

Original Article

Ulinastatin combined with magnesium isoglycyrrhizinate suppressed bleomycin-induced acute pulmonary fibrosis by inhibiting MMP-9, ICAM-1 and TGF- β 1 expressions

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Abstract: To investigate the effects of ulinastatin (UTI) combined with magnesium isoglycyrrhizinate (MgIG) on experimental rats with acute pulmonary fibrosis and its mechanism of prevention and treatment. Wistar female rats were randomly divided into: group I, sham operation group; group II, Solu-Medrol group; group III, UTI group; group IV, MgIG group; group V, UTI + MgIG group. Results among groups showed that a small amount of fibroblasts and capillary proliferation, some alveolar collapsed within the lesion were found in the sham operation group; serious damage to the alveolar structure, accompanied with fibrous tissue proliferation were detected on the lungs over time, fibrosis was also aggravated. In drug intervention groups at different time points, the degree of inflammatory cell exudation and fibrous tissue proliferation was reduced, among which the degree of lesion reduction was more obvious in group V. Significant reduction of alveolar catarrh and pulmonary fibrosis degrees were found in the other four drug intervention groups compared to that in group I, and more significant reduction of pulmonary fibrosis was found in group V. Furthermore, the expression levels of MMP-9, ICAM-1 and TGF- β 1 were all reduced in drug intervention groups when compared to group I, especially remarkably in group V. UTI combined with MgIG can effectively inhibit expression of MMP-9, ICAM-1 and TGF- β 1 in rats lung tissues of pulmonary fibrosis, reduce the degree of alveolitis and pulmonary fibrosis and decrease the secretion of proinflammatory cytokines and pulmonary fibrosis factors, and thereby reducing the synthesis and deposition of collagen and suppressing the bleomycin induced acute pulmonary fibrosis process.

Keywords: Acute pulmonary fibrosis, ulinastatin, magnesium isoglycyrrhizinate

Introduction

In recent years, the incidence of pulmonary fibrosis has shown an obvious upward trend, causing great concern to the worldwide [1-3]. Pulmonary fibrosis is a diffuse inflammatory disease caused by many kinds of reasons, which mainly invades the pulmonary interstitium [4]. The condition of primary pulmonary fibrosis assumes the continually progresses, corresponding five year survival rate was lower than 50%, and patients may finally die of respiratory failure [5]. At present, there is a lack of specific treatment methods, thus, glucocorticoid and immunosuppressive agents are still

the main clinical treatment. Therefore, there is an urgent need for new therapeutic drugs and methods considering side effects of long-term usage [6, 7].

Alveolar epithelial cell damage and basement membrane damage are important characteristics of pulmonary interstitial fibrosis during the early period [8]. Matrix metalloproteinase-9 (MMP-9) is a key enzyme in tissue remodeling, cell adhesion molecule-1 (ICAM-1) is an important pro-inflammatory factor, and transforming growth factor- β 1 (TGF- β 1) is the most important factor to induce fibrosis [9-11]. An extremely complex network among above three factors is

suggested to be involved in the process of the disease, responsible for the occurrence and development of interstitial fibrosis. The occurrence of pulmonary fibrosis is the imbalance of cytokine network regulation which leads to the release of a large number of chemokines, resulting in the activation of inflammatory cells aggregation [12]. Therefore, activation of cytokines, growth factors and matrix metalloproteinases may hence be critical in the formation of pulmonary alveolar inflammation and pulmonary interstitial fibrosis [13].

Ulinastatin (UTI) is a urinary trypsin inhibitor isolated from human urine, which can inhibit activities of a variety of proteases, glucose and lipid hydrolase, thus it has been widely used in the treatment of acute pancreatitis, hemorrhagic shock, myocardial infarction, severe infection, and involved in the inhibition of tumor invasion and metastasis [14, 15]. Studies have showed that UTI can inhibit the expression of IL-8, TNF- α and other inflammatory mediators in the process of acute lung injury, and can enhance the synthesis of alveolar macrophage and T cells for the secretion of IL-10, suggesting a potential protective and therapeutic effect on acute lung injury [16, 17]. Besides, previous evidence have also revealed that UTI can significantly reduce the degree of inflammation and fibrosis induced by paraquat poisoning in rats, inhibiting the excessive expression of TGF- β 1 in lung tissue [18].

Magnesium isoglycyrrhizinate (MgIG) is a glycyrrhizic acid of the fourth generation of new formulations [19]. A large number of studies have found that glycyrrhizic acid has a variety of clinical effects, including strong anti-inflammatory, antioxidant, anti-fibrosis, cell membrane stability, immune regulation, preventing cell apoptosis and increasing the production of endogenous steroids [20, 21]. It is now used in the prevention and treatment of liver fibrosis. Previous evidence regarding the protective effects of glycyrrhizic acid on pulmonary fibrosis induced by carrageenin in rats has proved that the glycyrrhizic acid can significantly decrease the level of ICAM-1, HYP, TNF- α , IL-1 β , and NF- κ B; besides, continuous administration of MgIG can significantly reduce the level of ICAM-1 and MMP-9 in serum of paraquat exposed rats [22].

Both UTI and MgIG have important effects, mainly manifested as inhibiting excessive

release of inflammatory mediators, antioxidant, anti-fibrosis, cell membrane stability, immune regulation, inhibiting paraquat poisoning induced pulmonary fibrosis. Both drugs have been proved to have inhibitory role of lung fibrosis induced by paraquat poisoning theoretically. However, there is no clinical report on the treatment of pulmonary fibrosis, besides, there is no experimental study on the treatment of pulmonary fibrosis in rats' model. In the study, under the background of the lack of effective drugs for the treatment of pulmonary fibrosis, two drugs with the effect of inhibiting inflammatory factors were combined and compared with those of hormones, looking forward to verify our hypothesis that the combined treatment of UTI and MgIG may strengthen the suppression of inflammation and anti-pulmonary fibrosis by regulating the activities of MMP-9, ICAM-1 and TGF- β 1, meeting or exceeding the curative effect of hormone treatment and without obvious side effect, so as to provide a new therapeutic methods for the prevention and treatment of pulmonary interstitial fibrosis.

Materials and methods

Experiment animals

A total of 75 Wistar female rats (12~16 weeks, weighing from 240 g~280 g with a mean weight of $[265.8 \pm 4.2]$ g) were obtained from the Experimental Animal Center of the Institute of Health and Medicine from The First Center Hospital of Tianjin, Graduate School of Medical University of Tianjin. All animals were housed at an environment with constant temperature and humidity, under a 12/12 h light/dark cycle before the experiment. Besides, all rats were fed on a standard diet with sterilized food and water. After obtaining prior approvals from the Institutional Animal Care and Use Committee of the First Center Hospital of Tianjin, Graduate School of Medical University of Tianjin, all animal care and experimental procedures were performed. Reasonable efforts were made to minimize the numbers of rats used in the study and to reduce animals' suffering from the experiment.

Animal model and experimental grouping

Rats were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium (0.004 ml/g). Under strict asepsis procedure, median incision of the neck was performed to expose

trachea. By puncture, intra-tracheal injection of 5 mg/kg bleomycin (BLM, concentration of 4 mg/ml) was performed slowly, and then, animal rotation was made to ensure a uniformed distribution of the medicine liquids in the body. An instillation of bleomycin was performed to establish a rat model of acute pulmonary fibrosis. Involved rats were randomly divided into 5 groups (15 rats in each group): (1) BLM group; (2) Methylprednisolone (MTH) group; (3) UTI group; (4) MglG group; (5) UTI + MglG group. Rats in BLM group were injected with equal volume of physiological saline (1 ml) intraperitoneally from the second day after surgery, once daily; rats in MTH group received intraperitoneal injection of MTH (4.5 mg/kg) once every day from the second day following operation; similarly, rats in UTI group were administrated with intraperitoneal injection of MglG (30 mg/kg) one time a day from the next day after surgery; in addition, in MglG group, rats were intraperitoneally injected with 20 thousand U/kg UTI from the following day postoperatively; and rats in UTI + MglG group were administrated with UTI + MglG (30 mg/kg and 20 thousand U/kg, respectively) in a way of intraperitoneal injection, also from the next day postoperatively.

Animal sacrifice and specimen collection

After right ventricular blood collection (3 to 4 ml), mice were sacrificed by dislocation of cervical vertebra 7, 14 and 28 days after surgery, respectively; lung specimens were collected and preserved for the following experiment. Detection of liver and kidney function was conducted after serum collection for the evaluation of drug safety. Furthermore, 5 rats in each group were killed immediately, 10% neutral buffer Faure Marin was injected into the lung by the right main bronchus, and following the expansion of pleural, 10% neutral buffered formalin was used to fix the specimen for 4 h. And then, conventional dehydration was performed, followed by transparent, dipping wax, embedded, continuous section in a thickness of 5 μ m, finally made of the paraffin sections for HE staining and observation. In accordance with the method promoted by Szapiel *et al.* for the evaluation of pulmonary alveolar inflammation and pulmonary fibrosis, degrees of alveolar catarrh was preset: (1) grade 0, 0 point, without alveolar catarrh; (2) grade I, 1 point, mild alveolar catarrh, mononuclear cell infiltration increased the alveolar interval, which was

confined to the local or pleural area only, with an area of less than 20% of the total lung, and the alveolar structure was normal; (3) grade II, 2 points, moderate alveolar catarrh, the affected area accounted for 20%~50% of the whole lung, and the proximal part of the pleural was more serious; (4) grade III, 3 points, serious alveolar structure, severe pulmonary alveolar inflammation with an area of more than 50%, pulmonary fibrosis was formed. In addition, pulmonary fibrosis was also pre-defined: (1) grade 0, 0 point, without significant change; (2) grade I, 1 point, minimal changes, the lesion was smaller than 20% of the whole lung; (3) grade II, 2 points, moderate alteration, lesions range from 20%-50% to the whole lung; (4) grade III, 3 points, severe changes, the lesion area accounted for more than 50% of the whole lung, accompanied with disordered alveolar structure.

Serum liver and kidney function test

The collected rat serum was analyzed by fully automatic biochemical analyzer (OLYMPUS AU, Olympus, Japan) to record and compare the changes of liver and kidney function indexes in rats. Measured indexes related to liver function of rats included alanine aminotransferase (ALT) and aspartate aminotransferase (AST); and indexes associated with kidney function involved blood urea nitrogen (BUN) and creatinine (Cr).

Detection of the MMP-9, ICAM-1 and TGF- β 1 expression by immunohistochemistry

Immunohistochemical assay was used to detect expression of MMP-9, ICAM-1 and TGF- β 1. Quantitative analysis was performed by using color imaging analyzer. MMP-9, ICAM-1 and TGF- β 1 rabbit polyclonal antibodies working concentration were preset as 1: 50, using PBS instead of the primary antibody as blank control. Slice thickness was 5 μ m, paraffin sections were dewaxed to water; a 3% H₂O₂ was used to close endogenous peroxidase, followed by microwave antigen repairing; and then, MMP-9, ICAM-1 and TGF- β 1 antibodies were dropped and saved at 4°C overnight; subsequently, added the second antibody, generally 3,3'-diaminobenzidine (DAB) staining were then conducted, and dehydrated with ethanol of gradient concentration, then cleared in xylene for transparent, finally neutral gum sealing piece. A

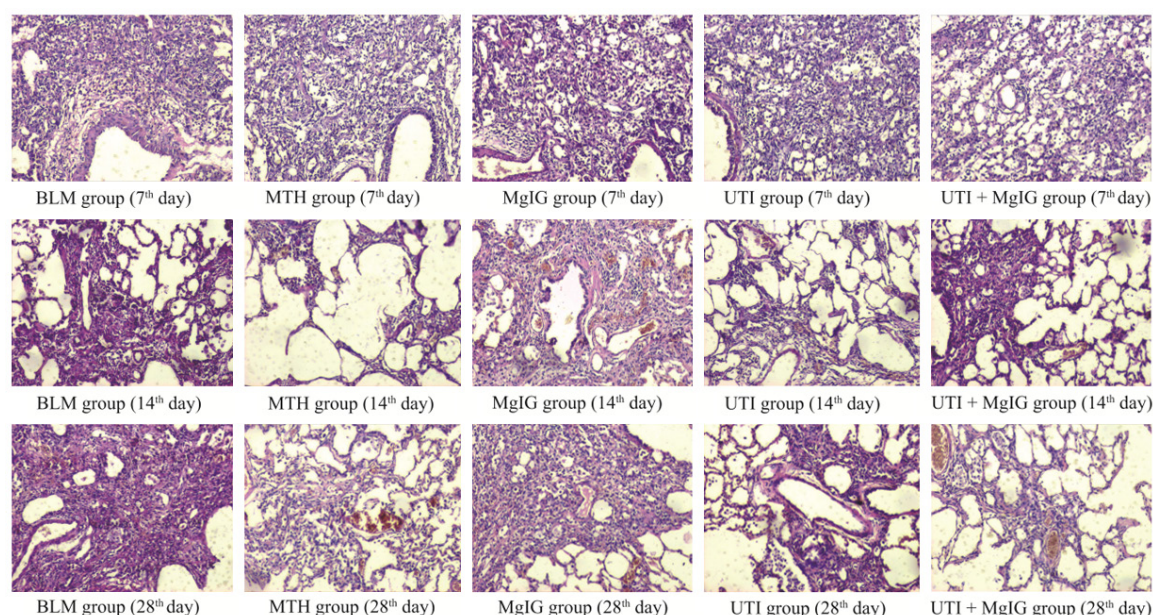


Figure 1. HE staining results (magnification: $\times 200$) among BLM group MTH group UTI group MglG group and UTI + MglG group on the 7th day, the 14th day, and the 28th day.

Table 1. Effects of UTI combined with MglG on degrees of alveolar catarrh and pulmonary fibrosis results in experimental rats with acute pulmonary fibrosis

Grouping	Cases (n)	Degrees of alveolar catarrh (points)			Degrees of pulmonary fibrosis (points)		
		The 7 th day	The 14 th day	The 28 th day	The 7 th day	The 14 th day	The 28 th day
BLM group	5	2.86 $\pm$ 0.50	2.60 $\pm$ 0.48	1.95 $\pm$ 0.43	0.87 $\pm$ 0.30	1.92 $\pm$ 0.52	2.98 $\pm$ 0.56
MTH group	5	2.57 $\pm$ 0.54*	2.55 $\pm$ 0.41*	1.87 $\pm$ 0.31*	0.60 $\pm$ 0.39*	1.49 $\pm$ 0.50*	2.34 $\pm$ 0.56*
UTI group	5	2.44 $\pm$ 0.40* [#]	2.20 $\pm$ 0.30* [#]	1.68 $\pm$ 0.24* [#]	0.58 $\pm$ 0.32*	1.35 $\pm$ 0.44*	2.26 $\pm$ 0.47*
MglG group	5	2.47 $\pm$ 0.36* [#]	2.16 $\pm$ 0.20* [#]	1.71 $\pm$ 0.21* [#]	0.54 $\pm$ 0.38*	1.30 $\pm$ 0.45*	2.48 $\pm$ 0.46*
UTI + MglG group	5	1.81 $\pm$ 0.40*	1.67 $\pm$ 0.37*	1.34 $\pm$ 0.25*	0.40 $\pm$ 0.34*	1.10 $\pm$ 0.38*	2.02 $\pm$ 0.42*

Note: UTI: ulinastatin; MglG: magnesium isoglycyrrhizinate. *, $P < 0.05$, a comparison of all drugs intervention groups (MTH group, UTI group, MglG group and UTI + MglG group) with the BLM group. [#], $P < 0.05$, a comparison of UTI + MglG group with other interventional groups (MTH group, UTI group and MglG group).

total of 5 field of vision from each slice ($\times 200$ times) were randomly read under the microscope, and analyzed with HMIAS-2000 high definition color medical image analysis system, taking the average absorbance value for statistical analysis.

Statistical analysis

All data were analyzed using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). Data was presented as mean $\pm$ standard deviation (SD) and were tested for normality using a modified Shapiro-Wilks test. Comparisons between groups were made using t test and comparison among groups by One-Way analysis of variance (ANOVA). Least significant difference (LSD)-t test was employed to make multiple compari-

sons regarding means value of groups. Correlation analysis adopted linear correlation analysis. A two-side P value of less than 0.05 was used to determine the significance.

Results

HE staining results among groups

In the BLM group, on the 7th day, alveolar septum widened, a large number of lymphocytes, neutrophils and macrophages infiltration could be seen in the alveolar and alveolar interval. Besides, there was a small amount of fibroblasts and capillary proliferation, some alveolar collapsed within the lesion. Furthermore, on the 14th day, alveolar inflammatory effusion reduced gradually, obvious thickened alveolar

Table 2. Effects of UTI combined with MglG on liver and kidney function in experimental rats with acute pulmonary fibrosis

Grouping	Liver function		Kidney function	
	ALT (U/L)	AST (U/L)	BUN (mmol/L)	Cr (mmol/L)
BLM group	45.14 $\pm$ 5.00	155.12 $\pm$ 25.27	7.67 $\pm$ 0.41	37.65 $\pm$ 1.65
MTH group	47.30 $\pm$ 5.12	163.26 $\pm$ 23.16	7.51 $\pm$ 0.39	35.44 $\pm$ 1.61
UTI group	46.32 $\pm$ 4.97	147.38 $\pm$ 26.00	7.53 $\pm$ 0.31	36.62 $\pm$ 1.71
MglG group	45.73 $\pm$ 5.34	153.80 $\pm$ 23.45	7.49 $\pm$ 0.36	35.99 $\pm$ 1.94
UTI + MglG group	46.25 $\pm$ 4.97	151.23 $\pm$ 24.61	7.24 $\pm$ 0.29	36.72 $\pm$ 1.80

Note: UTI: ulinastatin; MglG: magnesium isoglycyrrhizinate. ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; Cr: creatinine.

was observed in the damaged lungs, associated with increased number of fibroblasts in alveolar septum, and hyperplasia of pulmonary fibrous tissues. On the 28th day, serious damage to the alveolar structure was detected on the lungs, accompanied with fibrous tissue proliferation, fibrosis was also aggravated. Meanwhile, in drug intervention groups at different time points, the degree of inflammatory cell exudation and fibrous tissue proliferation was reduced compared with that in the BLM group in the same period, among which the degree of lesion reduction was more obvious in the UTI + MglG group when compared with that in the other drug intervention groups (MTH group, UTI group and MglG group). Corresponding imaging results were illustrated in **Figure 1**.

Meanwhile, degrees of alveolar catarrh and acute pulmonary fibrosis results were shown in **Table 1**. With respect to the degree of alveolar catarrh, on different time points, such degree was significantly lighter in all drug intervention groups than that in the BLM group, among which the UTI + MglG group, indicated apparent statistical difference compared with the BLM group (On the 7th day: $P = 0.006$; On the 14th day: $P = 0.004$; On the 28th day: $P = 0.025$, respectively); the degree of alveolar catarrh in the UTI group and MglG group were both higher compared with UTI + MglG group (On the 7th day: $P = 0.038$ and $P = 0.048$; On the 14th day: $P = 0.038$ and $P = 0.031$; On the 28th day: $P = 0.045$ and $P = 0.011$, respectively), and both lower compared with MTH group, but without obvious statistical differences (all $P > 0.05$). In addition, as for acute pulmonary fibrosis degree, on the 7th, 14th and 28th day, significant reduction of acute pulmonary fibrosis was fo-

und in the other four drug intervention groups than that in the BLM group, and more clear reduction of pulmonary fibrosis was found in the UTI + MglG group (On the 7th day: $P = 0.026$; On the 14th day: $P = 0.022$; On the 28th day: $P = 0.015$, respectively). Additionally, no noticeable statistical difference was

found in the comparison among MTH group, UTI group and MglG group (all $P > 0.05$).

Analysis of drug safety in vivo

Serum analysis of ALT, AST, BUN and Cr results found that there was no significant statistical difference in pairwise comparison among all the five experimental groups (all $P > 0.05$), suggesting that the application of UTI and MglG indicated none apparent toxic side effects. Detailed information was shown in **Table 2**.

Immunohistochemical results of MMP-9

MMP-9 positive expressions were primarily located on alveolar macrophages and bronchial epithelial cells at different time points; besides, the number of positive stained cells was increased on the 7th day and the staining intensity was strengthened, but was then gradually decreased over time. Immunohistochemical staining results were shown in **Figure 2** with respect to effects of UTI combined with MglG on expression levels of MMP-9 in experimental rats with acute pulmonary fibrosis on the 7th day, the 14th day, and the 28th day. Positive expression intensity of MMP-9 in each time point of the drug intervention groups (MTH group, UTI group, MglG group and UTI + MglG group) was lower than that of the BLM group. As shown in **Table 3**, expression levels of MMP-9 among groups was compared, on the 7th day, expressions of MMP-9 among all drug intervention groups were significantly lower than that in the BLM group, and significant differences were found when compared with MglG group and UTI + MglG group ($P = 0.043$ and $P = 0.008$, respectively); and within intervention groups comparison, MMP-9 level in the UTI + MglG

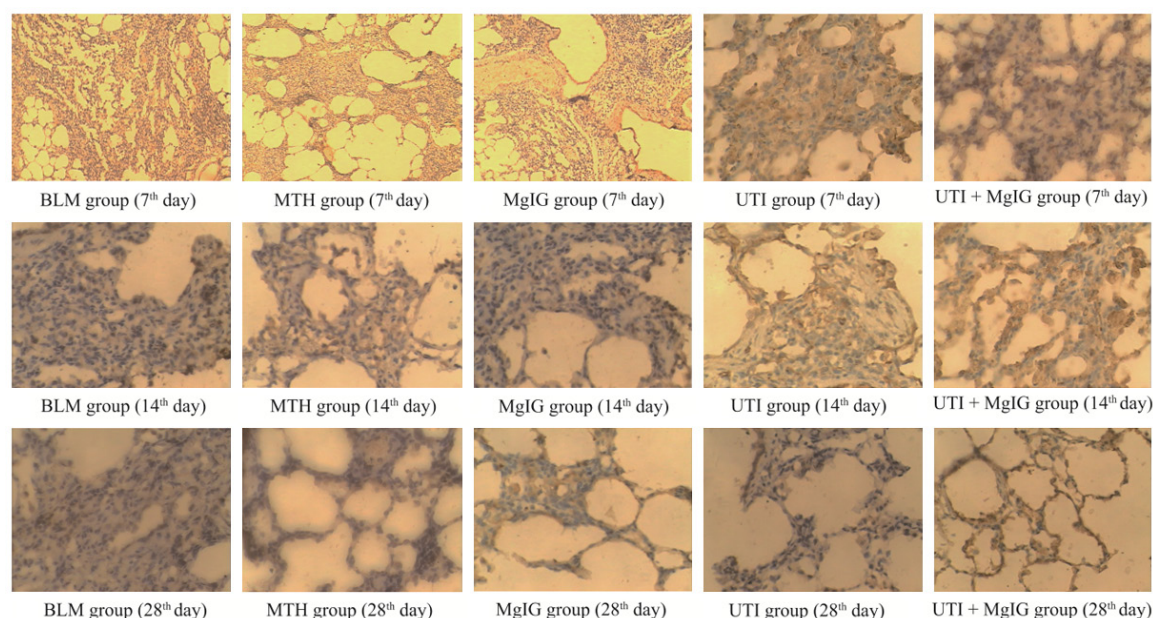


Figure 2. Effects of UTI combined with MglG on expression levels of MMP-9 in experimental rats with acute pulmonary fibrosis detected by immunohistochemistry (magnification: $\times 200$) on the 7th day, the 14th day, and the 28th day.

Table 3. Effects of UTI combined with MglG on expression levels of MMP-9 in experimental rats with acute pulmonary fibrosis

Grouping	Cases (n)	Expression levels of MMP-9		
		The 7 th day	The 14 th day	The 28 th day
BLM group	5	0.344 $\pm$ 0.029	0.390 $\pm$ 0.022	0.445 $\pm$ 0.039
MTH group	5	0.327 $\pm$ 0.025 [#]	0.376 $\pm$ 0.021 [#]	0.411 $\pm$ 0.029 [#]
UTI group	5	0.314 $\pm$ 0.026	0.359 $\pm$ 0.028 [#]	0.373 $\pm$ 0.021 ^{*,#}
MglG group	5	0.300 $\pm$ 0.029 [*]	0.352 $\pm$ 0.034	0.368 $\pm$ 0.032 [*]
UTI + MglG group	5	0.287 $\pm$ 0.022 [*]	0.321 $\pm$ 0.025 [*]	0.348 $\pm$ 0.034 [*]

Note: UTI: ulinastatin; MglG: magnesium isoglycyrrhizinate. *, $P < 0.05$, a comparison of all drugs intervention groups (MTH group, UTI group, MglG group and UTI + MglG group) with the BLM group. [#], $P < 0.05$, a comparison of UTI + MglG group with other interventional groups (MTH group, UTI group and MglG group).

group was significantly reduced than that in the MTH group, UTI group, and MglG group, with statistical difference when compared with that in the MTH group ($P = 0.028$). On the 14th day, MMP-9 expressions in the UTI group, MglG group and UTI + MglG group were all reduced when compared to the BLM group, and there was evident statistical difference between UTI + MglG group and BLM group ($P = 0.002$); and the expression level in the UTI + MglG group were also decreased comparing with that in the MTH group, UTI group and MglG group, indicated apparent statistical differences when compared with MTH group and UTI group ($P = 0.006$ and $P = 0.046$, respectively). Furthermore, on the 28th day, compared with that in the BLM

group, decreased MMP-9 expression level was found in the UTI group, MglG group and UTI + MglG group than other four groups ($P = 0.030$, $P = 0.009$ and $P = 0.003$, respectively). Beside, there were statistical differences in the comparison of UTI + MglG group with MTH group and UTI group ($P = 0.014$ and $P = 0.036$, respectively).

Immunohistochemical results of ICAM-1

Immunohistochemical staining images were shown in **Figure 3**, presented detailed information of different drugs interventions effects on expression levels of ICAM-1 in experimental rats with acute pulmonary fibrosis on the 7th day, the 14th day, and the 28th day. Yellow or brown yellow particles found in the alveolar epithelium of ICAM-1 were the positive expression location. ICAM-1 was mainly expressed in alveolar epithelial cells, inflammatory cells, bronchial epithelial cells at different time points, peaked on the 7th day and then gradually decreased over time. Positive expression intensity of ICAM-1 in each time point of the drug

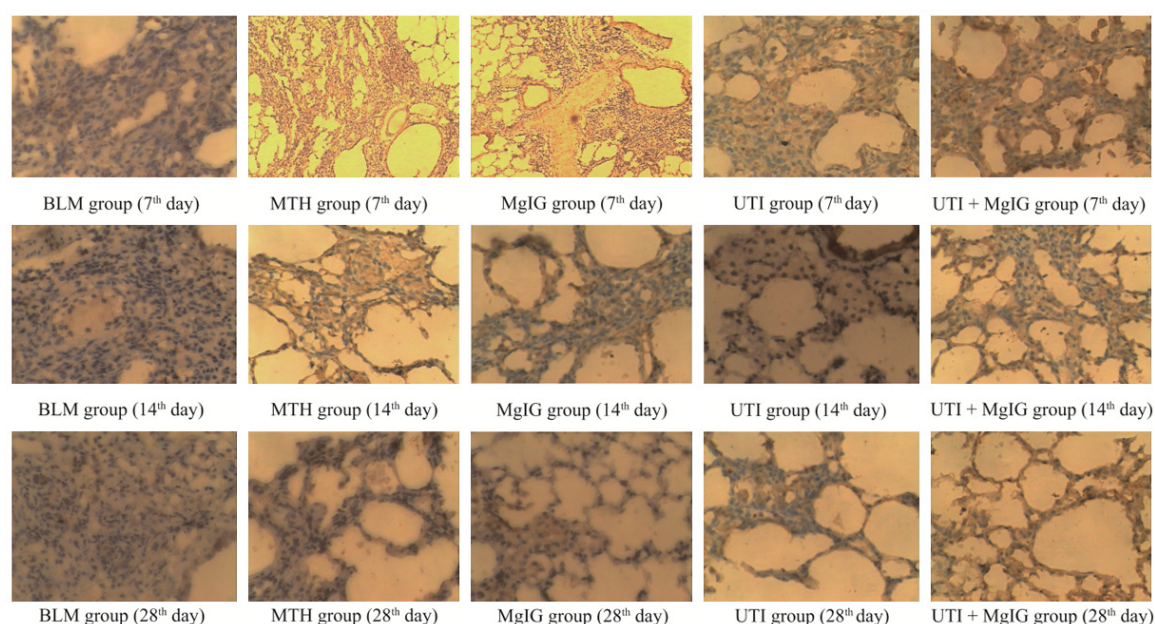


Figure 3. Effects of UTI combined with MgIG on expression levels of ICAM-1 in experimental rats with acute pulmonary fibrosis detected by immunohistochemistry (magnification: $\times 200$) on the 7th day, the 14th day, and the 28th day.

Table 4. Effects of UTI combined with MgIG on expression levels of ICAM-1 in experimental rats with acute pulmonary fibrosis

Grouping	Cases (n)	Expression levels of ICAM-1		
		The 7 th day	The 14 th day	The 28 th day
BLM group	5	0.166 $\pm$ 0.032	0.322 $\pm$ 0.021	0.343 $\pm$ 0.025
MTH group	5	0.145 $\pm$ 0.024 [#]	0.264 $\pm$ 0.027 ^{*,#}	0.309 $\pm$ 0.026 ^{*,#}
UTI group	5	0.130 $\pm$ 0.023	0.245 $\pm$ 0.022 [*]	0.284 $\pm$ 0.029 ^{*,#}
MgIG group	5	0.133 $\pm$ 0.019	0.238 $\pm$ 0.028 [*]	0.266 $\pm$ 0.027 ^{*,#}
UTI + MgIG group	5	0.117 $\pm$ 0.012 [*]	0.224 $\pm$ 0.026 [*]	0.237 $\pm$ 0.019 [*]

Note: UTI: ulinastatin; MgIG: magnesium isoglycyrrhizinate. *, $P < 0.05$, a comparison of all drugs intervention groups (MTH group, UTI group, MgIG group and UTI + MgIG group) with the BLM group. #, $P < 0.05$, a comparison of UTI + MgIG group with other interventional groups (MTH group, UTI group and MgIG group).

intervention groups was lower than that of the group I in the same period. In addition, in **Table 4**, as for the expression levels of ICAM-1 among groups, on the 7th day, expressions of ICAM-1 among all drug intervention groups were significantly lower than that in the BLM group, with statistical difference in UTI + MgIG group ($P = 0.013$); and within intervention groups comparison, ICAM-1 level in the UTI + MgIG group was significantly reduced than that in MTH group, UTI group, and MgIG group, with statistical difference between MTH group and UTI + MgIG group ($P = 0.048$). On the 14th day, ICAM-1 expressions in the MTH group, UTI group, MgIG group and UTI + MgIG group were all reduced

when compared to the BLM group ($P = 0.005$, $P = 0.001$, $P = 0.001$, and $P < 0.001$, respectively), and the expression level in the UTI + MgIG group were also decreased comparing with that in the UTI group and MgIG group without statistical differences (all $P > 0.05$). Furthermore, on the 28th day, decreased ICAM-1 expression

level was found in the UTI + MgIG group than other four groups, with statistical differences except with the MgIG group ($P < 0.001$, $P = 0.001$, and $P = 0.016$, respectively).

Immunohistochemical results of TGF- β 1

Yellow or brown yellow particles found in the cytoplasm of TGF- β 1 were the positive expression location. At the 7th day, TGF- β 1 was mainly expressed in alveolar macrophages and a few in fibroblasts. On the 14th day, TGF- β 1 expression in bronchus, bronchiole epithelial cells and fiber cells was significantly increased; on the 28th day, TGF- β 1 expression in the cytoplasm

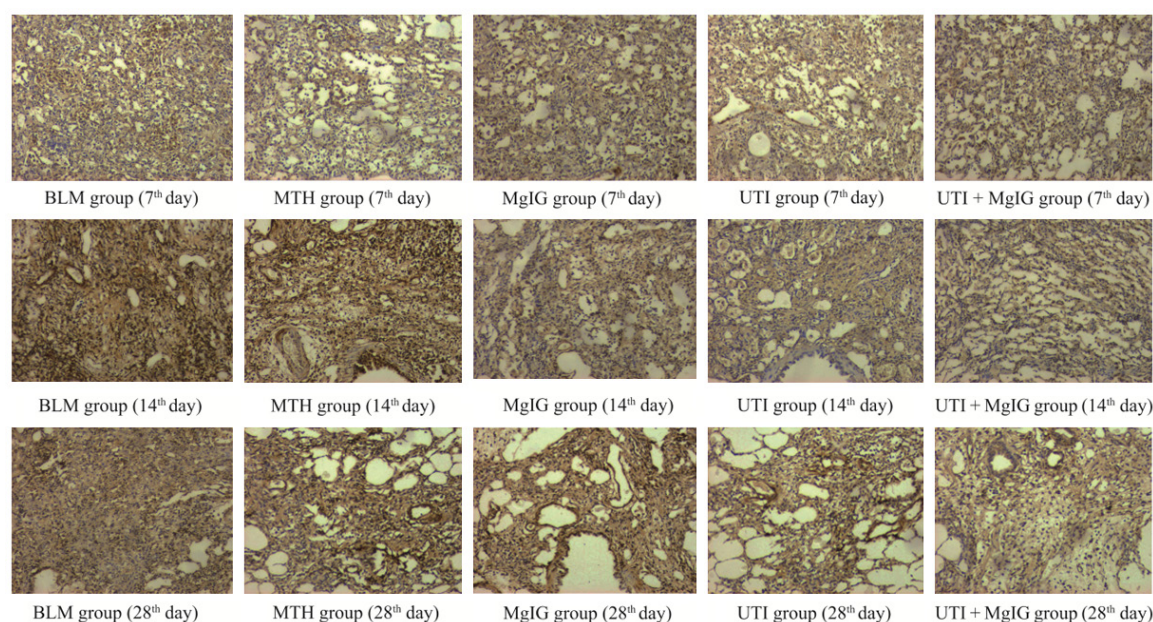


Figure 4. Effects of UTI combined with MglG on expression levels of TGF- β 1 in experimental rats with acute pulmonary fibrosis detected by immunohistochemistry (magnification: $\times 200$) on the 7th day, the 14th day, and the 28th day.

Table 5. Effects of UTI combined with MglG on expression levels of TGF- β 1 in experimental rats with acute pulmonary fibrosis

Grouping	Cases (n)	Expression levels of TGF- β 1		
		The 7 th day	The 14 th day	The 28 th day
BLM group	5	0.313 $\pm$ 0.025	0.340 $\pm$ 0.025	0.431 $\pm$ 0.026
MTH group	5	0.302 $\pm$ 0.027 [#]	0.313 $\pm$ 0.047 [*]	0.427 $\pm$ 0.029 ^{*,#}
UTI group	5	0.277 $\pm$ 0.024 ^{*,#}	0.299 $\pm$ 0.053 [*]	0.413 $\pm$ 0.25 ^{*,#}
MglG group	5	0.270 $\pm$ 0.022 ^{*,#}	0.287 $\pm$ 0.059 [*]	0.395 $\pm$ 0.026 ^{*,#}
UTI + MglG group	5	0.232 $\pm$ 0.023 [*]	0.272 $\pm$ 0.039 [*]	0.335 $\pm$ 0.039 [*]

Note: UTI: ulinastatin; MglG: magnesium isoglycyrrhizinate. ^{*}, $P < 0.05$, a comparison of all drugs intervention groups (MTH group, UTI group, MglG group and UTI + MglG group) with the BLM group. [#], $P < 0.05$, a comparison of UTI + MglG group with other interventional groups (MTH group, UTI group and MglG group).

was further increased. Importantly, the positive expression intensity of TGF- β 1 in each time point of the drug intervention groups was lower than that of the BLM group, the sham operation group, in the same period. Detailed immunohistochemical staining images were shown in **Figure 4**, to figure out the influence of different drugs intervention on expression levels of TGF- β 1 in experimental rats with acute pulmonary fibrosis on different time points. With respect to the expression levels of TGF- β 1 comparison among groups (as presented in **Table 5**), on the 7th day, expressions of TGF- β 1 among all drugs intervention groups (MTH group, UTI group, MglG group and UTI + MglG group) were signifi-

cantly lower than that in BLM group, indicating statistical differences among UTI group, MglG group and UTI + MglG group ($P = 0.049$, $P = 0.020$, and $P = 0.001$, respectively); and within drugs intervention groups comparison, such expression level in the UTI + MglG group also indicated a decreased tendency when compared to the MTH group, UTI group, and MglG group all revealing statistical differences ($P = 0.002$, $P = 0.016$, and $P = 0.028$, respectively). On the 14th day, TGF- β 1 expressions in the UTI group, MglG group and UTI + MglG group were all reduced when compared to the BLM group, with statistical differences among UTI group, MglG group and UTI + MglG group ($P = 0.047$, $P = 0.015$, and $P = 0.004$, respectively); and the expression level in the UTI + MglG group were also decreased comparing with that in the UTI group and MglG group, yet none obvious statistical differences were found (all $P > 0.05$). Furthermore, on the 28th day, decreased trend of TGF- β 1 expression levels was also found in

the UTI + MglG group when compared to the other four groups, showing statistical significance ($P = 0.002$, $P = 0.003$, $P = 0.006$, and $P = 0.021$, respectively).

Correlation analysis of MMP-9, ICAM-1 and TGF- β 1 in the lung fibrosis process

Correlation analysis results indicated that there were positive correlations of MMP-9, ICAM-1 and TGF- β 1 expression with the degrees of alveolar catarrh in the lung tissues, corresponding correlation coefficients were 0.763, 0.815 and 0.780, respectively (all $P < 0.05$). Furthermore, obvious positive correlations of MMP-9, ICAM-1 and TGF- β 1 expression with the score of pulmonary fibrosis were also observed, and their correlation coefficients were 0.532, 0.477, and 0.520, respectively (all $P < 0.05$).

Discussion

Both UTI and MglG have inhibitory role of inflammatory cytokines, but there were still without clinical evidence and experimental study of combination therapy in rats' model of pulmonary fibrosis [23, 24]. Etiological study shows that viral infection, abnormal expression of cytokines, autoimmunity, oxidative stress, inhalation of toxic substances are all correlated with the development of acute pulmonary fibrosis. The development of acute pulmonary fibrosis include three major steps, the immune and inflammatory response of the lung, the damage to the lung parenchyma, and the repair of the damaged alveoli. Chronic inflammation is the pathological basis and promotes the formation of pulmonary fibrosis. The present study is therefore conducted to investigate the effects of UTI combined with MglG on acute pulmonary fibrosis rats' model and its mechanism of treatment.

In the study, two major problems needed to be solved and corresponding solution were illustrated as follows: firstly, this study was designed to identify the effects of the administration of UTI combined with MglG against pulmonary fibrosis; accordingly, besides the construction of rats' model of pulmonary fibrosis by injection of bleomycin as the model control group [25], a known drug, Solu-Medrol, having anti-pulmonary fibrosis effect was used as a intervention control group, and finally UTI combined with MglG or their single usage were all proved to

have the same anti-pulmonary fibrosis effect. Secondly, with regard to the selection of observational indexes, ICAM-1 is an important pro-inflammatory factor, TGF- β 1 is the most important index for the induction of fibrosis, and MMP-9 is the key enzyme in tissue remodeling [26-28], which are the central parts in the development of pulmonary alveolar inflammation and pulmonary fibrosis.

To be specific, previous studies have confirmed that UTI and MglG can inhibit the excessive release of inflammatory mediators, exerting antioxidation, anti-fibrosis, stable cell membrane, immune regulation, and inhibition of paraquat poisoning induced pulmonary fibrosis [20, 29]. In the study, lymphocytes, neutrophils and other inflammatory cells infiltration were predominant during the early stage of Bleomycin induced lung injury, with the passage of time, the proliferation of fibroblasts and pulmonary fibrosis aggravated gradually. In the early stage (0~7 days) of administration, acute inflammation of alveoli was observed, and such acute inflammatory would be decreased accompanied with proliferation of fibroblasts and deposition of matrix collagen. It might be correlated with reasons that inflammatory cells in lung tissues produced a variety of cytokines and other molecules, stimulating fiber cell replication, proliferation and synthesis of collagen mainly extracellular matrix (ECM) components, leading to the accumulation of a large number of collagen [30]. Furthermore, in the late phase (14~28 days), normal structure of lung tissue and the alveoli were destroyed, and the large fibrotic tissue was formed; such process also indicated the successful establishment of pulmonary fibrosis model. Meanwhile, pulmonary alveolar inflammation and pulmonary fibrosis degrees in drug intervention groups at different time points were significantly reduced compared with that in the model control group and in the intervention control group, which was also more obvious in rats managed by UTI combined with MglG than the other drug intervention groups. Firstly, the establishment method of pulmonary fibrosis in rats by bleomycin was mature and with rich experience. Currently, it is well known that the standard agent for induction of experimental pulmonary fibrosis in animals is bleomycin. It causes inflammatory and fibrotic reactions within a short period of time. The initial elevation of pro-inflammatory cyto-

kines is followed by increased expression of pro-fibrotic markers with a peak around day 14, which has been mentioned in the study conducted by Moeller A [31]. Secondly, acute pulmonary fibrosis is a kind of disease with complicated etiology and pathogenesis, its main pathological changes are alveolar inflammation and interstitial fibrosis. From the present results, the combination of UTI with MglG might be workable for the treatment of pulmonary fibrosis to significantly reduce degrees of pulmonary alveolar inflammation and pulmonary fibrosis, and there was no obvious toxic-side effects, suggesting that UTI combined with MglG could have a role on pulmonary fibrosis. TGF- β 1 is a multifunctional cytokine, which is the most direct cell factor in the process of pulmonary fibrosis [32]. TGF- β 1 can stimulate the proliferation of fibroblasts in the lung tissue, but also to promote the synthesis of ECM, on the other hand to inhibit the new synthesis of MMP degradation, but also has the role of inhibiting the proliferation of alveolar epithelial cells [33]. ICAM-1 participates in cell adhesion and recognition, which can promote the activation of T cells and the infiltration of leukocytes from the blood vessels to inflammatory sites, essential for the inflammation and immune response [34]. As for the internal mechanisms, MMP-9, ICAM-1 and TGF- β 1 expression levels showed increased tendency in rats model of pulmonary fibrosis, and then inhibited in rats lung tissues after drugs intervention, suggesting that MMP-9, ICAM-1 and TGF- β 1 play critical roles in pathologic process of pulmonary fibrosis, mainly manifested as the promotion of inflammation and fibrosis, and UTI with MglG were indicated to take effect during such process by exerting effects of decreasing alveolitis and pulmonary fibrosis.

In conclusion, our findings support the view that UTI combined with MglG can effectively inhibit expression of MMP-9, ICAM-1 and TGF- β 1 in rats lung tissues of pulmonary fibrosis, inhibit the recruitment and activation of inflammatory cells in the lung, reduce the degree of alveolitis and pulmonary fibrosis, decrease the secretion of proinflammatory cytokines and pulmonary fibrosis factors, thereby reducing the synthesis and deposition of collagen and effectively suppressing the bleomycin induced pulmonary fibrosis process, providing new treatments for the prevention and treatment of pulmonary interstitial fibrosis.

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Disclosure of conflict of interest

None.

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