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Original article

The effects of low-molecular-weight heparin on lung and pulmonary artery injuries in acute pulmonary embolism rat model via platelet-derived growth factor- β



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ABSTRACT

Objective: To evaluate the effects of anticoagulant agent (low-molecular-weight heparin, LMWH) on the pulmonary artery intima hyperplasia of rats with acute pulmonary embolism (APE) by assaying platelet-derived growth factor- β (PDGF- β).

Methods: A total of 90 Sprague-Dawley rats were randomly assigned into the sham, APE, and LMWH groups with 30 rats in each group. The APE rat models were established by injecting autologous blood clots via external jugular veins. In each group, six mice were sacrificed at the 1st day (D1), 4th day (D4), 7th day (D7), 14th day (D14), and 28th (D28) subsequent to the induction of APE to collect the lungs. Right ventricle pressure (RVP) and mean pulmonary arterial pressure (mPAP) were measured. Western blot and RT-PCR analyses were used to assess PDGF- β expression at various time points. In addition, changes in lung pathology were evaluated using hematoxylin and eosin (H&E) staining and electron microscope.

Results: The overall success rate of establishing APE rat models was 85.7% (60/70). There was no difference in mPAP between the sham group and the APE group at the D1, D4, D7, and D14. However, at the D28, mPAP in the APE group was significantly higher than that in the sham group. There was no difference among the three groups regarding RVP. PDGF- β expression were decreased in the LMWH group at all time points compared with the sham and APE groups ($P < 0.01$). Furthermore, pulmonary embolism, alveolar wall necrosis and hemorrhage, and inflammation were significantly attenuated in the LMWH group compared with the sham and APE groups subsequent to the induction of APE.

Conclusion: LMWH attenuates lung and pulmonary artery injuries and improves prognosis. Decreased PDGF- β in the lungs may be the important factor in the effects observed.

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1. Introduction

Acute pulmonary embolism (APE) is a common cause of pulmonary hypertension (PH) contributing to right heart failure which has a high rate of mortality (Benotti and Dalen, 1984).

Traditionally, the degree of mechanical obstruction of the pulmonary vascular by embolic thrombi was considered to be the major determinant of the increase in PH (Fedullo et al., 2001). However, it has been recently shown that pulmonary vascular reconstruction in response to APE whose mechanism is mainly migration of vascular smooth muscle cells (SMCs) to the pulmonary artery intima and proliferation under the influence of cytokines (Wang et al., 2015; Liu et al., 2015; Zagorski et al., 2003).

It has been shown that heparin and low molecular weight heparin (LMWH) have resistant effects against SMC proliferation (Downing et al., 1998). Therefore we hypothesize that LMWH might be able to inhibit pulmonary artery intima hyperplasia. The objective of this study is to determine if APE and pulmonary artery intima hyperplasia are affected by LMWH.

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2. Materials and methods

2.1. Materials and experimental animals

2.1.1. Experimental animals

A total of 90 male Sprague-Dawley rats weighting 250 ± 20 g were purchased from Institute of Laboratory Animals of Chinese Academy of Medical Science. The mice were kept in Laboratory Animal Center of Peking Union Hospital and had free access to water and food. Those mice were randomly divided into the sham operation group, acute pulmonary embolism (APE) group, and low molecular heparin (LMWH) treatment group, with 30 rats in each group. 30 rats in each group were further divided into the 1st day (D1), the 4th day (D4), the 7th day (D7), the 14th day (D14), and the 28th (D28), with 6 mice in each group.

2.1.2. The major instruments and reagents

Cmias 2001B multifunctional color pathological image analysis system (Beihang University, China); IM1000 ECG monitor (COULD Inc, the United States); JE M1010 transmission electron microscopy (TEM) (JEOL Inc, Japan); Twin Block PCR thermal cycle system (ERICOMP Inc, the United States); GAA7001B gel image analysis system (UVI Inc, the United Kingdom). Fraxiparine (Low-Molecular-Weight Heparin Sodium Injection, 9500 IU anti-Xa/ml according to European pharmacopoeia, Sanofi Pharma, France). Rabbit anti-rat PDG- β polyclonal antibody (Wuhan Boster Biotechnology Company, China).

2.2. Methods

2.2.1. Establishment of rat APE model

The APE model was produced using the autologous blood clot method. Briefly, blood was drawn from the tail veins of the rats. After coagulation, blood clots were in 70°C water bath for 10 min and then cut into a size of $1.1\text{ mm} \times 2\text{ mm}$. 25 autologous blood clots followed by 2 ml normal saline were injected. At the 24 h post-operation, rats in the LMWH treatment group were given subcutaneous injection of LMWH (Fraxiparine, 0.01 ml/kg), Q12h, for 14 consecutive days. Rats in the sham and APE groups were given subcutaneous injection of the same amount of normal saline. All the rats had free access to water and food after operation.

2.2.2. Measurement of right ventricle pressure (RVP) and mean pulmonary arterial pressure (mPAP)

The cannula was inserted into the pulmonary artery through the right ventricle and placed tightly around the trunk of the pulmonary artery. The lungs were perfused with a peristaltic pump (Incibras, Sao Paulo, Brazil; 9 mL/min) by inserting the left ventricle into the left ventricle and ligating the pulmonary vein to the reservoir at the vertex of the heart. Another ligation is placed above the atrioventricular node to prevent the infusion mixture from flowing into the ventricle. The perfusate mixture was maintained at 37°C by means of a heat exchanger. Pulmonary arterial intubation pressure transducers (COBE, Arvada, CO) were measured at the horizontal position of pulmonary artery intubation and the mean pulmonary arterial pressure was measured from the side of the fluid intubation.

2.2.3. Expression of SMCs and PDGF- β in lung tissues

On day 1 (D1), day 4 (D4), day 7 (D7), day 14 (D14) and intramuscular injection of 10 aqueous chloral hydrate solutions were performed on the first day (D1), day 1 (day 6) D28). Next, lung tissue was quickly removed, and a small piece of lung was routinely embedded in paraffin for immunohistochemical staining of SMC and PDGF-[beta]. Four lung sections of each rat were analyzed,

and three high power fields were randomly selected from each site to calculate the mean. Each group of lung tissue was fixed with 10% formaldehyde for 24 h. The paraffin-embedded sections were cut into sections, stained with hematoxylin-eosin, and analyzed by an experienced pathologist under an optical microscope.

2.2.4. Measurement of pulmonary vessels

Five cross sections of pulmonary artery was selected for each mouse. Cmias2001B multifunctional true color pathological image analysis system was used to measure the vessel tube areas, lumen areas, wall areas, tube diameters, wall thickness, wall area to tube area ratios, lumen area to tube area ratios, vessel wall thickness, and vessel diameters. In order to make the size of the pulmonary artery not affect the comparison, wall area to tube area ratios and vessel wall thickness to tube diameter ratios were selected as statistical indexes.

2.2.5. Measurement of PDGF- β mRNA expression in lung tissues

RNA was extracted from lung tissues, PDGF- β was amplified using RT-PCR. PCR primers (Shanghai Sangon biotech company, Shanghai, China) included rat β -actin upstream primer 5'CCA AGG CCA ACC GCG AGA TGA C 3', downstream primer 5'AGG GTA cat GGT GGT GCC GCC AGA C 3'. PCR product length was 587 bp; rat PDGF- β upstream primer 5' GAT CCG CTC CTT TGA TGA TC 3', downstream primer 5'GTC TCA CAC TTG CAT GCC AG 3', PCR product length was 435bp.

PCR amplified products underwent 2% agarose gel electrophoresis and was scanned by UVI gel image scan and analyze system using UVI soft UVIB and Application V97.04 analysis software.

2.3. Statistical analysis

Statistical analysis was performed using SPSS 13.0. The data were expressed as the mean \pm standard deviation (SD). Differences were analyzed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test; values of $P < 0.05$ were considered statistically significant. The $2^{-\Delta\Delta\text{Ct}}$ method was used for the RT-PCR analysis.

3. Results

3.1. Establishment of rat APE model

A total of 70 rats were injected with autologous clot. Two rats died during anesthesia. Two rats died during injection. Four and two rats died 1 and 3 h post-injection respectively. Therefore, there were 60 rat APE models established. The success rate was 85.7%. All rats had shortness of breath, cyanosis, and increased heart rate after injection.

3.2. Measurement of RVP and mPAP

There was no difference in mPAP between the sham group and the APE group at the D1, D4, D7, and D14. However, at the D28, mPAP in the APE group was significantly higher than that in the sham group (33.17 ± 7.99 vs 24.80 ± 5.54 mmHg, $p = 0.08$) (Table 1). Regarding RVP, there were no differences among the three groups (Table 2).

3.3. Lung tissue pathology and pulmonary artery changes

In the sham group, the pulmonary vascular structure was normal. There was no bleeding and exudation in alveolar and no inflammatory cell infiltration in alveolar septum. At the D1, there were visible emboli and secondary thrombosis found in pulmonary

Table 1
mPAP among the three groups ($\bar{x} \pm s$, mmHg).

	d ₁ (n = 6)	d ₄ (n = 6)	d ₇ (n = 6)	d ₁₄ (n = 6)	d ₂₈ (n = 6)
Sham	25.5 ± 4.65	31.4 ± 8.53	30.6 ± 3.91	25 ± 5.83	24.8 ± 5.54
APE	26.8 ± 1.30	28.6 ± 2.51	30.2 ± 3.63	26 ± 7.56	33.17 ± 7.99
LMWH	28.6 ± 2.70	30.4 ± 8.26	29.6 ± 4.45	27.5 ± 3.62	25 ± 4.29

Table 2
RVP among the three groups ($\bar{x} \pm s$, mmHg).

	d ₁ (n = 6)	d ₄ (n = 6)	d ₇ (n = 6)	d ₁₄ (n = 6)	d ₂₈ (n = 6)
Sham	44.5 ± 3.54	50.25 ± 4.5	52 ± 3.54	41.2 ± 5.85	46.6 ± 11.24
APE	56.2 ± 14.53	47.6 ± 1.52	48.5 ± 7.05	45.83 ± 8.86	55.5 ± 15.35
LMWH	50.2 ± 7.85	52.6 ± 12.64	50.8 ± 5.54	47.33 ± 4.63	47.5 ± 3.99

arteries in the APE group. At the D4, there was partial dissolution of thrombus and a lot of white blood cells accumulated (Fig. 1); At the D7, most thrombi were dissolved, a few of them which did not dissolve had polymorphonuclear cells and macrophages infiltration and angiogenesis; At the D14, only a few thrombi were not dissolved; At the D28, all thrombi were completely dissolved. Pulmonary artery intima occurred hyperplasia from the D4 until the D28. There was severe vascular stenosis (Fig. 2); At the D1, there were a lot of alveolus exudation and infiltration of inflammatory cells in alveolar septa; At the D4 and D7, alveolar edema was relieved. But there were still a large number of inflammatory cells accumulated in alveolar septa; At the D14 and D28, alveolar structure was basically normal. Interstitial inflammatory cells decreased, but were still more than that in the sham group. The pulmonary artery intimal hyperplasia and vascular stenosis were much less in the LMWH group than the APE group; At the D1, alve-

olar exudation and interstitial inflammatory cell infiltration in the LMWH group were very similar with the APE group. At the D4, alveolar exudation was not obvious. There was still infiltration of inflammatory cells in alveolar septa, which was less severe than the APE group. The pulmonary artery wall area to tube area ratios and wall thickness to tube diameter ratios are shown in Tables 3 and 4.

3.4. Electron microscope results

The pulmonary artery smooth muscle cells (SMCs) in the sham group were spindle-shaped. There were very few organelles in the cytoplasm. The pulmonary artery SMCs in the PTE group were dumbbell-shaped. The cytoplasm was abundant. The muscle fiber was decreased. And some of the vacuoles were degenerated (Fig. 3). The morphology of pulmonary artery SMCs in the LMWH group was normal (Fig. 4).

3.5. PDGF- β immunohistochemistry results

PDGF- β was primarily expressed in the pulmonary artery SMCs. A small amount of it was expressed in alveolar macrophages. There was no expression in the pulmonary vascular endothelial cells. The expression reached the peak at the 4th day after pulmonary embolism (Fig. 5A) and gradually decreased after one week. There was a small amount of PDGF- β expression in the pulmonary artery of the sham group, but no expression in the lung tissue at all.

3.6. PDGF- β protein expression in lung tissues

There was significant increase of PDGF- β protein expression from the 1st to the 14th day after pulmonary embolism which actually reached its peak at the 7th day ($P < 0.05$). The expression level at the 28th day was similar with that of the sham group. The expression levels in the LMWH group were less than the APE group at the 4th, 7th, and 14th days ($P < 0.05$), but were still more than the sham group ($P < 0.05$) (Fig. 5C).

3.7. PDGF- β mRNA expression in lung tissues

There was a small amount of PDGF- β mRNA expression in lung tissue of the sham group. The expression was significantly increased in the APE group and reached the peak 4 days after pulmonary embolism ($P < 0.01$) but decreased at the 7th and 14th days which were still higher than the sham group ($P < 0.05$) until the 28th day. PDGF- β mRNA expression in the LMWH group declined at the 4th, 7th, and 14th days compared with the APE group ($P < 0.05$) (Fig. 5B and D).

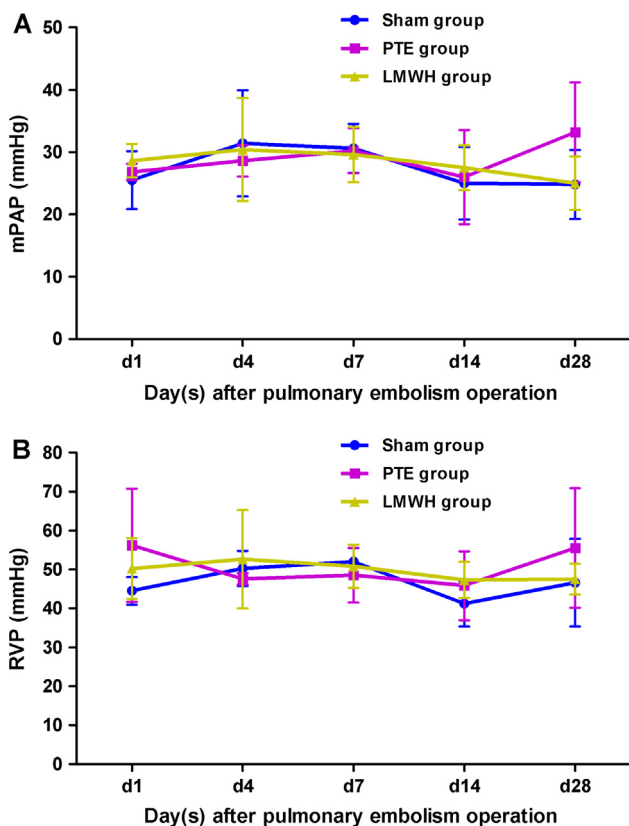


Fig. 1. The changes of mPAP and RVP after pulmonary embolism operation. There was no difference among three groups at the all time points.

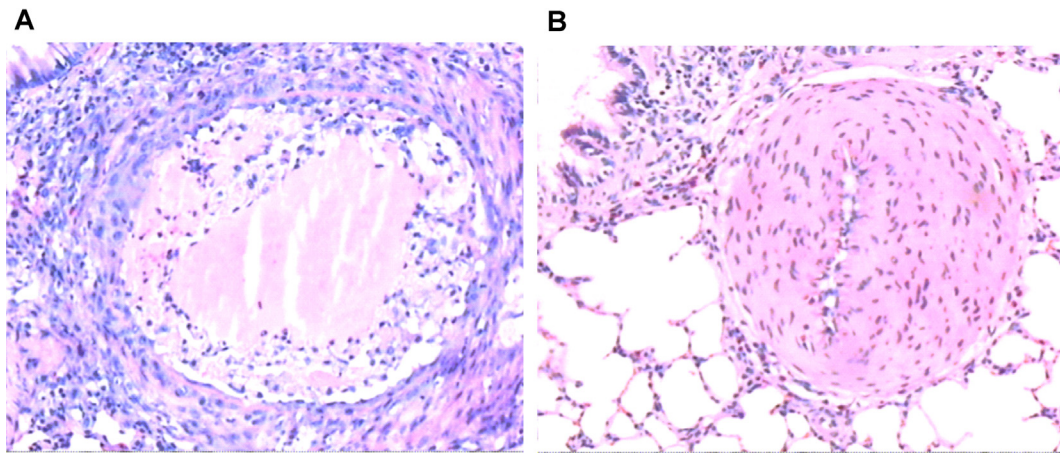


Fig. 2. The cross sections of lungs (HE \times 100). (A) Four days after APE, thrombi was partially dissolved. There were a lot of white blood cells infiltration. (B) 14 days after APE, there were pulmonary artery intima hyperplasia and vascular stenosis.

Table 3
Wall area/tube area ratios among the three groups ($\bar{x} \pm s$).

	d ₁ (n = 6)	d ₄ (n = 6)	d ₇ (n = 6)	d ₁₄ (n = 6)	d ₂₈ (n = 6)
Sham	0.68 \pm 0.15	0.65 \pm 0.08	0.63 \pm 0.02	0.60 \pm 0.08	0.56 \pm 0.03
APE	0.65 \pm 0.12	0.78 \pm 0.07 [*]	0.71 \pm 0.03 [*]	0.71 \pm 0.07 [*]	0.61 \pm 0.03 [*]
LMWH	0.52 \pm 0.03	0.68 \pm 0.09 [#]	0.64 \pm 0.06 [#]	0.55 \pm 0.11 [#]	0.52 \pm 0.05 [#]

^{*} p < 0.05 APE vs sham.

[#] p < 0.05 LMWH vs APE.

Table 4
Wall thickness/tube diameter ratios among the three groups ($\bar{x} \pm s$).

	d ₁ (n = 6)	d ₄ (n = 6)	d ₇ (n = 6)	d ₁₄ (n = 6)	d ₂₈ (n = 6)
Sham	0.39 \pm 0.10	0.31 \pm 0.01	0.35 \pm 0.04	0.34 \pm 0.06	0.30 \pm 0.02
APE	0.40 \pm 0.10	0.50 \pm 0.10 [*]	0.42 \pm 0.04 [*]	0.43 \pm 0.05 [*]	0.35 \pm 0.03 [*]
LMWH	0.30 \pm 0.03	0.40 \pm 0.05 [#]	0.36 \pm 0.04 [#]	0.31 \pm 0.09 [#]	0.28 \pm 0.04 [#]

^{*} p < 0.05 APE vs sham.

[#] p < 0.05 LMWH vs APE.

4. Discussion

This study used autologous blood clot method to establish the rat pulmonary embolism model which had several advantages including small trauma, fixed embolus size and number to guarantee the success and stability of the APE model. The success rate of our method was 85.7% which demonstrated that it was a successful method.

Without underlying lung diseases, the blocking area of pulmonary vascular bed is strongly correlated with mPAP, RVP, and PaO₂ (Henke et al., 2005). In general, when 25–30% pulmonary vascular bed is blocked, mPAP rises in certain degree; when 50% pulmonary vascular bed is blocked, mPAP rises significantly; when 75% pulmonary vascular bed is blocked, mPAP will rise to result in right heart failure (Ware and Heistad, 1993). In our experiment, the average mPAP was not increased significantly due to relatively small blocked area of pulmonary vascular bed and relatively good cardiopulmonary function of rats at the baseline. Although the pathology showed that there was pulmonary artery intimal hyperplasia 28 days after pulmonary embolism, mPAP does not rise significantly suggesting the pulmonary artery intima hyperplasia caused by pulmonary vascular remodeling after an acute embolism events does not play a major role in the formation of pulmonary hypertension. To the contrary, recurrence of pulmonary embolism and uncomplete dissolution of some embolus resulting in

pulmonary vascular reconstruction is the key factors that lead to chronic pulmonary hypertension.

Eagleton et al. (2002) found that neutrophils began to infiltrate the pulmonary artery wall 3 h from APE for 2 days and returned to baseline at 8 days after APE. One day after APE, macrophages accumulated to the peak, and 4 days after APE returned to baseline. APE is also associated with APE 4 days after the obvious pulmonary artery intimal hyperplasia, sustained 14 days (Smulders, 2000). In our study, we found that white blood cells including neutrophils and macrophages infiltrated pulmonary wall beginning at 1 day after APE, gradually increased as embolus was dissolving, and was persistent by 7 days after APE, which was consistent with Eagleton et al.'s results. In addition, we found that white blood cells was present not only in pulmonary artery wall and embolus but also in distant pulmonary tissues suggesting that PE-caused inflammation might be more general than expected.

Previous study showed that 66% patients had incomplete recovery of lung perfusion 3 months after APE which was even treated with antithrombotic agents. We hypothesize that pulmonary vascular reconstruction is the key factors that result in lung perfusion deficiency. Our study showed that compared to the sham group, the wall area/tube area ratio and wall thickness/tube diameter ratio of the PTE group were significantly higher, apparent at 4 days after APE and persisting through 28 days. After LMWH treatment, the wall area/tube area and wall thickness/tube diameter ratios

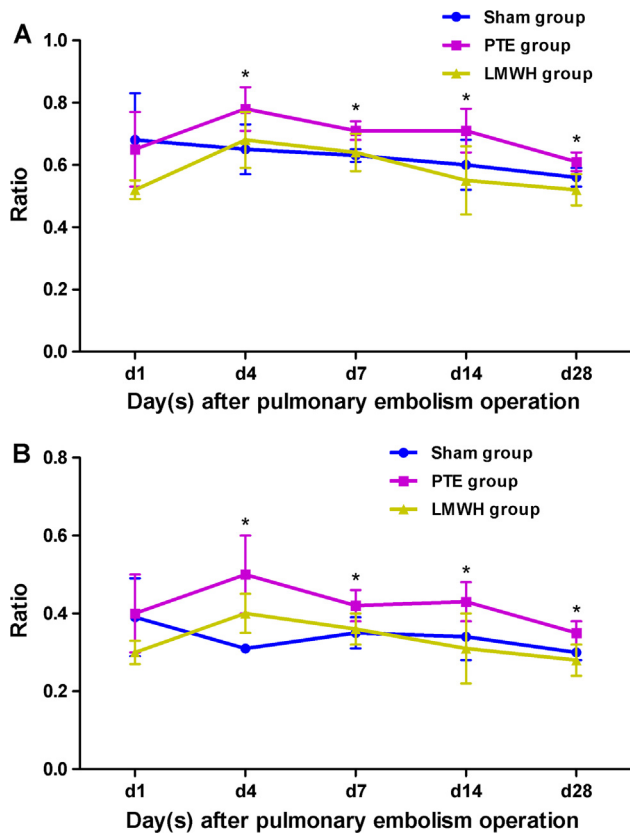


Fig. 3. The changes of wall area/tube area ratios and wall thickness/tube diameter ratios after pulmonary embolism operation. (A) The changes of wall area/tube area ratios. * compared with the sham and LMWH groups, the PTE group was significant higher, $P < 0.05$; (B) the changes of wall thickness/tube diameter ratios. * compared with the sham and LMWH groups, the PTE group was significant higher, $P < 0.05$.

were significantly decreased than the PTE group. The above findings showed that APE resulted in pulmonary artery intimal hyperplasia which was inhibited by LMWH.

Vascular smooth muscle cells (VSMCs) phenotype has the characteristics of diversity and variability. This study found that pulmonary artery VSMCs translated from deflation phenotype to synthetic phenotype after APE, peaked at 14 days after APE. APE can cause release of a variety of cytokines and inflammatory mediators including TXA_2 , AT-II, PDGF, and bFGF etc. Under the stimulus of above factors, pulmonary artery smooth VSMCs

migrate to intima and translate into synthetic phenotype. In addition, extracellular matrix deposition resulted in pulmonary artery intima hyperplasia. This study showed that LMWH can reduce the degree of pulmonary artery stenosis after APE suggesting LMWH inhibits the translation of pulmonary artery VSMCs from deflation phenotype to synthetic phenotype.

Studies have shown that PDGF is associated with hypoxic pulmonary VSMCs proliferation and post-PTCA restenosis. Haruno et al. (1999) found that arterial endothelium PDGF- β expression level significantly increased 14 days after balloon injury on rat artery. Our study showed that PDGF- β mRNA and protein expression levels significantly increased within 28 days after pulmonary embolism.

PDGF- β is primarily expressed by pulmonary artery VSMCs and alveolar macrophages. Tanizawa et al. (1996) reported that PDGF- β expression mainly is located in the α -actin negative spindle cells and macrophages at the early stage of post-PTCA. When the neointima was composed mainly of α -actin positive smooth muscle cells, PDGF- β expression significantly decreased. Our findings are consistent with the above results regarding the location and timeline of PDGF- β expression.

This study suggests that LMWH can reduce pulmonary artery intimal hyperplasia after APE, therefore has the anti-proliferation effects on VSMCs. This may be due to PDGF-B and thrombin promoting the proliferation of VSMCs. Rothman et al. (1994) studied the effects of thrombin and PDGF on rat pulmonary arterial smooth muscle cell line (PAC1). Over 72 h, thrombin (1 unit/ml) resulted in $102 \pm 12\%$ increase in protein synthesis per cell. PDGF stimulated proliferation as evidenced by an increase in cell number (doubling in 5 days). Therefore, thrombin caused cell hypertrophy while PDGF caused cell proliferation. They cooperate with each other to translate VSMCs from deflation phenotype to synthetic phenotype (Zhao and Ashraf, 2016; Sullivan et al., 2001; Huang et al., 2015).

In conclusion, this study found that rats had severe pulmonary artery intima hyperplasia 4–28 days after APE. The underlying mechanism might be increase of synthesis and release of cytokines including PDGF- β under whose influence VSMCs translate from deflation phenotype to synthetic phenotype resulting in neointimal formation. LMWH can inhibit PDGF- β mRNA and protein expression, thus improve hypoxemia after APE, reduce the proliferation-promoting effects of PDGF- β on pulmonary artery VSMCs to inhibit pulmonary artery intimal hyperplasia after APE.

There are several limitations of this study. First of all, a lot of factors are involved in the formation of intima hyperplasia after APE. We only conducted preliminary studies on PDGF-B. Further studies should be done on other cytokines including MCP-1,

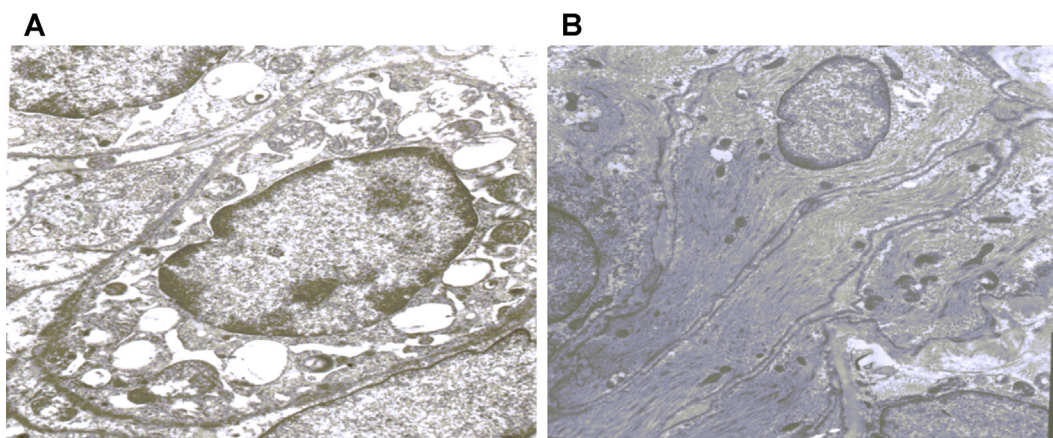


Fig. 4. The images from electron microscope. (A) 14 days after APE, the nucleoli of pulmonary artery smooth muscle cells enlarged, cytoplasm was abundant. Rough endoplasmic reticulum and mitochondria increased. Part of the vacuolar degenerated. $\times 6000$; (B) 14 days after APE, pulmonary artery SMCs morphology was normal in the LMWH group $\times 5000$.

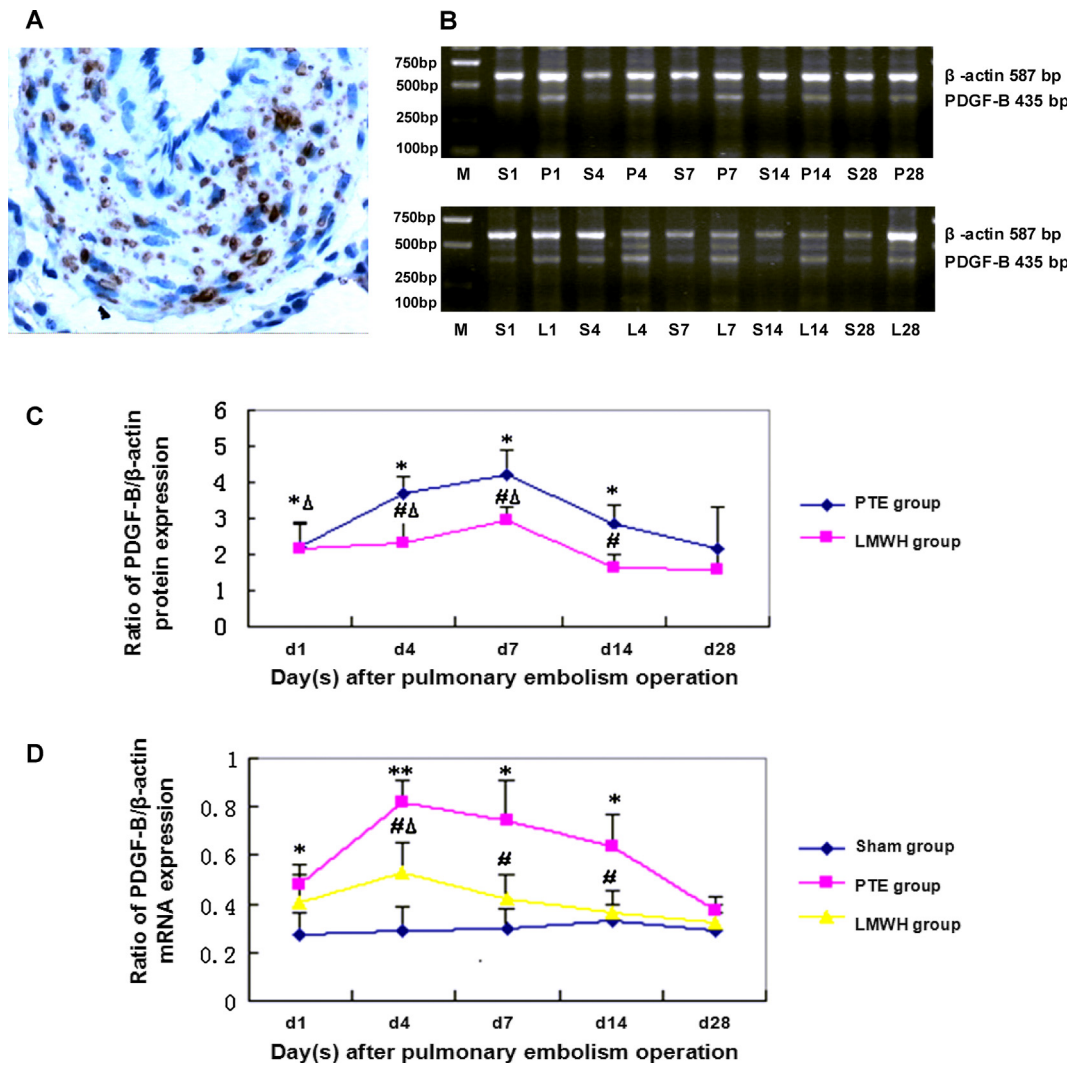


Fig. 5. Expression of PDGF-B. (A) 4 days after APE, there were a lot of PDGF-B expression in pulmonary artery wall. $\times 400$; (B) expression in the sham and APE groups at the different time points. Expression in the LMWH and PTE groups at the different time points. M, S, P, L represent DNA marker, Sham, APE, and LMWH respectively 1, 4, 7, 14, 28 represent the days post pulmonary embolism $^*p < 0.05$ PTE vs sham; $^{\#}p < 0.05$ LMWH vs PTE; $\Delta p < 0.05$ LMWH vs sham; (C) expression of PDGF-B protein in the LMWH and PTE groups, $^*p < 0.05$ PTE vs sham; $^{\#}p < 0.05$ LMWH vs PTE; $\Delta p < 0.05$ LMWH vs sham; (D) expression of PDGF-B mRNA among the three groups, $^*p < 0.05$ PTE vs sham, $^{**}p < 0.01$ PTE vs sham; $^{\#}p < 0.05$ LMWH vs PTE; $\Delta p < 0.05$ LMWH vs sham.

ET-1, and AT II, etc. Second, due to the differences between species, the role of PDGF - B in the process in human pulmonary vascular remodeling might be different from it in rats. Therefore whether LMWH can inhibit pulmonary arterial intima hyperplasia in human being needs further investigation.

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