Effects of Chinese herbal medicine Shenlong Decoction on mRNA expressions of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 in lung tissue of rats with pulmonary fibrosis induced by bleomycin

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Objective: To observe the expressions of matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in rats with pulmonary fibrosis (PF) induced by bleomycin, and to explore the mechanisms of Shenlong Decoction in preventing and treating PF.

Methods: A total of 230 Wistar rats were divided into normal control group, untreated group, prednisone group, and low-, medium- and high-dose Shenlong Decoction groups. Wistar rats were intratracheally injected with bleomycin to induce PF. From the 2nd day, rats in the normal control and untreated groups were lavaged with normal saline (NS), and rats in the other groups were lavaged with prepared Shenlong Decoction by the same amount. Hematoxylin-eosin (HE) staining and Masson staining were used to observe pathological changes in lung tissue at different time points, and to evaluate whether the model was successfully induced. Expressions of MMP-2 and TIMP-1 mRNAs in rats’ lung tissue were measured by reverse transcription-polymerase chain reaction (RT-PCR).

Results: Expressions of MMP-2 and TIMP-1 mRNAs in lung tissue of rats were observed from all groups at each time point. In comparison with the normal control group, on the 7th day, the transcription levels of MMP-2 and TIMP-1 mRNAs, especially the former, of the untreated group increased significantly (\(P<0.05\) or \(P<0.01\)). On the 14th day, the transcription levels of MMP-2 and TIMP-1 mRNAs kept rising, especially the latter (\(P<0.05\) or \(P<0.01\)). On the 28th day, the transcription level of MMP-2 decreased a little, while the transcription level of TIMP-1 mRNA did not stop increasing (\(P<0.05\) or \(P<0.01\)). Compared with the untreated group, decrease of the transcription levels of MMP-2 and TIMP-1 mRNAs were observed in the treatment groups, especially the former, and this effect continued to the 28th day with the medium-dose Shenlong Decoction group decreasing most obviously (\(P<0.05\) or \(P<0.01\)).

Conclusion: Shenlong Decoction may inhibit the expression of MMP-2 mRNA by up-regulating the expression of TIMP-1 mRNA so as to slow the progression of PF.

Keywords: pulmonary fibrosis; bleomycin; matrix metalloproteinase-2; tissue inhibitor of metalloproteinase-1; rats

One of the most important pathogeneses causing pulmonary fibrosis (PF) is the accumulation of extracellular matrix (ECM) as a result of the imbalance between its synthesis and degradation\(^{11}\). Matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) are main enzymes with the function of regulating the synthesis and degradation of ECM. O’Connor et al\(^{[12]}\) revealed that MMP plays a role in the degradation of basement membrane in the early injury stage; in advanced stage of the disease, ECM begins to deposit due to the inhibited expression of MMP. It is possible to...
treat PF by maintaining the balance between MMP and TIMP. This experiment, by means of reverse transcription-polymerase chain reaction (RT-PCR), observes the dynamic changes of the MMP-2 mRNA and TIMP-1 mRNA in lung tissue of PF rat model induced by bleomycin injection and discusses the mechanisms of Shenlong Decoction (SLD) in preventing and treating PF.

1 Materials and methods

1.1 Experimental materials

1.1.1 Animals A total of 230 healthy male Wistar rats, weighing 180～210 g, animal license No. SCXK2003-0016, were provided by the Experimental Animal Centre of Shenyang Medical College.

1.1.2 Drugs and reagents Bleomycin injection (8 mg/bottle) was produced by Harbin Bolai Pharmaceutical Co., Ltd, batch No. 06060201; Shenlong Decoction is composed of Huangqi (Radix Astragali Mongolici) 25 g, Shashen (Radix Adenophorae Strictae) 30 g, prepared Shudihuang (Radix Rehmanniae) 10 g, Dilong (Phereetima Aspergilum) 10 g, Danggui (Radix Angelicae Sinensis) 15 g, Chuanxiong (Rhizoma Chuanxiong) 15 g, and Gancuo (Radix Glycyrrhizae) 10 g, and was prepared by Drug Preparation Room of the Second Affiliated Hospital of Liaoning University of Traditional Chinese Medicine; prednisone acetate tablets (5 mg/tablet) were produced by Tianjin Tianyao Pharmaceutical Co., Ltd, batch No. 051108. TRizol reagent was produced by Invitrogen Company of the United States; M-MULV reverse transcriptase was produced by Promega Company of the United States; PCR amplification Taq enzyme was produced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

1.1.3 Instruments CX41-1202 optical microscope (Olympus Company, Japan); PE9600 PCR Instrument (Perkin Elmer Company, USA); DYCP-33A electrophoresis tank (Beijing Liuyi Instrument Factory, China); Tanon Gel gel image analysis system (Tanon Science & Technology Co., Ltd, China).

1.2 Experimental methods

1.2.1 Preparation of PF model With the methods of Szapel et al[1], 3～5 min after intraperitoneal injection of pentobarbital sodium (0.25 mL) of 2% mass percentage concentration, each rat fell lethargic and sluggish under narcosis (narcosis should be enhanced by administering ether in the event that its effect got weak). Then it was fixed on a rat plate in supine position with head and limbs fixed and fur of the neck cut. The skin of its neck was sterilized by iodide fluoride and cut open with a 1-cm incision by aseptic operation. The trachea got exposed by stripping off the overlying tissue layer by layer and then was passed underneath by a bent-nosed plier of ophthalmology which slightly kept lifting it until the trachea was separated away from the tissue. Lift the headend of the rat plate to angulate a 30～35 degree angle to the horizontal. The needle (gauge 7) which had been sanded to arc-shaped and obtuse in advance should be punctured between the cricoid cartilages at 15 degree to the horizontal and was proved to reach inside the trachea if there was an empty feeling. Give the rat bleomycin injection (0.2 mL, about 4 mg/kg) and then air (0.2 mL) for 2 or 3 times, meanwhile rotate the rat plate 1～2 min to make the drug well-distributed in the lung.

1.2.2 Grouping and medication After one-week adaptive feed, 36 rats were selected by random number table from a total of 230 Wistar rats into normal control group and were fed with standard diet. PF was induced in rest of the rats and they were randomized into 5 groups including untreated group, prednisone group, and low-, medium- and high-dose Shenlong Decoction groups, with 36 rats in each group. Rats from these 5 groups were checked weekly to observe the change of their weight to adjust the dosage of drugs. From the 2nd day after modeling, rats from normal control group and untreated group were intragastrically administered with normal saline (1 mL/kg), while the rest rats were intragastrically administered with prepared isovolumetric drugs: 5.4 mg/kg prednisone for the prednisone group, 0.6 g/mL crude drug for the low-dose Shenlong Decoction group, 1.2 g/mL crude drug for the medium-dose Shenlong Decoction group, and 2.4 g/mL crude drug for the high-dose Shenlong Decoction group (according to body surface area conversion table between humans and mice[4], surface area ratio of 200 g rat and 70 kg adult human is 0.018). Intragastrically administration was performed each day. On the 7th, 14th, and 28th days, 12 rats were sacrificed each time to obtain their lung tissue. Rats should be supplemented when being unsuccessfully modeled or unexpectedly died.

1.2.3 Preparation of samples Sacrifice each rat under narcosis in supine position, open its abdominal cavity along the midline of abdomen to have a full exposure of aorta abdominalis, and bleed the rat to death. Under aseptic condition, take part of its left lobe (50 mg) by thoracotomy, and put the sample into an Eppendorf pipe which was immediately preserved in liquid nitrogen. Meanwhile, the right principal bronchus was ligated and cut off from the hilus pulmonis. The right upper lobe was carefully perfused with paraformaldehyde solution (3 mL) of 4% mass percentage concentration by a volumetric infusion pump under constant 25-cm hydraulic pressure. The lung tissue was sliced into samples with the volume of 1.5 cm × 1.5 cm × 1 cm each and was put into paraformaldehyde solution 30 min later to get fixed. After one week,
the samples were stained with hemotoxylin-eosin (HE) and Masson and observed under an optical microscopy to evaluate whether the preparation of these samples was successfully done or not [31].

1.2.4 RT-PCR detection

1.2.4.1 Methods of RT-PCR Total RNA were extracted according to the instructions of TRizol reagent. Primers were provided by Beijing Genomics Institute. The upstream and downstream sequences of the primer MMP-2 were 5'-CCATGTCTTCCCCTTCAC-3' and 5'-CGATGCCCACAAGACATG-3' respectively with amplified fragment length of 391 bp, and the reaction conditions were: 94°C 90 s, 56°C 1 min, 72°C 1 min, 30 cycles. The upstream and downstream sequences of the primer TIMP-1 were 5'-TCCTCCGAAACTCATCGAGAC-3' and 5'-ATGCTCTGTTGAGCCTTCT-3' respectively with amplified fragment length of 391 bp, and the reaction conditions were: 94°C 45 s, 56°C 1 min, 72°C 1 min, 30 cycles. The β-actin adopted was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with upstream sequence as 5'-ACCACAGTCCATGCCATAC-3', downstream sequence as 5'-TCTCCACACCTGTGGCTGTA-3', and amplified fragment length of 500 bp, and the reaction conditions were: 94°C 45 s, 54°C 1 min, 72°C 1 min, 30 cycles.

1.2.4.2 Semi-quantitative analysis of the products

Electrophoresis products of RT-PCR on the agarose gel, load each sample (5 μL) into a well, then carry out densitometric scanning with agarose gel electrophoresis imaging, and an analysis system was used to detect the expression level of the target gene.

1.3 Statistical analysis

SPSS 10.0 statistical software was used to analyze the data. Values were expressed as $\bar{x} \pm s$. Comparison of data among multiple groups were analyzed by one-way ANOVA, while comparison between every two groups were analyzed by SNK-q test. Differences were considered significant if $P$ value was less than 0.05.

2 Results

2.1 Pathological changes of lung tissues in untreated rats Numerous inflammatory cell infiltration was observed in the alveolar and interstitial space of the lung by HE staining, mainly macrophages, lymphocytes, and a few neutrophils. Mild edema became serious until the 7th day, with the structural confusion of the lung tissue, and obvious swelling and slight widening in alveolar septum. From the 7th day, the inflammatory infiltration was alleviated in alveolar space. Alleviation of pulmonary alveolitis was observed on the 14th day; the alveolar septum got thinner with a few lymphocytes and macrophages and proliferation of fibroblasts; alveolar space became narrowed with scattered proliferation of pulmonary interstitial collagenous fiber and fibrosis appeared. On the 28th day, severe PF was observed in lung tissue of the untreated group with structural disruption, atrophy and disappearance of alveoli and a small amount of inflammatory cells infiltrated; collagen deposition and fibrosis in lung tissue were observed with the diffusion of fibrous tissue appearing as patch or streak (Figure 1). It was observed by Masson staining that collagen fibers increasingly appeared in the pulmonary interstitial space, bronchus wall, and alveolar septa in the untreated group. On the 7th day, collagen fibers were mainly distributed around the bronchi and small vessels and those around interstitial pulmonary space were short. On the 14th day, collagen fibers increased considerably around the bronchi and small vessels; a few fascicular collagen fibers were observed around the bronchi; collagen fibers in the pulmonary interstitial space appeared as fasciculation or platelet. On the 28th day, numerous collagen fibers were observed around the bronchi, bronchi and small vessels; collagen fibers proliferated, extended to the pulmonary interstitial space and deposited, and appeared as fasciculation or sheet (Figure 2).

![Figure 1](Light microscopy, ×200)

A: 7th d; B: 14th d; C: 28th d.
2.2 Expressions of MMP-2 and TIMP-1 mRNAs in different groups after modeling. There were expressions of MMP-2 and TIMP-1 mRNAs in lung tissue of rats in all groups at each time point. In comparison with the normal control group, on the 7th day, transcription levels of MMP-2 and TIMP-1 mRNAs, especially the former, increased significantly in the untreated group ($P < 0.05$ or $P < 0.01$); on the 14th day, the transcription levels of MMP-2 and TIMP-1 mRNAs, especially the latter, kept rising ($P < 0.05$ or $P < 0.01$); on the 28th day, the transcription level of MMP-2 mRNA decreased a little, while the transcription level of TIMP-1 mRNA increased ($P < 0.05$ or $P < 0.01$). Compared with the untreated group, the groups receiving medications had a decrease in the transcription levels of MMP-2 and TIMP-1 mRNAs, especially the MMP-2 mRNA, and this effect continued to the 28th day with the medium-dose Shenlong Decoction group decreasing most ($P < 0.05$ or $P < 0.01$). See Table 1, Table 2 and Figure 3.

### Table 1  Expression of MMP-2 mRNA in lung tissue of rats in different groups at different time points

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>7 d</th>
<th>14 d</th>
<th>28 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>12</td>
<td>0.591±0.031</td>
<td>0.584±0.064</td>
<td>0.575±0.014</td>
</tr>
<tr>
<td>Untreated</td>
<td>12</td>
<td>0.580±0.074**</td>
<td>0.891±0.032**</td>
<td>0.846±0.023**</td>
</tr>
<tr>
<td>Prednisone</td>
<td>12</td>
<td>0.539±0.058△△</td>
<td>0.845±0.086△△</td>
<td>0.774±0.027△△</td>
</tr>
<tr>
<td>Low-dose SLD</td>
<td>12</td>
<td>0.772±0.032△△</td>
<td>0.865±0.023△△</td>
<td>0.835±0.071**</td>
</tr>
<tr>
<td>Medium dose SLD</td>
<td>12</td>
<td>0.655±0.016△△</td>
<td>0.845±0.004△△</td>
<td>0.761±0.034△△</td>
</tr>
<tr>
<td>High-dose SLD</td>
<td>12</td>
<td>0.687±0.052△</td>
<td>0.837±0.017△</td>
<td>0.802±0.051△△</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$, vs normal control group; △ $P < 0.05$, △△ $P < 0.01$, vs untreated group; △ $P < 0.05$, vs low-dose SLD group.

### Table 2  Expression of TIMP-1 mRNA in lung tissue of rats in different groups at different time points

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>7 d</th>
<th>14 d</th>
<th>28 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>12</td>
<td>0.555±0.024</td>
<td>0.541±0.064</td>
<td>0.555±0.039</td>
</tr>
<tr>
<td>Untreated</td>
<td>12</td>
<td>0.246±0.043*</td>
<td>0.992±0.017*</td>
<td>1.555±0.095*</td>
</tr>
<tr>
<td>Prednisone</td>
<td>12</td>
<td>0.794±0.056△</td>
<td>0.845±0.015△</td>
<td>0.890±0.048△△△</td>
</tr>
<tr>
<td>Low-dose SLD</td>
<td>12</td>
<td>0.795±0.080△△</td>
<td>0.855±0.051△△</td>
<td>0.883±0.038△△△</td>
</tr>
<tr>
<td>Medium dose SLD</td>
<td>12</td>
<td>0.712±0.074△△</td>
<td>0.869±0.042△△</td>
<td>0.878±0.038△△△</td>
</tr>
<tr>
<td>High-dose SLD</td>
<td>12</td>
<td>0.832±0.075*</td>
<td>0.891±0.053△△</td>
<td>1.138±0.075△△△</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$, vs normal control group; △ $P < 0.05$, △△ $P < 0.01$, vs untreated group; △ $P < 0.05$, vs low-dose SLD group; △△△ $P < 0.05$, vs medium-dose SLD group.
3 Discussion

PF is one of the eventual consequences of various chronic pulmonary diseases. Its pathological changes in lung tissue in the early stage are mainly inflammatory cell infiltration, and edema and congestion of the lung tissue; in the later stage, its pathological changes in lung tissue are mainly proliferation of collagen fibers, displacing the normal tissue and resulting in dysfunction of the lung.

Injections of bleomycin into trachea can induce models with pathological changes similar to those of human’s pulmonary interstitial fibrosis. The pulmonary alveolitis and fibrosis of this disease are ever-changing with the progression of time, which can be divided into 4 phases: (1) the damage and edema of the lung tissue (1 to 3 days after giving medicine); (2) inflammatory reaction and proliferation of type-ll pneumocytes (3 to 7 days after giving medicine); (3) proliferation of pulmonary interstitial cells (7 to 14 days after giving medicine); (4) diffuse fibrosis in pulmonary interstitial space (14 to 28 days after giving medicine). Its pathological features are lung injury in the early stage and the ensuing pulmonary fibrosis. The results of staining in this study correspond to the pathological progression of PF.

PF is characterized by remodeling of lung tissue and over deposition of ECM. A main pathological cause of PF is the imbalance between the synthesis and degradation of ECM. Degradation of ECM is mainly regulated by MMP and TIMP. MMP degrades most components of ECM, which is activated in the early stage of lung injury and fibrosis. Increased secretion and enhanced activity of MMP dissolves and disrupts basilemma and ECM. TIMP is a primary inhibitor of MMP; each type of TIMP inhibits MMP specifically by forming composite with types of MMP via covalent bond by 1:1 ratio. Overproduction of TIMP can aggravate the fibrosis. With the alleviation of pulmonary inflammatory reaction, the production and activation of MMP-2 decrease, while the expression of TIMP-1 continues to increase. Imbalance between MMP-2 and TIMP-1 and accumulation of ECM result in PF.

PF is thought to belong to lung atrophy from the perspective of traditional Chinese medicine (TCM). The nature of this disease is deficiency in origin and excess in superficiality. The origin is dual deficiency of qi and yin in both lung and kidney, and the superficiality is blood stasis in lung collaterals. The treatment principle is tonifying the lung and replenishing the kidney and dissipating stasis to free collaterals. The prescription Shenlong Decoction adopted several herbs such as Huangqi (Radix Astragali Mongolici), Danggui (Radix Angelicae Sinensis), Chuanxiong (Rhizoma Chuanxiong) and Dilong (Phretema Aspergilum) in Buyang Huanwu Decoction from Yi Lin Gai Cuo (Correction on the Errors of Medical Works) by Wang Qingren. In the light of the theory in TCM that same treatment for different diseases, the 4 herbs above in combination with Shashen (Radix Adenophorae Strictae) and Danggui (Radix Angelicae Sinensis), could tonify qi and nourish yin, dissipate stasis to free collaterals, and nourish and activate blood. The results of RT-PCR demonstrates that from the 7th day to the 14th day, Shenlong Decoction can decrease the transcription level of MMP-2 mRNA through inhibiting synthesis and activation of MMP-2 mRNA so as to alleviate the injury in inflammation stage, which is relatively
mild and can be repaired before the pulmonary
alveolitis transforms to fibrosis, leaving the lung
structurally intact and the fibrosis significantly
relieved; this effect does not disappear until the
28th day when the pathological change is mainly
PF. During the pulmonary alveolitis stage in PF,
Shenlong Decoction can decrease the transcription
level of TIMP-1 mRNA to inhibit the production
deposition of ECM by keeping a balance of
MMP-2 and TIMP-1. In the fibrosis stage, Shen-
long Decoction decreases the level of TIMP-1
probably by reducing the synthesis of TIMP-1
mRNA thus reducing its inhibitory effect on
degradation of ECM and slowing the progression
of fibrosis. The influence of Shenlong Decoction on
MMP-2 mRNA is probably reducing the transcrip-
tion level of TIMP-1 mRNA, which correspondingly
reduces the inhibitory effect on degradation of
TIMP-1 mRNA and ECM. Reducing of ECM
deposition causes decrease of MMP-2 which
promotes degradation of ECM. This demonstrates
that Shenlong Decoction may decrease the expres-
sion levels of MMP and TIMP and maintain a
dynamic balance of them.

To sum up, the imbalance between MMP and
TIMP may be one of the mechanisms that causes
PF, and no effective therapeutic method is found
by now. TCM prescriptions showed some effects
on regulating the ECM deposition and inhibiting
the PF progression, which may become an important
method to prevent and treat PF.

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**目的**：观察二氧化硫致肺纤维化大鼠肺组织中基质金属蛋白酶 2 (matrix metalloproteinase-2, MMP-2) 和金属蛋白酶组织抑制剂 1 (tissue inhibitor of metalloproteinase-1, TIMP-1) mRNA 的表达，探讨中药复方参龙煎剂防治大鼠肺纤维化的作用机制。

**方法**：230 只健康雄性 Wistar 大鼠随机分为正常对照组、模型组、波尼松组和参龙煎剂低、中、高剂量组，采用气管内注入二氧化硫的方法建立 Wistar 大鼠肺纤维化模型。正常对照组和模型组大鼠第 2 日起每日予生理盐水灌胃，其余各组大鼠按配好的参龙煎剂以等容氨基酸灌胃。苏木精-伊红染色和 Masson 染色观察不同时点大鼠肺组织病理变化，以评价模型是否成功。逆转录聚合酶链反应检测大鼠肺组织 MMP-2 和 TIMP-1 mRNA 的表达水平。

**结果**：各组大鼠肺组织在各个时间点均有 MMP-2 和 TIMP-1 mRNA 表达。与正常对照组大鼠比较，模型组大鼠在第 7 天时 MMP-2 和 TIMP-1 mRNA 基因转录水平均显著增高，尤以 MMP-2 mRNA 增加明显，差异均有统计学意义（P＜0.05 或 P＜0.01）；在第 14 天时，MMP-2 和 TIMP-1 mRNA 的基因转录表达继续增高，但 TIMP-1 mRNA 增加更显著，差异均有统计学意义（P＜0.05 或 P＜0.01）；第 28 天时，MMP-2 已有所下降，但 TIMP-1 mRNA 表达仍持续性增高，差异均有统计学意义（P＜0.05 或 P＜0.01）。与模型组大鼠比较，治疗组大鼠第 7 天 MMP-2 和 TIMP-1 mRNA 基因转录水平降低，尤其是 MMP-2 的表达降低更明显，第 28 天时这种降低作用仍持续存在，以参龙煎剂中剂量组大鼠表现最明显，差异均有统计学意义（P＜0.05 或 P＜0.01）。

**结论**：参龙煎剂可能是通过上调 TIMP-1 mRNA 的表达来抑制 MMP-2 mRNA 表达水平，从而起到延缓肺纤维化的作用。

**关键词**：肺纤维化；二氧化硫；基质金属蛋白酶 2；金属蛋白酶组织抑制剂 1；大鼠