rhCygb reverses bleomycin-induced established pulmonary fibrosis in rats

Li Zhen  
Southern Medical University

Lin Zhongshun  
Southern medical university

Wang Ping  
Southern medical university

Wenqi Dong (jenny08@smu.edu.cn)  
Southern Medical University  https://orcid.org/0000-0003-4930-8279

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Abstract

Purpose

Recombinant human cytoglobin (rhCygb) has been demonstrated to anti-inflammation, anti-oxidation, anti-fibrosis in liver and kidney in animal disease models. However, the effect of rhCygb on the progression of pulmonary fibrosis is still unclear. The aim of this study was to investigate the therapeutic effect of rhCygb in the bleomycin (BLM)-induced pulmonary fibrosis rats.

Methods

We tested whether rhCygb would reverse lung fibrosis (at day 28 and 56) in Sprague-Dawley rats treated with bleomycin. Bleomycin (5mg/kg) resulted in fibrosis by CT and serum measurement at day 7. Effects of rhCygb treatment on morphological and CT imaging of the lung, as well as serial serum levels of biomarkers with progressive lung fibrosis were tested at day 28 and 56.

Results

In BLM-treated rats (7 days), the well-established lung fibrosis was evidence by changes in rat lungs with CT images and serum levels of hyaluronic acid (HA), laminin (LN), procollagen III N-terminal peptide (PIIINP) and type IV collagen (IVC).After treatment with rhCygb for 49 days, we found significantly decreasing in HA, LN, PIIINP and IVC levels almost to controls. Hydroxyproline (HYP), CT mean lung density (MLD) and Masson collage volume fraction (CVF) were also significantly reduced. Furthermore, CT MLD positively correlated with serum levels of HA, LN, PIIINP, IVC, and HYP, and especially with CVF.

Conclusion

rhCygb may be a new potential medicine for reversing lung fibrosis in the future.

Background

Idiopathic pulmonary fibrosis (IPF) is rare but fatal lung disease, which is characterized by chronic alveolar wall and interstitial fibrosis of lung tissue, causing progressive decline in lung function and respiratory failure.[1] Most patients with IPF may combine with pulmonary hypertension, emphysema or obstructive sleep apnea; the estimated median survival time is 2–5 years following diagnosis.[2] Typical high-resolution computed tomography (HRCT) of IPF displays fibrotic changes in both of the basal and peripheral lungs, with reticular opacities, honeycomb changes and ground-glass opacities.[3] An Official American Thoracic Society Workshop Report confirmed that there was a consensus opinion of the intratracheal BLM model as “the best-characterized animal model available for preclinical testing”. Although the molecular pathogenesis remains incompletely elucidated, an updated pathological mechanism is advocated that epithelial injuries and subsequent aberrant repair play key roles in its development and progression. As a result of alveolar epithelial cells damage, they secrete several
mediators to stimulate the migration, proliferation of fibroblasts and myofibroblasts, and the deposition of excessive extracellular matrix (ECM), leading to remodeling of a so-called “honeycomb” lungs.\[4\]

Extracellular matrix is a highly dynamic macromolecular structure that provides both three-dimensional tissues and function support to organ.\[5\] In the lung, the ECM is generally composed of fibrillar proteins, glycoproteins, glycosaminoglycans, and the basement membrane laminin. There are still some variants of ECM constituents which have not been characterized.\[6\] The lung ECM is confined to contribute to health and disease; thus, serum ECM molecules have been used to evaluate the progression of fibrosis and patient prognosis.\[7\–\8\] Yilang et al showed significantly higher serum levels of ECM molecules in IPF patients, involving laminin (LN), type IV collagen (IVC), procollagen III N-terminal peptide (PIIINP), and hyaluronic acid (HA), compared with healthy individuals; they pointed out that serum levels of LN, IVC, PIIINP and HA might indicate IPF progression and might be indicators for the severity of IPF.\[9\]

Cytoglobin (Cygb) is a member of the globin family, which was discovered in hepatic stellate cells in 2001.\[10\] The biological and pathophysiological importance has increased progressively in the past decade. It is associated with many physiological functions, including cytoprotection against oxidative and nitrosidative stress,\[11\–\12\] fibrotic stimulation,\[13\–\15\] and tumour suppression.\[16\–\18\] Recombinant human cytoglobin (rhCygb) is a biologically synthesized protein. It has been reported to be an active oxygen scavenger as well as a functional enzyme like NADH oxidase and catalase.\[19\]

In the previously study, we found rhCygb had the efficacy of preventing atherosclerosis, protecting against chronic liver disease and liver fibrosis.\[20\–\23\] Therefore, in this study, we intended to investigate the efficacy of rhCygb in a lung fibrosis rat model induced with BLM.

### Methods

Male rats (Sprague-Dawley, SD) with an average weight of 180–220 g were purchased from the Guangdong Medical Laboratory Animal Center, China. Care of the animals in this investigation was done according to the guidelines approved by the Chinese Association of Laboratory Animal Care. Rats were housed in groups of 5 per cage under constant temperature (20 ± 2°C) and humidity (70%) with a 12 h light-dark cycle and had free access to food and water. The animals were acclimatized for 1 week prior to the experiments. All animal protocols were approved by the Ethics Committee for Animal Research of Southern Medical University.

To induce pulmonary fibrosis, rats were anesthetized with 2% pentobarbital (50 mg/kg body weight) and placed on an intubation stand facing upward at an angle of approximately 30-35 by using an elastic string carefully positioned under the animal’s front incisors. The tongue was gently pulled out with forceps and the trachea was intubated. BLM-treated rats were intratracheally instilled with 5 mg/kg bleomycin sulfate (Nippon Kayaku, Tokyo, Japan ) solution in 0.9% saline as previously described \[24\], and slowly instilled through the catheter into the trachea. The control rats simultaneously received the same volume of 0.9% saline solution without bleomycin.
SD rats were randomly allocated to the following groups:

- SD rats receiving a single intratracheal instillation of saline solution and sacrificed after 56 days (n = 11);
- SD rats receiving a single intratracheal instillation of BLM (5 mg/kg) and sacrificed after 56 days (n = 11);
- SD rats receiving a single intratracheal instillation of BLM (5 mg/kg), and 7 days later, treated with rhCygb (4 mg/kg, daily, by subcutaneous) for 49 days (n = 12);
- SD rats receiving a single intratracheal instillation of BLM (5 mg/kg), and 7 days later, treated with DXMS (3 mg/kg, daily, by subcutaneous) for 49 days (n = 11);

A general schedule of the treatment is shown in Figure 1. The rats were scanned with multi-slice spiral CT at day 7 and 56. Finally, the rats were sacrificed and specimens of lung were harvested and processed for subsequent analyses. Furthermore, the rats were subjected to measurement of biomarkers at day 7, 28 and 56. Finally, rats were sacrificed with a sealed euthanasia device, before that, carbon dioxide was put into the device, so that the rats could enter anesthesia faster with reduced fear and pain. Then specimens of lung were harvested and processed for subsequent analyses. Furthermore, the rats were subjected to measurement of biomarkers at day 7, 28 and 56.

**Drug**

rhCygb was produced according to the method described previously. It was dissolved in phosphate buffer saline and administered at 4 mg/kg subcutaneously. Selection of this dose of rhCygb was based on our previous literature.

**Computed tomography analysis**

Seven days after BLM treatment, CT scan was performed for assessment of BLM-induced changes in lung density. Rats were scanned at 7 and 56 days postoperatively. The rats were placed in the prone position, under 2% vol. isoflurane anesthesia, and then underwent plain CT scan with multi-slice spiral CT (SOMATOM Emotion 16; Siemens, Erlangen, Germany). Plain CT scan conditions were as follows: 80 kV with 500µA, a pitch of 0.7 mm, slice thickness of 0.7 mm and acquisition time of 32 s, 512 × 512 matrix, FOV 9.6 cm, and 1000 projections per scan. Images were reconstructed and analyzed. Mean lung density (MLD) for the entire lung volumes for each rat was calculated using the Somaris/5VB10B software. The MLD was assessed with quantitative determination of the overall lung Hounsfield units (Hu). Before scanning, the MLD for air was calibrated as −1000, while the water was 0.

**Histological assessment of lung injury**

At day 56, rats were sacrificed after the CT scan were performed and the lungs were carefully isolated to histological analysis. The lungs were fixed with neutral buffered formalin (10%), dehydrated and embedded in paraffin, and then sectioned at 5 µm thickness. The slices were stained with hematoxylin
and eosin (H&E) and Masson trichrome staining (Sigma-Aldrich, Saint Louis, Unite States), and observed under a microscope (Seepack TX510, Shenzhen, China) for assessing lung injury. Masson trichrome staining was performed to detect left lung fibrosis. Four fields of each sample were randomly selected and collagen volume fraction (CVF) was assessed by Image-Pro Plus 6.0 to represent the degree of fibrosis in the left lung. CVF (%) refers to the percentage of the area stained positive for collagen relative to the total area in a field of view.\textsuperscript{[26]}

**Hydroxyproline content**

To obtain a quantitative measure of lung collage at the end of 56 days, hydroxyproline as the major component of collagen was measured in the lung tissues using the Hydroxyproline Assay Kit (Nanjing jiancheng bioengineering institute, Nanjing, China). After euthanizing the rat and harvesting the lung tissue, 30-60 mg (wet weight) of tissue was taken and put into the glass vial. One ml hydrolysate was added to the vial, capped tightly and hydrolyzed at 60°C for 20 min. The hydrolysate was then centrifuged at 3500 rpm for 10 min according to the instructions of HYP assay kit. Absorbance was measured at 560 nm by a spectrophotometer, and HYP content of lung tissue was calculated according to the manufacture’s protocol.

**Measurement of serum levels of HA, LN, PIIINP, IVC**

On day 7, 28 and 56, blood samples were collected from the veil and serum was separated by centrifugation at 4°C and stored at -20°C for future use. The levels of HA, LN, PIIINP and IVC in serum were determined using a chemiluminescence immunoassay kit (Shenzhen new industry biomedical engineering Ltd., Shenzhen, China) according to the protocol by automatic chemiluminescence analyzer (Shenzhen new industry biomedical engineering Ltd., Shenzhen, China).

**Statistical Analysis**

Statistical analysis was performed with the statistical analysis software Graph Pad Prism 7 (GraphPad Software, San Diego, CA ). The experimental results were given as mean ± standard errors of mean. Group comparisons were performed by one-way or two-way ANOVA followed by the Bonferroni post hoc multiple comparison test. Results were considered statistically significant at \( P < 0.05 \).

**Results**

**Establishment of BLM-induced pulmonary fibrosis model in rats**

CT is a routine, rapid and non-invasive method for diagnosis and characterisation of IPF.\textsuperscript{[27]} To evaluate the BLM-induced rat model at day 7, CT was used to analysis the marked change in lung. Compared to control rats, the lungs of BLM-treated rats indicated parenchymal opacity, with a main features of a peripheral, predominantly basal pattern of coarse reticulation (Figure 2). Moreover, the CT MLD for BLM-treated rats was -(704.78±19.76) Hu (n=11), and for the control rats the MLD was
-(401.71±22.29) Hu (n=34) (Table 1). ECM molecules including LN, IVC, PIIINP, and HA are the main components of lung collagen and associated with pulmonary fibrosis.[28-30] The serum levels of LN, IVC, PIIINP and HA in BLM-treated rats significantly increased compared with control rats (Table 1). Together with the CT results, it showed that the IPF rat model was well established.

**rhCygb reversed BLM-induced histopathological and fibrotic changes in the lung**

After the establishment of IPF rat model, the rats were separated into BLM, rhCygb+ BLM (4 mg/kg) and DXMS+ BLM group (3 mg/kg). In figure 3A, CT scan images at day 56 are shown the differences in fibrotic and non-fibrotic lung in BLM-treated and control groups, while rhCygb had improved lung architecture with observable reduced collagen deposition and loss of air spaces. Rats received DXMS also had effect on the lungs as compared to that of BLM-treated rats.

Morphologically, compared with the smooth surface of control lung, BLM-treated rats showed the rough, ugly surface of the lung with gray fibrous nodules. On the other hand, rhCygb obviously improved pulmonary morphology in rats with no fibrous nodules (Figure 3B).

H&E staining showed normal lung tissue structure and intact alveolar cavity in the control group. In the BLM-treated group, there was significant alveolar cavity collapse. The alveolar septum, deposition with hemoglobin, was thickened. The alveolar septum was less thickened in the rhCygb+BLM and DXMS+BLM groups (Figure 3C).

Masson trichrome staining (Figure 3D) revealed the lung tissue of the control group had no obvious fibroblast foci. In the BLM-treated group, BLM enhanced pulmonary alveolus inflammation and broadened alveolar septa. Moreover, there was a lot of collagen deposition in lung tissue and the pulmonary fibrosis was severe. In the rhCygb and DXMS treatment groups, the alveolar wall was less thickened and inflammatory cell infiltration decreased. Additionally, there were decreased fibroblasts in lung tissues. However, microthrombus were found in the bronchus of the DXMS treatment group, as compared to control. The Masson CVF results (Figure 3E) showed that the average percentage of lung fibrosis in the control group was 1.86 ± 1.51 %. In contrast, rats treated with BLM resulted in a higher degree of lung fibrosis, which was 40.33 ± 2.08 %. In the rhCygb and DXMS treatment groups, the CVF was 20.81 ± 1.65 % and 30.76 ± 1.57 %, respectively. The comparison between each group was statistically significantly different (Control vs. BLM group, \( P < 0.001 \); BLM group vs. rhCygb+BLM group, \( P < 0.001 \); BLM group vs. DXMS+BLM group, \( P < 0.001 \)).

Hydroxyproline levels in lung tissue of BLM-treated group were significantly higher than that in control group (\( P < 0.05 \)). In the rhCygb and DXMS treatment, HYP levels were obviously lower than that of BLM group (\( P < 0.05 \)). Moreover, there was significant difference in HYP level between the rhCygb and DXMS groups (\( P < 0.05 \)) (Figure 3F). This result was consistent with the Masson staining results.

The CT MLD acquired by experiments was shown in Figure 3G. Since most part of the lung is composed by air, the MLD of control lung is tendency towards to -800 Hu. The CT MLD in BLM-treated group was
significantly higher than that in control group ($P < 0.001$). In the rhCygb and DXMS treatment, the CT MLD were obviously lower than that of BLM group ($P < 0.001$).

**Effect of rhCygb on HA, LN, PNP and C serum levels**

The levels of HA, LN, PNP and C of rats at day 7, 28 and 56 were measured in blood corresponding to the CT analysis in Figure 4. With a longer process of fibrosis, the HA, LN, PNP and C levels increased from 7 days to 56 days. Serum HA, LN, PNP and C levels in BLM-treated group were approximately 2.14, 1.61, 1.69, and 1.48 folds of those in the controls, respectively. Both rhCygb and DXMS significantly reduced HA, LN, PNP and C levels in blood of pulmonary fibrosis rats ($P < 0.001$). And at day 56, there was no significant difference in HA, LN, PNP levels between rhCygb and control groups except C levels ($P > 0.1$).

**Correlation of HA, LN, PNP and C serum levels, HYP and CVF to CT MLD**

Positive correlations were observed between CT MLD and LN, IVC, PIIINP, and HA levels, HYP and CVF (Figure 5). The HA, LN, PNP and C levels exhibited moderate correlations with CT MLD ($R^2 = 0.4103, P < 0.001$; $R^2 = 0.627, P < 0.001$; $R^2 = 0.6995, P < 0.001$; $R^2 = 0.7489, P < 0.001$; respectively). HYP also showed a moderate correlation with CT MLD ($R^2 = 0.7127, P < 0.001$). Masson CVF(%) showed very strong correlations with CT MLD ($R^2 = 0.9376, P < 0.001$).

**Discussion**

The establishment of BLM-induced lung fibrosis model

The model of BLM-induced lung fibrosis has played a pivotal role in the search for antifibrotic agents for treating pulmonary fibrosis.$^{[31]}$ An Official American Thoracic Society Workshop Report confirmed that there was a consensus opinion of the intratracheal BLM model as “the best-characterized animal model available for preclinical testing”.$^{[32]}$ Nevertheless, sex and age differences in mice might affect the experiment results. Young mice, aged 8-12 weeks, has been reported to undergo spontaneous resolution of BLM-induced pulmonary fibrosis, while this phenomenon is not obtained in aged mice.$^{[33]}$ Thus, the aged male mice are recommended for experiment research, which may provide a more clinically relevant model of IPF. In our study, adult male SD rats were used in the BLM-induced pulmonary fibrosis model.

Recently, some researchers point out that therapies are usually administered within < 7 days following BLM exposure, which may represent a stage of inflammation or early fibrosis; so the therapeutic effects may through prevention of the inflammatory cascade rather than reversal of fibrosis, and this is in contrast to the clinical situation when fibrosis has already been established.$^{[34]}$ Thus, more recent studies have begun to explore administration of drugs after 7 days.$^{[35,36]}$ However, those papers were based on the hypothesis that lung fibrosis was induced 3 or 4 weeks after bleomycin instillation without diagnosing the establishment of lung fibrosis model. Surprisingly, Choi reported that more than half of
bleomycin-instilled mice did not show lung fibrosis at 3 weeks or even 6 weeks by micro-CT. So it is very essential to develop assessment methods or tools for establishment of IPF animal models. CT is used to evaluate the IPF as a non-invasive technique in humans, with the feature of honeycombing or traction bronchiectasis and a reticular abnormality consistent with fibrosis present in a basal and peripheral predominance. Until now, with increasing technical possibilities, micro-CT provides high-resolution anatomical images of small animals, and it allows repeated measurements, which avoid animal euthanasia. Interestingly, in our study, we confirmed that a fibrosis phase in the BLM-induced fibrosis model has been well established at 7 days by spiral CT, showing alveolar septal thickening, interstitial fibrosis and honeycombing. This may be due to the animal breed, gender, dose, and administration affecting the results. Peng reported that bleomycin induced a dose-dependent increase in lung fibrosis, and significant fibrosis was observed in the groups of mice with higher dose. These observations would support our hypothesis that testing of candidate (rhCygb or DXMS) during the stage of late fibrosis is likely to translate into a therapeutic benefit in the clinic, evidence that comes from the clinical situation in which treatment is initiated after onset of symptoms and when fibrosis has already been established.

Furthermore, the combination of clinical parameters and biological markers has been studied in order to achieve more accurate results regarding the prognosis of IPF. Finding biomarkers for IPF has been a central challenge for a long time and would aid the fulfillment of the need for noninvasive diagnostic of patients with IPF. Yiliang et al investigated serum levels of HA, LN, PIIINP, IVC in 323 patients and 160 healthy controls, and found that serum HA, LN, PIIINP, IVC levels were all significantly higher in the patients with IPF than in the control groups and had a significant positive association with HRCT score in patients with IPF. However, whether serum levels of HA, LN, PIIINP and IVC could reflect the development and progression of IPF, a serial measurement of the serum levels of HA, LN, PIIINP and IVC at different disease stage is required. In the present study, we tested elevation of HA, LN, PIIINP, IVC serum levels at 7, 28 and 56 days of the same rats in BLM-treated group when the lung fibrosis obviously occurred. Surprisingly, compared with the progressive serum levels of HA, LN, PIIINP and IVC, the CT MLD of BLM-treated rats at 56 days almost equal to that at 7 days. Hence, we suggest that it is necessary to assess the progress of fibrosis in the BLM-induced fibrosis model using CT combined with biomarker measurement.

**Reversed effect of rhCygb on BLM-induced lung fibrosis**

Although we have previously reported that rhCygb displays antioxidative, anti-inflammatory, and antifibrotic properties in vitro and in animal models of liver fibrosis, its efficacy against the pulmonary fibrosis in BLM-induced rats has never been studied. Herein, we test of the candidate (rhCygb and DXMS) in the well-established pulmonary fibrosis rat model evident by the CT diagnosis combined with biomarker measurement including HA, LN, PIIINP, and IVC.

In the current study, we found that rats treated with rhCygb had improved lung architecture than BLM-treated group, with observable reduced collage deposition and loss of air spaces in CT imaging, and the
CT MLD were obviously lower than that of BLM group. It is known that CT MLD provide prognostic estimation of disease severity in patients with IPF and other ILDs and high MLD values may indicate severe IPF.[25] Consistent with these beneficial effects of rhCygb (4 mg/kg) treatment in our lung fibrosis rats, we found a marked attenuation of the HA, LN, PⅢNP and IVC levels in serum and HYP concentration in rat lungs compared to BLM-treated rats.

Recent advances targeting ECM production and repair have provided novel approaches that could be used to treat chronic lung diseases. The ECM basement membrane matrix is composed of nonfibrillar collagens (e.g., collagens IV and V), LN, and proteoglycans, and the lung's interstitial connective tissue is composed of complex networks of fibrillar collagen (e.g., collagens I and III) as well as HA and proteoglycans. Excessive accumulation of ECM molecules within the interstitial matrix is thought to underlie the pathogenesis of IPF.[41,42] In clinic research, serum LN, IVC, PⅢNP, and HA significantly increased in the patients (323 patients) with IPF or CTD-ILD compared with the healthy controls. After treatment, the survived patients had significantly lower serum LN, IVC, PⅢNP, and HA than the dead patients.[9] In the current study, we found there was no significant difference in HA, LN, PⅢNP levels between rhCygb+BLM group and control group at 56 days, so we inferred that the reversing effect of rhCygb may be involved in the regulation of ECM turnover.

This study also has several limitations. The molecular mechanism underlying the reversing effect of rhCygb in lung fibrosis remains unknown; and the anti-fibrotic effects of rhCygb was investigated only against the rat model, future works will be done to validate the effects of rhCygb using other animal IPF models. However, we believe that the experiment costs, time and labor could be lessened due to the non-invasive CT radiologic and biomarkers measurement. We will further verify the reversing lung fibrosis effects of rhCygb in other models in our future study.

**Conclusions**

In conclusion, our results indicate that rhCygb plays a significant reversing role in bleomycin-induced lung fibrosis rats. This beneficial effect is mediated by decreasing the EMC molecules of HA, LN, PⅢNP and IVC levels near to the control levels. Our data also indicate that rhCygb lower the CT MLD, HYP and Masson CVF, which reflect the progress of lung fibrosis. CT MLD positively correlates with serum levels of HA, LN, PⅢNP, IVC, and HYP, and especially with CVF. Our findings encourage CT combined with biomarkers should be performed to evaluate whether or not lung fibrosis model is established.

**Abbreviations**

BLM  bleomycin

rhCygb  recombinant human cytoglobin

DXMS  dexamethasone
Declarations

Ethics approval and consent to participate

All experimental procedures were performed in accordance with the institutional guidelines approved by the Chinese Association of Laboratory Animal Care. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Southern Medical University (Guangzhou, China).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

DWQ and LZ conceived and designed research; LZ and LZX performed experiments; WP analyzed data; DWQ and LZ edited and revised manuscript; DWQ approved nal version of manuscript. All authors have read and approved the manuscript.

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**Table**

| Group | n | HA (ng/mL)  | LN (ng/mL) | PIIINP (ng/mL) | IVC (ng/mL) | MLD (μm) |
|-------|---|-------------|------------|----------------|-------------|-----------|
| Control | 11 | 411.26±225.73 | 70.84±2.70 | 71.21±10.45 | 305.61±49.42 | -(704.78±19.76) |
| BLM | 34 | 668.36±63.30*** | 103.69±6.27*** | 106.55±4.91*** | 421±12.73*** | -(401.71±22.29)*** |

| | HA/CT | LN/CT | PIIINP/CT | IVC/CT |
|---|---|---|---|---|
| R² | 0.4097 | 0.8463 | 0.865 | 0.7918 |
| p | <0.0001 | <0.0001 | <0.0001 | <0.0001 |

*** p <0.0001
Figures

Figure 1

Schematic diagram of the experimental design Rats were intratracheal injected with BLM, 7 days later, CT and biomarkers were measured to confirm the establishment of lung fibrosis in rats. Then the BLM-treated rats were divided into BLM, rhCygb+BLM (4 mg/kg, daily, s.c.) and DXMS+BLM (3 mg/kg, daily, s.c.) groups. Blood samples were collected at day 7, 28 and 56. At last, rats were sacrificed and the parameters were studied.

Figure 2
Computed tomography images of control rat (left) and BLM-treated rat (right) Left is normal findings at day 7 after intratracheal saline instillation. Right is peribronchial wall thickening, consolidation and ground glass attention on CT image at day 7 after intratracheal BLM instillation.

Figure 3

Morphological, histologic analysis of lungs at day 56 after administration of treatments (A)
Representative images of CT between different treatment groups. BLM-treated rats show diffuse
peibromchial wall thicking and ground glass opacity in the lung. (B) Representative images showing gross lung morphology of rats. (C) Representative photomicrographs of lung sections stained with H&E (200×). (D) Representative images of lung sections stained with masson trichrome (200×). (E) The collagen volume fraction of lung. Data represents ab mean ±SEM; n = 7–11 per group. significantly higher than control group (P < 0.0001); significantly lower than BLM group (P < 0.0001); csignificantly higher than rhCygb+BLM group (P < 0.0001). (F) Hydroxyproline content. ab Data represents mean ±SEM; n = 7–11 per group. significantly higher than control group (P < 0.05); significantly lower than BLM group (P < 0.05); csignificantly higher than rhCygb+BLM group (P < 0.05). (G) Mean lung density a of CT. Data represents mean ±SEM; n = 7–11 per group. significantly higher than control group (P < 0.0001); bsignificantly lower than BLM group (P < 0.0001); csignificantly higher than rhCygb+BLM group (P < 0.0001).
Figure 4

Effect of rhCygb on serum levels of HA, LN, PIIINP and IVC of rats at day 7, 28 and 56

![Graphs showing the correlation between CT-MLD and HA, LN, PIIINP, IVC, HYP, and Masson CVF with R^2 values and P-values](image)

Figure 5

Correlations of serum levels of HA, LN, PIIINP, IVC, HYP and Masson CVF compared with CT MLD in experimental rats including control, BLM, rhCygb+BLM and DXMS+BLM groups.
Supplementary Files

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- supplement10.pdf