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Original Article

Effects of the Shenfuyixin granule on hemodynamics and angiogenesis in rats with pulmonary hypertension

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Abstract: Objective: To observe the effects of the Shenfuyixin Granule on hemodynamics and angiogenesis in rats with pulmonary hypertension, and to study the relationship between hemodynamics and angiogenesis in rats with pulmonary hypertension. Method: 30 male Wistar rats were randomly divided into 3 groups, the blank group, the model group and the Shenfuyixin granule group, with 10 rats in each group. The model group and the Shenfuyixin granule group received subcutaneous injection of monocrotaline (60 mg/kg) to establish the pulmonary arterial hypertension model. The pulmonary hemodynamic parameters were measured by the modified technique of right cardiac catheter in rats after 28-day medicine gavage, and the mean pulmonary artery pressure was calculated. The right ventricular hypertrophy index was measured at sacrifice of the rats and represented by the ratio of the weight of right ventricular to the sum of weight of light ventricular and septum, described as RV/(LV+S). Finally, the right lower lung tissues were taken to measure the pulmonary microvascular density. Results: In the model group, the pulmonary artery mean pressure and RV/(LV+S) were significantly higher than that of the Shenfuyixin granule group ($P<0.05$), while the results for the pulmonary vascular density were opposite. Conclusion: The Shenfuyixin granule can significantly reduce pulmonary artery pressure and right ventricular index in rats with pulmonary hypertension, contribute to the increase of microvessel density in lung tissue, and facilitate the treatment of pulmonary hypertension.

Keywords: Pulmonary hypertension, Shenfuyixin granule, pulmonary hemodynamics, angiogenesis

Introduction

Pulmonary arterial hypertension (PAH) is a highly fatal disease in heart, lung and blood vessel, featuring that pulmonary vascular remodeling induces progressive increase of resistance which eventually results in patients' death for right heart failure [1-3]. Its clinical expressions are dyspnea, fatigue, dizziness, chest pain, hemoptysis, etc., similar to "lung inflation" and "heart failure" in medicine and the basic pathology is Qi deficiency and blood stasis, accumulation of fluid caused by insufficiency of Yang. Traditional drug therapy to the pulmonary arterial hypertension is mainly on treating right ventricular dysfunction and primary thrombus formation in pulmonary artery, that is, targeting drug therapy for oxygen inhalation, diuretic, Cardiac and anticoagulation and pulmonary vascular dilatation. The targeting drugs can not only significantly improve patients' symptoms and their life quality, but also extend their life span, while the target drug is expensive and brings high incidence of drug resistance and larger side effects to patients [4-6]. Therefore, it is practically significant for the prevention and treatment of pulmonary hypertension to develop Chinese medicine.

At present, the study mainly focuses on pathological mechanism, such as the spasm, reconstruction or occlusion of the pulmonary artery and the decrease of the number of pulmonary artery [7]. For chronic pulmonary heart disease, the pathological changes of lung are masculinization of arterioles, abnormal smooth muscle bundles in the vascular intima pulmonary and abnormal hyperplasia of the pulmonary artery elastic fiber and collagen fiber. Non-muscle masculinization of pulmonary arterioles is mainly caused by increase of pulmonary blood flow, and the main cause of muscular artery

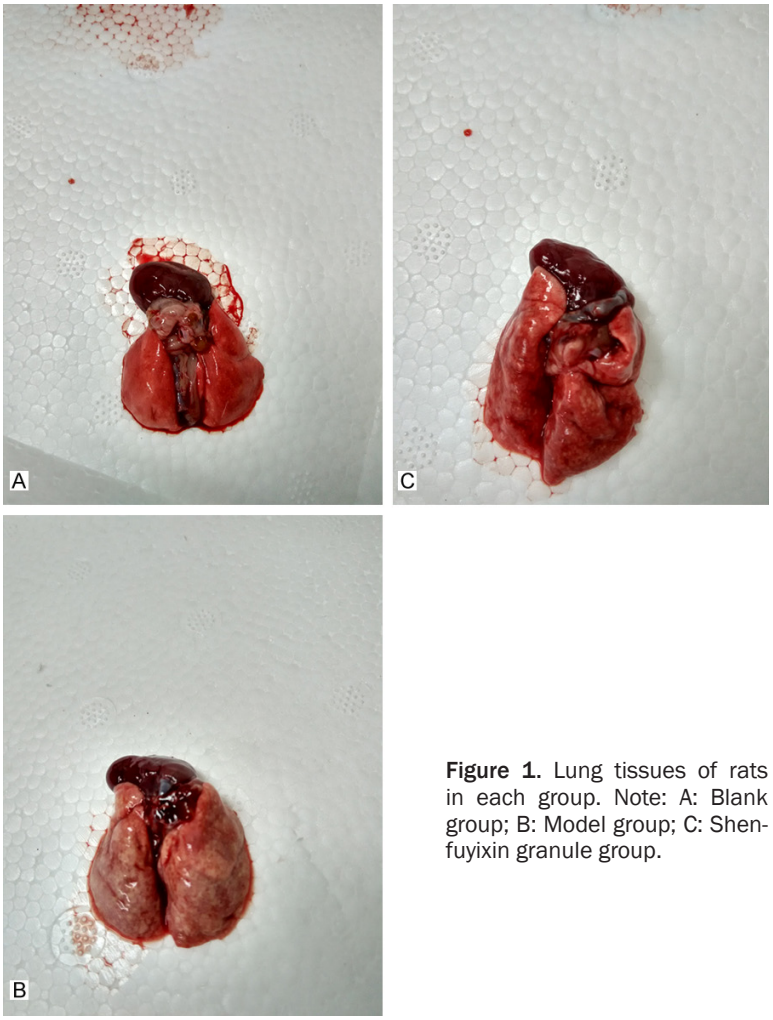

Figure 1. Lung tissues of rats in each group. Note: A: Blank group; B: Model group; C: Shenfuyixin granule group.

intima-media hypertrophy is the increase of pulmonary artery pressure; besides, the increase of the pulmonary vascular resistance also leads to a reduction in peripheral pulmonary arteries in numbers. Abnormal hyperplasia or small vascular occlusion, or both are the main reasons for the decrease in the number of pulmonary arteries. The mechanism of non-muscle masculinization of pulmonary arterioles primarily is the aggregation of the endothelial cells into the lumen and their interaction with platelets, releasing thromboxane A (TXA), which will result in vasoconstriction, and blood vessels shutting down or thrombus embolism of platelet fibrin [8]. Therefore, it is of great significance to study whether angiogenesis alleviates the symptoms of pulmonary hypertension.

In this study, the rat model of pulmonary hypertension induced by monocrotaline was selected to measure indicators like right ventricular systolic pressure (RVSP), right ventricular diastolic pressure (RVDP), mean pulmonary artery pressure (mPAP), pulmonary vascular resistance (PVR), cardiac output (CO), cardiac index (CI), right ventricular hypertrophy index (RVHI), pulmonary microvascular and other indicators, aiming to observe the effects of Shenfuyixin Granule on hemodynamics and angiogenesis in rats with pulmonary hypertension, and to further understand the relationship between hemodynamics and angiogenesis in pulmonary hypertension rats.

Materials and methods

Reagents, drugs and instruments

The monocrotaline was purchased in Sigma Company (the United States); the Shenfuyixin granule was provided by Shaanxi BuChang Pharmaceutical Group; Rabbit anti rat CD34 primary antibody was offered by Wuhan Boster Biological Technology., LTD, and Goat anti Rabbit secondary antibody SABC test kit by Wuhan Boster Biological Technology. LTD; PE-50 polyvinyl chloride conduit, temperature probe and cardiac output guide wire (MLT1402 T-type Ultra-Fast Thermocouple Probe) were all purchased in Shanghai instrument International Trading Co., Ltd.

Experimental grouping and the establishment of animal model

30 male Wistar rats (purchased in Laboratory Animal Center, Zhengzhou University), weighing from 220 to 280 g, were randomly divided into blank group, model group and Shenfuyixin granule group, with 10 rats in each group. And rats in model group and Shenfuyixin granule group were given the hypodermic injection with 1% monocrotaline to the neck and back by 60 mg/kg to establish the model of pulmonary hypertension, and the blank group rats were

Table 1. Comparison of mPAP, RVSP, RVDP and PVR among groups ($\bar{x} \pm s$)

Group (n=10)	mPAP (mmHg)	RVSP (mmHg)	RVDP (mmHg)	PVR (mmHg·min/mL)
Blank group	16.8±1.31	11.5±1.74	3.1±1.61	0.11±0.01
Model group	32.2±1.51*	28.4±1.80*	7.3±1.12*	0.34±0.02*
Shenfuyixin granule group	21.0±1.53Δ	18.4±1.45Δ	5.3±1.32Δ	0.18±0.02Δ

Annotation: *indicates that "*P*<0.05" in the comparison between model group and blank group; Δindicates that "*P*<0.05" in the comparison between Shenfuyixin granule group and model group.

Table 2. Comparison of CO, CI and RV/(LV+S) among groups ($\bar{x} \pm s$)

Group (n=10)	CO (mL/min)	CI (L/(min·m^2))	RV/(LV+S) (%)
Blank group	136.4±5.17	1.2±0.12	26.4±0.02
Model group	91.8±4.43*	0.7±0.05*	43.8±0.01*
Shenfuyixin granule group	122.6±4.57Δ	0.9±0.05Δ	31.1±0.02Δ

Annotation: *indicates that "*P*<0.05" in the comparison between model group and blank group; Δindicates that "*P*<0.05" in the comparison between Shenfuyixin granule group and model group.

not treated; 28 days later, the rats in the Shenfuyixin granule group were given Shenfuyixin granules by lavage daily (according to the clinical dosage of 60 kg adults at 105 g/kg per day and the conversion coefficient of 6.17 for control rats, the daily dosage of rats were determined as 3.7 g/kg). Rats in the blank group and model group were given the same amount of normal saline by lavage. The rats in all three groups were given daily lavage for 28 days. The experiment has obtained approval from animal ethical committee.

Determination of mean pulmonary artery pressure and cardiac output

After the 28-day lavage, anaesthetized by injection of 9% chloral hydrate (3 mL/kg) to the abdominal cavity, all three groups of rats were fixed on the mouse plate with the supine position before being given a neck incision for tracheal intubation and their right external jugular vein were separated; then a V type incision was made with an eye scissors for inserting PE-50 catheter whose anterior segment was improved [9]; this catheter went through the right atrium, the right ventricle, and to the pulmonary artery; finally pressure sensor was connected with biological detectors recording the results. And RVSP, RVDP and mPAP of each rat were calculated, according to which the PVR was indirectly calculated. After measuring the pressure of the pulmonary artery, CO of the rats should be measured by thermal dilution method. First, the PE-50 catheter was pulled back into the superior vena cava, and then similarly, the left carotid arteries were separated before being given an incision to insert a probe with heart displacement guide wire. This guide wire was inserted from the carotid artery to the ascending aorta, meanwhile, the ice salt water of 0.2~0.3 mL was injected into the right jugular vein simultaneously. The temperature curve on the recorder was observed to calculate the CO and CI of the rats.

Measurement of right ventricular hypertrophy index

After being killed, rats' hearts were taken out and the top atrial tissues were cut out, its right ventricle (RV) and left ventricular and septum (LV+S) were detached along the edge of the ventricular septum, and then after dried with filter paper, RV and (LV+S) were weighed, and their ratio was RVHI.

Measurement of microvascular density in lung

Nowadays, endothelial cell markers, such as CD31, VIII factor and CD34, are commonly used to assess microvessel density. CD34 is a highly glycosylated I type transmembrane protein, an adhesion molecule in cadherin family and also a kind of vascular endothelial cell marker with higher sensitivity and specificity [10, 11]. The left lower lung tissues of the death rats were taken and soaked in the 4% poly formaldehyde solution, dehydrated and cleared in graded ethanol and xylene, embed with paraffin, cut into pieces of 4 um thickness, conventionally dewaxed for aqueous phase, and sealed with 3% H_2O_2 conducted antigen retrieval, and sealed with 5% BSA; then gG-HRP CD34 primary antibody (1:200) was added before saving the lung

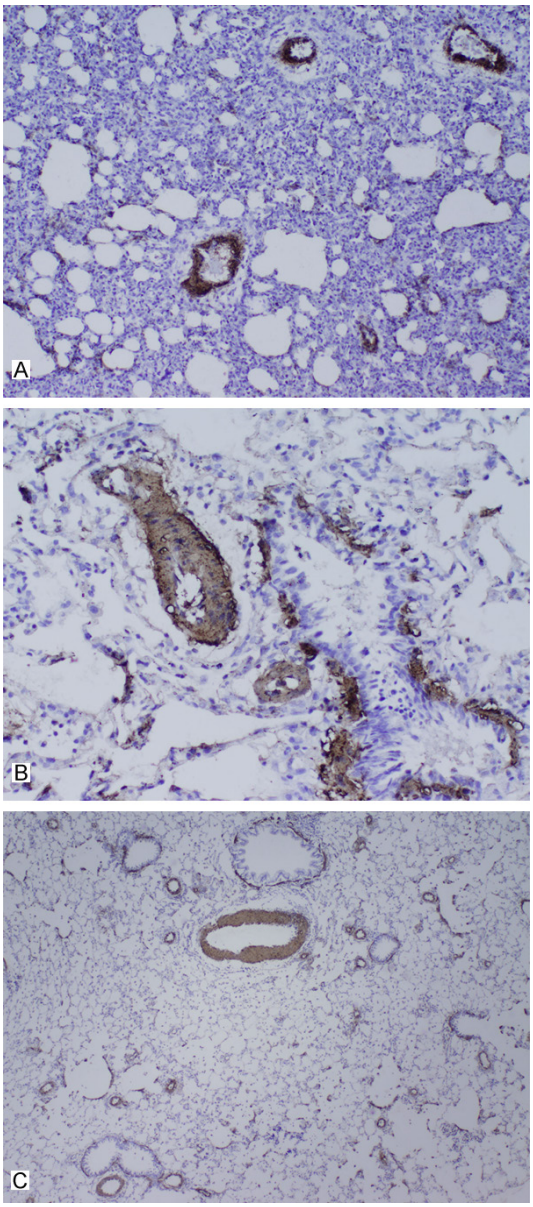

Figure 2. CD34 immunohistochemistry of lung tissue (1:200). Note: A: Blank group; B: Model group; C: Shenfuyixin granule group.

tissues for one night at 4°C; goat anti-rabbit antibody was added before preserving the lung tissues at 30°C for 20 min; SABC was added before keeping the lung tissues at 30°C for 20 min; then they were conducted DAB coloration and hematoxylin counter-staining followed by being mounted and examined under microscope. Weidner method was used in microvessel count [12], namely, when dealing with the embedded lung sections, immunohistochemical method was employed to do antigen staining towards CD34 factors; next, the region that gathers more vessels was identified at low power, 5 microvessel counts under 200 magnification were continuously read and recorded by converting the vessel density into per/mm^2, then the mean we got was MVD.

Statistical methods

The data results were expressed by mean ± standard deviation ($\bar{x} \pm s$), and the statistical software SPSS 19.0 was adopted. Single factor analysis of variance was used to perform the group comparison. $P<0.05$ indicates that there exists statistical difference.

Results

The characteristics of rats

Blank group: The fur, activity and spirit of the rats were as usual. When their chests were open, the color of the hearts and lungs were pink and elastic. Model group: The fur of rats was dark yellow and dull. Besides, they had less activities, poor appetite and weight loss, thickened right ventricles. Their hearts and lungs were dark red with poor elasticity and diffuse congestion points. Compared with the model group, the rats in the Shenfuyixin granule group had smoother fur, more frequent activities, better spirit, more stable respiratory and less congestion scattered at the lung, as shown in **Figure 1**.

mPAP, RVSP, RVDP, PVR, CO, CI, and RVHI

Compared with the blank group, RVSP, RVDP, mPAP, RV/(LV+S) and PVR in model group were significantly elevated ($P<0.05$), while CO and CI were lower ($P<0.05$). Compared with model group, RVSP, RVDP, mPAP, RV/(LV+S) and PVR in Shenfuyixin granule group decreased ($P<0.05$), while CO and CI were higher ($P<0.05$). Results are shown in **Tables 1** and **2**.

Microvessel density (MVD) of lung tissue

Results showed that both the MVD values of Shenfuyixin granule group and model group were lower than that of blank group ($P<0.05$), and the MVD value of Shenfuyixin granule group was significantly higher than that in model group ($P<0.05$), as shown in **Figures 2**, **3** and **Table 3**.

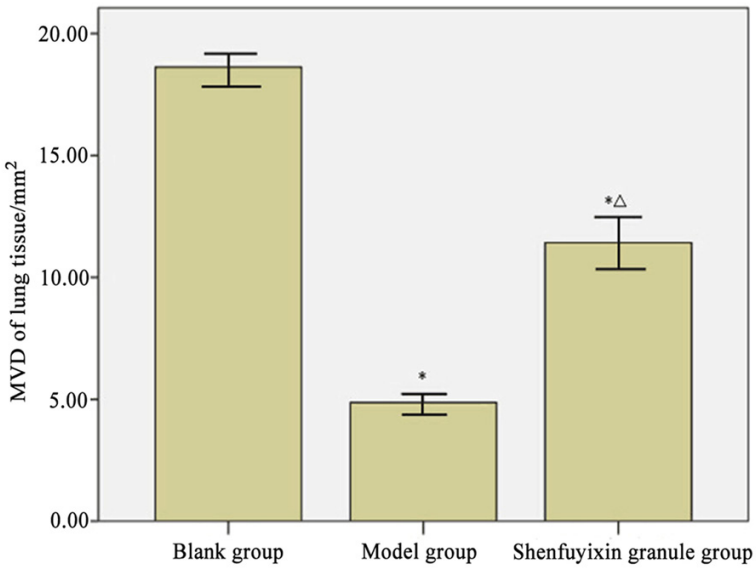

Figure 3. MVD of lung tissues (number/mm^2).

Table 3. Comparison of microvessel count among groups (number/mm^2) ($\overline{x} \pm s$)

Group	n	MVD (number/mm^2)
Blank group	10	18.61±0.77
Model group	10	4.86±0.52*
Shenfuyixin granule group	10	11.42±1.45$^{\Delta}$

Annotation: *indicates that "*P*<0.05" in the comparison between model group and blank group; $^{\Delta}$indicates that "*P*<0.05" in the comparison between Shenfuyixin granule group and model group.

Discussion

PAH is a kind of malignant pulmonary disease caused by many reasons, which is mainly characterized by the progressive rising of pulmonary arterial pressure and pulmonary vascular resistance. The principle of Chinese traditional treatment is mainly "warming Yang and benefiting Qi, promoting blood circulation and inducing diuresis". Shenfuyixin granule (formerly Xinshuaikang granule) is developed by the old famous herbalist doctor with years' experience in Henan University of Chinese Medicine. In its formulation, there is ginseng to supplement Qi, monkshood and cassia twig to warm Yang, salvia miltiorrhiza and motherwort to improve blood circulation for removing blood stasis, polyporus, alisma, and pepperweed seed to dispel dampness and promote diuresis, as well as jujube and villous amomum to fortify the spleen and disinhibit dampness. All of these fully embody the treatment principle of "warming Yang and benefiting Qi, promoting blood circulation and inducing diuresis". The research results show that Shenfuyixin granule can not only significantly reduce the ANP and BNP levels in the plasma [13] but also decrease the mass index number of the left ventricular and regional myocardial AngII [14]. What's more, it has the effect of delaying or reversing the myocardial remodeling [15]. Salvia miltiorrhiza, radix paeoniae rubra and motherwort in the formulation can promote blood circulation to remove blood stasis. Published researches show that salvia miltiorrhiza can promote myocardial angiogenesis [16] and radix paeoniae rubra can promote tumor angiogenesis [17]. As such, salvia miltiorrhiza and radix paeoniae rubra can promote pulmonary angiogenesis.

Rats in model group and Shenfuyixin granule group were injected monocrotaline, which simulated the change of pulmonary hypertension both in appearance and hemodynamics. In addition, the symptoms were basically in line with the symptoms of deficiency of lung Qi and phlegm stasis syndrome in traditional Chinese medicine, suggesting that the preparation of animal model for the experiment was successful. One week after injection of monocrotaline, the rats, except those in the blank group, all reduced activity and food intake, and cowered in different degrees. Two weeks later, their fur was dry and their activities were significantly reduced. They had weight loss and even wheezing symptoms. Three weeks later, the symptoms became more serious. Their noses and lips have cyanosis, and some even had severe right heart failure or death. After the drug intervention, the spirit of rats in Shenfuyixin granule group was improved. Moreover, they have smoother hair, more activities, and steady breath.

The results of this study show that the related indexes of hemodynamics in the Shenfuyixin granule group were significantly lower than

those of the model group, which has statistical significance ($P<0.05$). The change trend of mPAP is consistent with the results of studies by Huang et al. [18] who use sildenafil treatment and Ogawa et al. [19] who adopt right cardiac catheterization, suggesting that Shenfuyixin granule can effectively reduce the pulmonary artery pressure in rats with pulmonary hypertension. The CO and CI in Shenfuyixin granule group were higher than those of the model group, which is consistent with the results of that Fan Youfei [20] uses Vardenafil treatment to promote CI and CO and decrease mPAP and PVR, that Sun Yunjuan [21] uses Sildenafil treatment which obviously decreases mPAP and elevates CI, and that Jiang et al. [22] use fasudil to decrease mPAP and elevate CO. In this experiment, the indicators measured in the Shenfuyixin granule group are in line with the experiment results of Lijun Dong [23], indicating that Shenfuyixin granule can obviously improve the pulmonary arterial pressure and heart function of the rats.

At the same time, the microvessel density in Shenfuyixin granule group was significantly higher than that of model group. Using Qizhizhoufei granule in rats, Huang Renhe et al. [24] prove that MVD is an important index reflecting angiogenesis. Consistent with the experiment that Lin Qun et al. [25] use MSCs transplantation to improve the lung microvessel density of pulmonary hypertension rats, this result suggests that Shenfuyixin granule's effect on hemodynamic parameters of hypertension rats has a relationship with its role in promoting angiogenesis.

To sum up, Shenfuyixin granule has significant effect on hemodynamic indicators of rat with pulmonary hypertension that is induced by monocrotaline and it effectively alleviates symptoms of pulmonary hypertension, but the specific mechanism needs to be further studied.

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Disclosure of conflict of interest

None.

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