery wall in the hypoxia group was significantly thickened, with narrowed lumen, and cells were arranged in different ways, showing the characteristics of pulmonary artery remodeling (Fig. 3B). The WT%, WA% and LA% deriving from the pulmonary arteriole analysis were summarized in Fig. 3. The mean WT%, WA% and LA% in the hypoxia group were 43.50 ± 1.85%, 58.66 ± 2.07% and 40.96 ± 2.50% respectively, WT% and WA% were significantly increased compared with the values in the normoxia group ($P < 0.05$), while LA% was significantly decreased compared with the normoxia group ($P < 0.05$). Rat pulmonary artery thickening was reduced and pulmonary artery remodeling was reversed in the ECH (30 mg/kg) group (Fig. 3C), with an average WT%, WA% and LA % of 24.49 ± 1.84%, 44.80 ± 2.15% and 55.51 ± 2.13%, respectively, compared with the hypoxia group (all $P < 0.05$).

3.3. ECH regulated pulmonary artery function

The results of pulmonary artery perfusion (Fig. 4) showed that the maximum contraction percentage induced by NE (1 μmol/L) (NE-induced E_{max}) in the control group was 135.57 ± 6.32%. However, the maximum effect of pulmonary artery contraction in the hypoxia group was 47.38 ± 5.66% ($P < 0.05$ *vs.* normoxia group, n = 8). The treatment with ECH (3.75 mg/kg, 7.5 mg/kg, 15 mg/kg, 30 mg/kg, and 40 mg/kg) significantly increased the maximum vasoconstriction percentage induced by NE (1 μmol/L) ($P < 0.05$ *vs.* hypoxia group, n = 8), and the effect was dose-dependent within the range of 3.75~30 mg/kg.

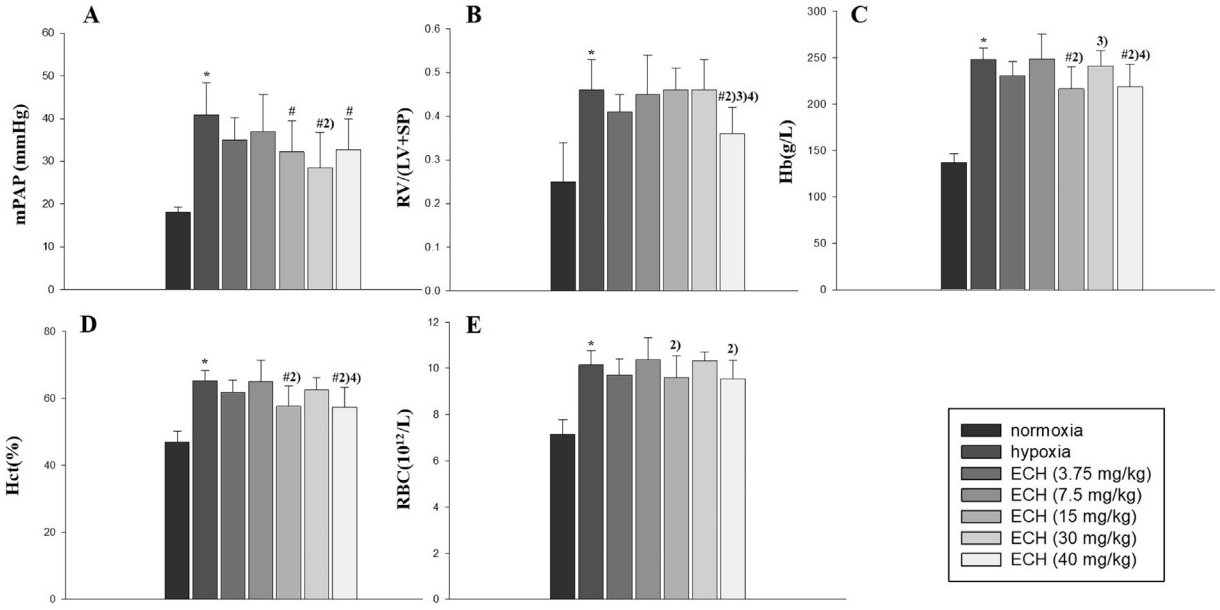
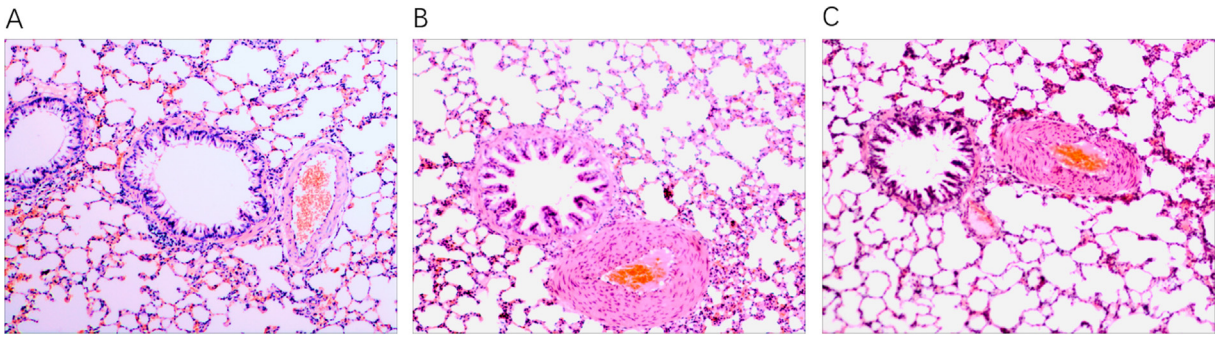


Fig. 2. Effect of ECH on HPH rats. Effect of ECH on mPAP (A), RV/(LV + SP) (B), Hb (C), Hct (D), RBC (E). *$P < 0.05$ *vs.* normoxia, #$P < 0.05$ *vs.* hypoxia, 2) $P < 0.05$ *vs.* ECH (7.5 mg/kg), 3) $P < 0.05$ *vs.* ECH (15 mg/kg), 4) $P < 0.05$ *vs.* ECH (30 mg/kg), n = 10.



	normoxia group (100×)	hypoxia group (100×)	ECH (30 mg/kg) group (100×)
WT%	13.80±1.74	43.50±1.85*	24.49±1.84#
WA%	31.65±1.80	58.66±2.07*	44.80±2.15#
LA%	68.39±1.93	40.96±2.50*	55.51±2.13#

Fig. 3. HE-stained lung tissues in each group. (A) Pulmonary artery wall in the normoxia group was complete and smooth, with neat and dense cell distribution, thin and uniform vessel wall with no thickening of the muscular layer. (B) Pulmonary artery wall in the hypoxia group was significantly thickened, with narrowed lumen, and cells were arranged in different ways, showing the characteristics of pulmonary artery remodeling. (C) Pulmonary artery thickening in rats of the ECH (30 mg/kg) group was reduced and pulmonary artery remodeling was reversed. *$P < 0.05$ *vs.* normoxia group, #$P < 0.05$ *vs.* hypoxia group, n = 10.

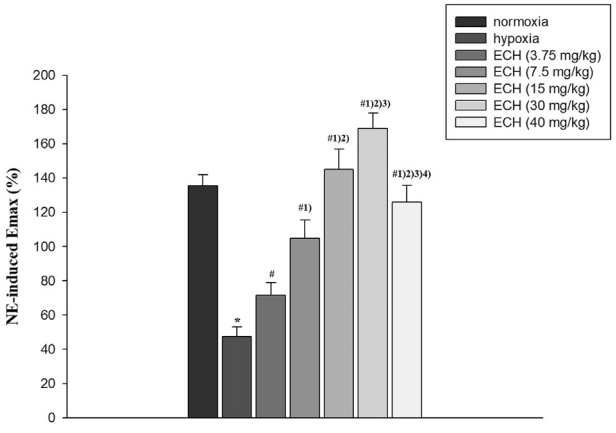


Fig. 4. Effect of ECH on NE-induced E_{max} in pulmonary arteries of HPH rats. *$P < 0.05$ *vs.* normoxia, #$P < 0.05$ *vs.* hypoxia, 1) $P < 0.05$ *vs.* ECH (3.75 mg/kg), 2) $P < 0.05$ *vs.* ECH (7.5 mg/kg), 3) $P < 0.05$ *vs.* ECH (15 mg/kg), 4) $P < 0.05$ *vs.* ECH (30 mg/kg), n = 8.

Furthermore, a vasorelaxant effect induced by different concentrations of ACh (10^{-8}~10^{-4} mol/L) was found. The results showed that ECH at different doses improved the response of pulmonary arteries to ACh in hypoxic animals to different extents, especially at ACh 10^{-7} mol/L and ACh 10^{-6} mol/L in a dose-dependent manner (Fig. 5). The ACh concentration–response curve was shifted to the right by the hypoxic treatment compared with the normoxia control group (Fig. 6), and the EC_{50} of the hypoxia group was significantly increased ($P < 0.05$ *vs.* normoxia group, n = 8) (Fig. 6). The ACh concentration–response curve was shifted to the left by the treatment with different doses of ECH compared with the hypoxia group (Fig. 6), and the EC_{50} of each drug treatment group was decreased to different degrees (Fig. 6).

Finally, the pulmonary artery reactivity to SNP was measured. Our results demonstrated that the pulmonary artery of hypoxic animals treated with different doses of ECH (3.75 mg/kg, 7.5 mg/kg, 15 mg/kg, 30 mg/kg, and 40 mg/kg) showed different reactivity to different concentrations of SNP (10^{-8}~10^{-4} mol/L). Different ECH doses improved the pulmonary artery reactivity of hypoxic animals to SNP, especially in the case of SNP at 10^{-8} mol/L and 10^{-4} mol/L, showing a certain dose-dependence (Fig. 7). The hypoxic treatment resulted in the shift of the concentration–effect curve of SNP to the right (Fig. 8), and the EC_{50} of the hypoxia group was significantly increased compared with the normoxia control group ($P < 0.05$) (Fig. 8), suggesting that hypoxia reduced pulmonary artery sensitivity of rats to the vasodilator SNP. The concentration–effect curve of the vasorelaxant effect induced by SNP was shifted to the left by different doses of ECH compared with the effect in the hypoxia group (Fig. 8), and the EC_{50} of each drug treatment group was decreased to different degrees (Fig. 8).

4. Discussion

Our results showed that ECH reduced mPAP, Hb, Hct and right ventricular hypertrophy index of HPH rats. Our previous study also indicated that ECH not only induced the vasorelaxation of the rat pulmonary artery, but also inhibited hypoxia induced-proliferation of rat PASMCs in a concentration-dependent manner.[8,9] All the results provided evidence that ECH might have the ability to prevent HPH.

At first, the rat HPH model was successfully set up. The hypobaric hypoxia chamber was used to simulate the altitude of 4500 m, and the results showed that mPAP, RV/(LV + SP), Hb, Hct, RBC, mean WT% and WA% were all enhanced compared to those in the normoxia group, suggesting that the rat HPH model was successfully obtained.

The pathological mechanism of HPH remains unclear. Although early studies suggested that enhanced pulmonary vasoconstriction lead to HPH, more and more studies indicate that in hypoxic condition, pulmonary vascular remodeling is the most important cause of HPH.[18,19] Our present results showed that after 28 days of low-pressure hypoxic treatment at a simulated altitude of 4500 m, pulmonary artery proliferation and remodeling in rats were the primary causes of pulmonary hypertension. After 28 days of treatment with ECH, pulmonary artery remodeling was significantly improved, which was consistent with the previous results of

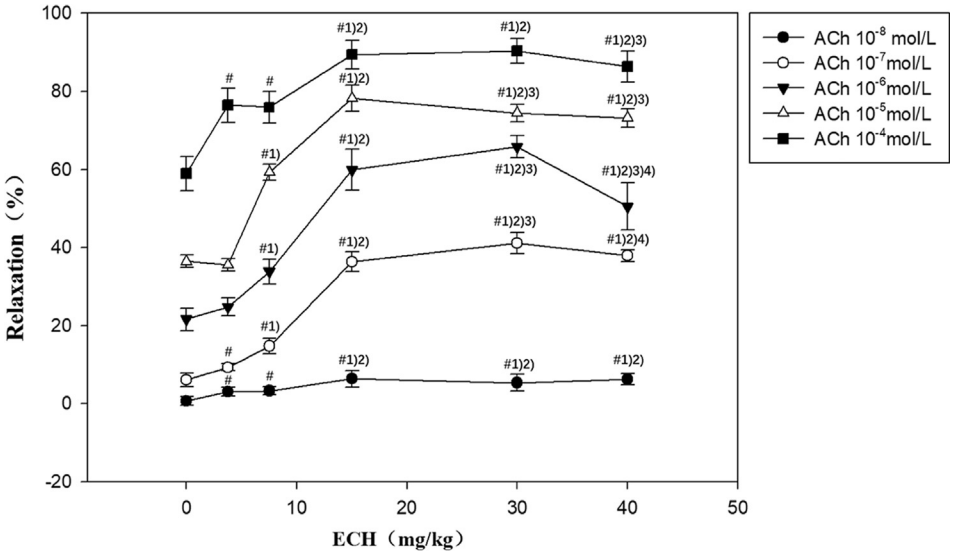
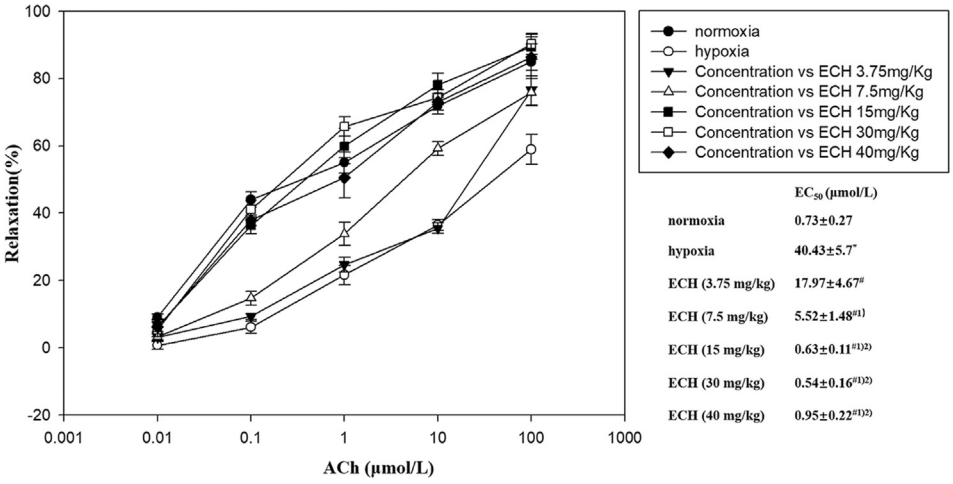


Fig. 5. Dose-effect curves of ECH under different concentrations of ACh (10^{-8}~10^{-4} mol/L) in pulmonary arteries of HPH rats. ECH at different doses improved the response of pulmonary arteries to ACh in hypoxic animals to different extents. $^{\#}P < 0.05$ *vs.* hypoxia + ECH (0 mg/kg), $^{1)}$ $P < 0.05$ *vs.* hypoxia + ECH (3.75 mg/kg), $^{2)}$ $P < 0.05$ *vs.* hypoxia + ECH (7.5 mg/kg), $^{3)}$ $P < 0.05$ *vs.* hypoxia + ECH (15 mg/kg), $^{4)}$ $P < 0.05$ *vs.* hypoxia + ECH (30 mg/kg), n = 8.



	EC_{50} (μmol/L)
normoxia	0.73±0.27
hypoxia	40.43±5.7*
ECH (3.75 mg/kg)	17.97±4.67#
ECH (7.5 mg/kg)	5.52±1.48#1)
ECH (15 mg/kg)	0.63±0.11#1)2)
ECH (30 mg/kg)	0.54±0.16#1)2)
ECH (40 mg/kg)	0.95±0.22#1)2)

Fig. 6. Effect of different doses of ECH on ACh concentration–response curves in pulmonary arteries of HPH rats. The ACh concentration–response curve was shifted to the right by the hypoxic treatment compared with the normoxia control group. The ACh concentration–response curve was shifted to the left by the treatment with different doses of ECH compared with the hypoxia group. $^{*}P < 0.05$ *vs.* normoxia, $^{\#}P < 0.05$ *vs.* hypoxia, $^{1)}$ $P < 0.05$ *vs.* ECH (3.75 mg/kg), $^{2)}$ $P < 0.05$ *vs.* ECH (7.5 mg/kg), n = 8.

our research group.[8] Thus, ECH might improve hypoxia-induced remodeling of rat pulmonary arteries through the inhibition of hypoxia induced-proliferation of rat PASMCs. Our previous study indicated that ECH suppresses the contraction of rat pulmonary artery through the reduction of intracellular Ca^{2+} levels.[9] Calcium overload is not only one important cause of abnormal vasoconstriction in pulmonary arteries but it also possesses a key role in the proliferation of cells.[20,21] Therefore, the effect of ECH improving hypoxia-induced remodeling in rat pulmonary arteries might be associated with the reduction of calcium overload. The effect of ECH on calcium overload in rat PASMCs under hypoxic condition will be analyzed in our further study.

Additionally, pathological changes of pulmonary artery remodeling include the hypertrophy and proliferation of pulmonary arterial smooth muscle cells and endothelial cells.[22–24] Therefore, it is necessary to further investigate the changes in the endothelium and smooth muscle layers of the pulmonary arteries in chronic hypoxic condition. Based on this assumption, the improvement of the function of the endothelium and smooth muscle layers of the pulmonary arteries seems to represent a key point in the treatment of HPH.

Since it is well known that NE induces a rapid change in the tension of vessel rings in the normal pulmonary artery, NE was used to evaluate the vasoconstriction.[25,26] Our present study proved that ECH could improve pulmonary arterial function. For example, the treatment with ECH significantly increased the maximum vasoconstriction percentage induced by NE, with a dose-dependent effect in the range of 3.75~30 mg/kg, thus suggesting that ECH enhanced the reactivity of pulmonary artery under hypoxia. This may be associated with the increase of receptor expression and the restoration of pulmonary arterial structure.

Vascular function also refers to vasorelaxation, thus, ACh and SNP were used to detect the relaxation percentage of the pulmonary artery. Our results showed that the hypoxic treatment

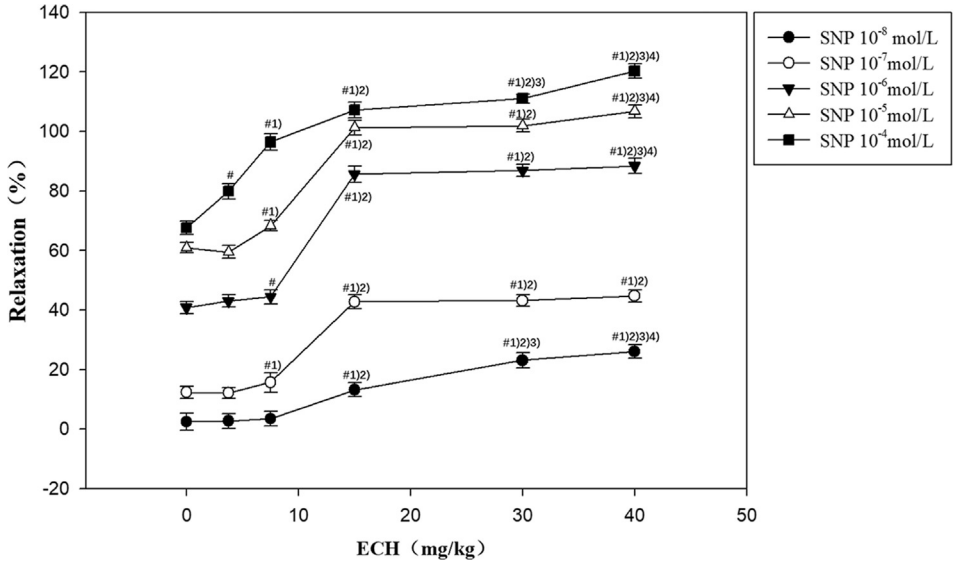
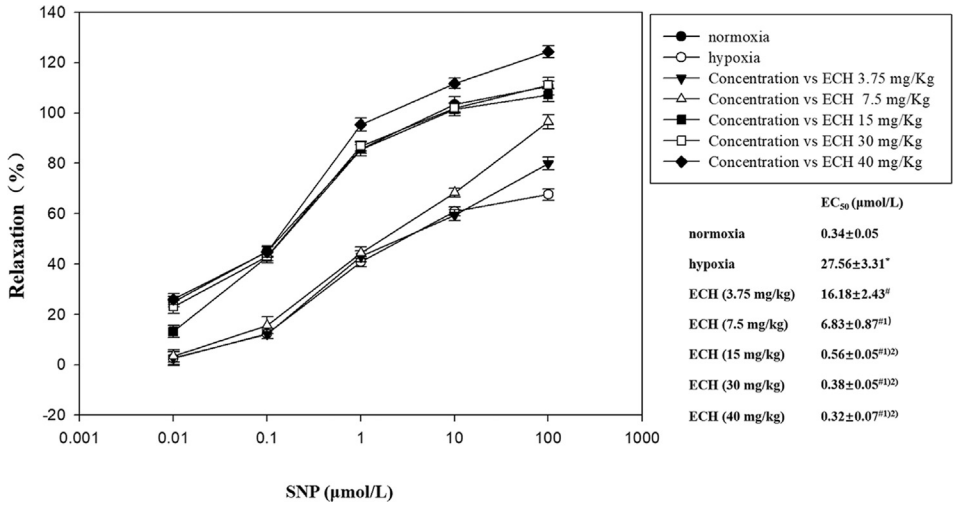


Fig. 7. Dose-effect curves of ECH under different concentrations of SNP (10^{-8}~10^{-4} mol/L) in pulmonary arteries of HPH rats. Different doses of ECH improved the pulmonary artery reactivity of hypoxic animals to SNP. $^{\#}P < 0.05$ *vs.* hypoxia + ECH (0 mg/kg), $^{1)}$ $P < 0.05$ *vs.* hypoxia + ECH (3.75 mg/kg), $^{2)}$ $P < 0.05$ *vs.* hypoxia + ECH (7.5 mg/kg), $^{3)}$ $P < 0.05$ *vs.* hypoxia + ECH (15 mg/kg), $^{4)}$ $P < 0.05$ *vs.* hypoxia + ECH (30 mg/kg), n = 8.



	EC_{50} (μmol/L)
normoxia	0.34±0.05
hypoxia	27.56±3.31*
ECH (3.75 mg/kg)	16.18±2.43#
ECH (7.5 mg/kg)	6.83±0.87#1)
ECH (15 mg/kg)	0.56±0.05#1)2)
ECH (30 mg/kg)	0.38±0.05#1)2)
ECH (40 mg/kg)	0.32±0.07#1)2)

Fig. 8. Effect of different doses of ECH on SNP concentration–response curves in pulmonary arteries of HPH rats. The hypoxic treatment resulted in the shift of the concentration–effect curve of SNP to the right compared with the normoxia control group. The concentration–effect curves of the vasorelaxant effect induced by SNP were shifted to the left with different doses of ECH compared with the hypoxia group. $^{*}P < 0.05$ *vs.* normoxia, $^{\#}P < 0.05$ *vs.* hypoxia, $^{1)}$ $P < 0.05$ *vs.* ECH (3.75 mg/kg), $^{2)}$ $P < 0.05$ *vs.* ECH (7.5 mg/kg), n = 8.

impaired the vasorelaxant effect induced by different concentrations of ACh and SNP. ACh and SNP have an effect on the endothelium and vascular smooth muscle, respectively.[27,28] Endothelial dysfunction is the core pathophysiologic process in pulmonary hypertension.[29,30] Endothelial cells play a major role in the regulation of vascular tone and barrier, blood coagulation, nutrient and electrolyte uptake, leukocyte trafficking and neovascularization of the hypoxic tissue.[31] On the other hand, the vascular smooth muscle cell is also a key cell type that plays a major role in the development of pulmonary hypertension.[32] Vascular smooth muscle cells are highly specialized cells that participate in vessel remodeling and in the regulation of vascular tone in physiological and hypoxic conditions.[33] Our results suggested that hypoxia might impair the endothelial and smooth muscle structures and functions of the pulmonary arteries. Thus, ECH might improve the function of endothelium and smooth muscle layer in pulmonary arteries under hypoxic condition. Furthermore, the structure of the endothelium and smooth muscle layers might be repaired by ECH under hypoxia. One of the key mechanisms used by ECH was the inhibition of hypoxia induced-proliferation of rat PASMCs, resulting in the improvement of remodeling of rat pulmonary arteries. In other words, ensuring the integrity of the structure can ensure the normal function. Of course, this aspect needs to be confirmed by further studies. Additionally, our previous study indicated that ECH induces vasorelaxation, which is associated with the relaxation of PASMCs in HPH rats.[9] Thus, ECH might regulate pulmonary arterial function through the relaxation of PASMCs and the improvement of the remodeling of rat pulmonary arteries.

The most remarkable aspect was that the lowest dose of ECH to reduce the mean pulmonary artery pressure was 15 mg/kg in HPH

rat models. Nevertheless, the lowest effective dose for ECH to improve pulmonary artery function was 3.75 mg/kg, which was only a quarter of the above one. This evidence represented one more proof that the improvement of pulmonary artery function might be one of the important mechanisms by which ECH prevented HPH.

Furthermore, our present study showed that ECH might prevent HPH through improving pulmonary blood flow and blood viscosity. The exposure to chronic hypoxia leads to an increase in the erythrocyte number, hemoglobin concentration and hematocrit values.[34] Hemoglobin is important determinants of blood viscosity and arterial oxygen content.[35] It induces the reduction of NO bioavailability, resulting in persistent vasoconstriction.[36] In addition, hemoglobin causes inflammation, vascular tissue injury and endothelial damage, mainly due to its interaction with superoxide and hydrogen peroxide to increase reactive oxygen species (ROS) formation and lipid peroxidation. In our previous study, ECH was found to induce rat pulmonary artery vasorelaxation by opening the NO-cGMP-PKG-BK_{Ca} channels.[9] Under hypoxia condition, there is a negative correlation between hemoglobin concentration and NO production. In addition, under hypoxia, increased hemoglobin concentration induces the increase of blood viscosity and the reduction of NO concentration, the latter leading to vasoconstriction in pulmonary arteries. Finally, increased blood viscosity and vasoconstriction led to the increase of mPAP. ECH might enhance NO production through the reduction of hemoglobin concentration, resulting in rat pulmonary artery vasorelaxation and decreased blood viscosity, finally inducing the reduction of mPAP. Increased hematocrit causes an increase in blood viscosity, which eventually induces an increase in pulmonary artery pressure.[37] Our results showed that ECH could reduce hematocrit. These results indicated that one of the mechanisms used by ECH to reduce the mean pulmonary artery pressure in hypoxic pulmonary hypertension rats was the reduction in blood viscosity by reducing Hb and Hct. Otherwise, the observed changes in vasoactivity might be a consequence of the reduced blood viscosity depending on the reduced Hb and Hct.

Moreover, the third and fifth dose (15 and 40 mg/kg) of ECH decreased Hb and Hct in HPH rat models. However, the decrease of Hb and Hct by ECH was not dose-dependent, and the exact mechanism remains elusive. It should be noted that ECH was administered on the same day the rats were placed into the hypobaric hypoxia chamber. Therefore the acute responses of these rats, such as acclimation to the sudden increased altitude, stress, and reduced food intake and sleep, were likely to affect the absorption and metabolism of ECH. Thus, further studies are required to investigate the pharmacokinetics and pharmacodynamics of ECH in HPH rat model.

Sample size in vascular perfusion assay was different with that in other parts. A small number of the rats died during the experiment due to various reasons. It is likely that the acute responses of these rats, such as acclimation to the suddenly increased altitude, stress, as well as reduced food intake and sleep. Therefore, the sample size of the rats in vascular perfusion assay was 8 instead of 10.

5. Limitations of this study

In this study the drug was administered once a day, thus, the altitude of the hypobaric hypoxia chamber was lowered to 3500 m, and the experimenter needed to enter the hypobaric hypoxia chamber to administer the drug and change the bedding. This resulted in a defect in the experiment because of the involvement of reoxygenation in rats. The influence of reoxygenation on the experiment was minimized. The altitude was lowered to 3500 m, not to the lowest elevation. More importantly, the establishment of the rat model of HPH by the measurement of mPAP, RV/(LV + S), Hct, Hb and RBC was finally evaluated. The changes of the above indexes indicated the success of the established model.

6. Conclusion

In conclusion, this work demonstrated for the first time that ECH could prevent hypoxic pulmonary hypertension by regulating pulmonary artery function, and improving vascular remodeling and blood viscosity.

Declaration of competing interest

The authors declare no conflict of interests.

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