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Gastric Acid and Pepsin Work Together in Simulated Gastric Acid Inhalation Leading to Pulmonary Fibrosis in Rats

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Background: The clinical association between gastroesophageal reflux disease (GERD) and idiopathic pulmonary fibrosis (IPF) has been known for many years, but it is still unclear. The present study investigated the association between experimentally simulated aspiration and pulmonary fibrosis.





Material/Methods A total of 120 male Sprague-Dawley rats were randomly divided into a negative control group, a bleomycin group, and 3 simulated aspiration groups. The bleomycin group was administered a one-time intratracheal injection of bleomycin, whereas the 3 simulated aspiration groups were treated either with an intratracheal instillation of gastric fluid combined with pepsin, with pepsin alone, or with hydrochloric acid, all twice a week, and the negative control group was administered normal saline twice a week. Lung tissues were collected to evaluate pathological changes and the mRNA expression levels of connective tissue growth factor (CTGF), type I collagen, and transforming growth factor.

Results: The results demonstrated that the degree of fibrosis in the early stage was low in each of the 3 simulated aspiration groups, but gradually increased over time. The expression levels of the downstream factor of fibrosis, CTGF, and type I collagen also reflected this trend.

Conclusions: The study demonstrates that aspiration of gastric contents can cause pulmonary fibrosis, and mixed aspiration of pepsin and gastric fluid can accelerate this process. This study provides strong evidence in support of a potential association between human GERD and IPF.

MeSH Keywords: **Fibrosis • Gastric Acid • Gastric Juice • Inhalation**

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Background

Idiopathic pulmonary fibrosis (IPF) is most commonly observed in idiopathic interstitial pneumonia, with a morbidity rate of 14–42.7 per 10 000 people and a median survival of 3 years. Pirfenidone and nintedanib are the only effective therapies currently available, but these 2 drugs do little to improve the prognosis. The natural course of IPF is regarded as a progressive process characterized by continuously decreasing pulmonary function over time [1]. However, the definitive mechanism of IPF has yet to be fully elucidated, and many patients develop acute aggravation for unknown reasons after experiencing a stationary phase [2].

Gastroesophageal reflux disease (GERD) is associated with multiple respiratory system diseases, such as chronic cough, asthma, obstructive sleep apnea syndrome, and chronic obstructive pulmonary disease [3]; however, its role in pulmonary fibrosis and lesion formation is often overlooked. As indicated in previous studies, the majority of patients with IPF also have GERD [4–6]. Extensive clinical application of methods such as 24-h esophageal pH monitoring in recent years has gradually improved our understanding of the potential influence of GERD on IPF. Consequently, it has been speculated that the acid inhalation caused by GERD may be associated with pulmonary fibrosis [7]. Numerous studies in the literature have reported an association between IPF and GERD [8–12] since the first report on the clinical association between GERD and pulmonary fibrosis was published by Mays et al. in 1976 [13]. Raghu et al. [14] conducted a similar statistical study in which 65 patients were shown to have been diagnosed with IPF, and the morbidity rate of GERD was as high as 87% [14]. Gao et al. [15] conducted similar research in a Chinese IPF population, and reported a GERD morbidity rate of ~62.3%.

Repeated aspiration results in repeated damage to the pulmonary parenchyma, which leads to the genesis and development of pulmonary fibrosis over time. In addition, repeated aspiration is also regarded as a possible cause of acute IPF aggravation [11,12].

The majority of published studies have suggested that IPF is associated with repeated minor aspiration of gastric acid (the main ingredient of which is hydrochloric acid) induced by GERD; however, as gastric juice contains not only hydrochloric acid, but also pepsin, mucus, and food particles, the effects of repeated inhalation of each ingredient on pulmonary injury have yet to be elucidated. In an animal experiment, Popper et al. [16] reported that acid instilled into the airway can induce pulmonary injury. Alveolar cells may be damaged under acidic conditions, resulting in reduced production of pulmonary surfactants. In addition, extravasation of protein-rich plasma can further aggravate pulmonary tissue damage.

Some researchers have reported that repeated inhalation of gastric acid in rat lungs (predominantly of hydrochloric acid) can cause inflammatory cell infiltration, bronchiolitis obliterans, or even pulmonary fibrosis [17]. A histological study of lung specimens from experimental animals indicated that increased giant cell infiltration, lymphocytic bronchiolitis, bronchiolitis, increased inflammatory cytokines in lung lavage fluid, and the formation of fibrosis may also occur [18].

Given that gastric fluid contains a complex mixture of ingredients, whether gastric fluid (hydrochloric acid), pepsin, or their combination influences the process of pulmonary fibrosis development has yet to be fully elucidated. Reports from previously published clinical studies have indicated that conventional anti-acid therapy did not improve the prognosis for patients with fibrosis [19–21], raising the 2 important questions: 1) Does repeated aspiration of multiple gastric fluid ingredients promote the pulmonary fibrosis process? and 2) What are the differences in the pathological and physiological features of rat pulmonary fibrosis models induced by conventional bleomycin treatment? The present study attempted to answer these questions.

We devised a series of experiments to discover the mechanisms associated with the pathological and physiological pulmonary changes induced by experimental inhalation of acid/pepsin in terms of histology, protein expression, and mRNA expression. The effects of hydrochloric acid (the main ingredient of gastric acid) and pepsin, as well as their combined effects, on pulmonary injury were observed through multiple methods, including pathological slice analysis, half-quantitative immunohistochemical protein identification, and semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR), to analyze their roles during pulmonary fibrosis development and to describe the possible pathological and physiological mechanisms.

Material and Methods

Reagents

Bleomycin A₅ was purchased from Tianjin Taihe Pharmaceutical Co. (Tianjin, China). The Invitrogen® semi-quantitative RT-PCR reagent, TRIzol®, the Superscript™ III Reverse Transcriptase kit, and the oligo(dT) were all obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA), and the PCR kit (Taq™) was provided by Takara Biotechnology Co. (Dalian, China). The rat monoclonal antibody against transforming growth factor β₁ (TGF-β₁) was purchased from R&D Systems, Inc. (Minneapolis, MN, USA), the rat monoclonal antibody against connective tissue growth factor (CTGF) was obtained from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA), and the type I collagen monoclonal antibody was provided by Sigma-Aldrich (now

a brand of Merck KGaA, Darmstadt, Germany). The PV-6000 two-step method immunohistochemical kit was purchased from Beijing Zhongshan Jinqiao Biological Company, Beijing, China), and pepsin was provided by Wuhan Boster Biological Technology (Wuhan, China).

Construction and grouping of the animal models

A total of 120 healthy male Sprague-Dawley (SD) rats (provided by the Laboratory Animal Center of Nanjing Medical University) weighing 250–300 g were selected and randomly divided into 5 groups: the negative control group (group N), the bleomycin group (group B), the pepsin combined with gastric fluid group (group P+G), the pepsin group (group P), and the hydrochloric acid group (group A), with 24 rats in each group. All experiments involving animals and tissue samples were performed according to the guidelines of the National Institutes of Health and Nanjing Medical University, and all procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (Nanjing, China).

Gastric fluid collection

The experimental rats were fasted for 12 h and subsequently sedated with an intraperitoneal injection of ketamine (100 mg/kg) and Xylazine (0.75 mg/kg). The abdomens were cut open to ligate the distal esophagus, and a small opening was created in the duodenum above the pyloric sphincter. Subsequently, 5 ml of distilled water was injected into the stomach through a channel connected to the small opening, and the gastric contents were collected, centrifuged (5°C, 2500 RPM) 20 min later, and frozen immediately at –80°C prior to use [22].

Pepsin and hydrochloric acid preparation

Pepsin was prepared to yield a finished product in liquid form at a concentration of 2.5 µg/ml and a pH in the range of 1.5–2 [22–24]. Generic hydrochloric acid was diluted with distilled water to achieve a solution with a pH of 1.5 [22–24].

Methods of aspiration simulation

For group N, intratracheal instillation with 2 ml normal saline was performed twice a week through tracheal intubation, whereas for group B, a one-time intratracheal injection of bleomycin (5 mg/kg) was administered. For group P+G, 2.5 µg/ml pepsin was mixed with the collected gastric fluid at a proportion of 1: 1, and intratracheal instillation (0.5 ml/kg) was performed twice a week through tracheal intubation. For group P, intratracheal instillation (0.5 ml/kg) with 2.5 µg/ml pepsin was performed twice a week through tracheal intubation, whereas for group A, intratracheal instillation (0.5 ml/kg) with HCl was performed twice a week through tracheal intubation.

Sampling

Six rats in each group were randomly sacrificed on the 7th, 14th, 28th, and 56th days following modelling via abdominal aorta exsanguination after having administered an intraperitoneal injection of ketamine combined Xylazine for anesthesia. Subsequently, the chest was cut open to collect the lung tissues, which were rinsed with normal saline several times. The superior lobe of the right lung was fixed in 10% formalin, followed by conventional paraffin embedding, hematoxylin and eosin (HE) staining, Masson trichrome staining, and immunohistochemical staining.

Lung tissue pathological analysis

HE and Masson trichrome staining of the lung tissues were performed following conventional dehydration with alcohol of various concentrations, paraffin embedding, and slicing. The severities of pulmonary alveolitis and pulmonary fibrosis were evaluated in accordance with the methods described by Szapiel et al. [25]. Pulmonary alveolitis was classified into 4 grades: (1) nonpulmonary alveolitis (0), which was assigned a grade of 0; (2) mild pulmonary alveolitis (+), which was assigned grade of 1, and was characterized by a normal alveolar structure and a broadening alveolar septum due to mononuclear cell infiltration restricted to local and proximal pleural regions affecting <20% of the whole lung; (3) moderate pulmonary alveolitis (++), which was assigned a grade of 2, and affected 20–50% of the whole lung, with greater severity in the proximal pleural region; and (4) severe pulmonary alveolitis (+++), which was assigned a grade of 3, and affected >50% of the lung, with evident mononuclear cell infiltration, as well as consolidation due to occasional bleeding within the alveolar space. Pulmonary fibrosis was classified into 4 grades: (1) nonpulmonary fibrosis (–), which was assigned a grade of 0; (2) mild pulmonary fibrosis (+), which was assigned a grade of 1, and affected <20% of the lung, with fibrosis involving the pleura and the subpleural pulmonary interstitium and an evidently disordered alveolar structure; (3) moderate pulmonary fibrosis (++), which was assigned a grade of 2, and affected 20–50% of the whole lung, with the local fibrosis region extending beyond the pleura; and (4) severe pulmonary fibrosis (+++), which was assigned a grade of 3, and affected >50% of the lung, with evident fusion as well as cystic air cavities of various sizes.

Expression of TGF-β₁ mRNA in lung tissues

Semi-quantitative RT-PCR was used for detection, and the TRIzol® method was utilized to extract the total lung tissue RNA, followed by reverse-transcription to synthesize cDNA, as well as PCR amplification. The primers were synthesized by Shanghai Shengggong Biological Engineering Technology

Service Co. (Shanghai, China), and the sequences were as follows: (i) TGF- β_1 , forward primer: 5'-GCTAATGGTGACCGCAAC-3'; and reverse primer: 5'-GCAGTGAGCACTGAAGCGA-3', with a target fragment length of 340 bp; and (ii) β -actin, forward: 5'-GAGACCTCAACACCCAGC-3'; and reverse: 5'-CACAGAGTACTTGCCTCAG-3', with a target gene fragment length of 654 bp. The PCR reaction conditions were as follows: TGF- β_1 : Pre-degeneration for 4 min at 94°C, 45 s at 94°C, 45 s at 56°C, and 60 s at 72°C for 35 loops, followed by extension for 10 min at 72°C and termination; and β -actin: Pre-degeneration for 4 min at 94°C, 45 s at 94°C, 45 s at 56°C, and 60 s at 72°C for 35 loops, followed by extension for 10 min at 72°C and termination. The amplification products were subjected to 1% agarose gel electrophoresis (including 0.5 μ g/ml ethidium bromide), and scanning and analysis were performed using a gel imaging and analysis system. The absorbance ratio of TGF- β_1 to β -actin amplification products was regarded as the relative mRNA level of each specimen.

Determination of the expression levels of CTGF and type I collagen in lung tissue

The immunohistochemical DAKO EnVision™ two-step method (Agilent Technologies, Inc., Santa Clara, CA, USA) was used, and the expression levels of CTGF and type I collagen were measured according to the manufacturer's protocol. The key steps were as follows. The paraffin sections were dewaxed by conventional method, and then rehydrated; subsequently, blocking of the peroxidase was performed with 3% H₂O₂/formaldehyde solution. Repair of the antibodies was accomplished with EDTA repair buffer (pH 8.0) under conditions of high temperature and high pressure, followed by instillation of the anti-CTGF and anti-type I collagen monoclonal antibodies, and preservation in a refrigerator at 4°C overnight. The following day, the sections were incubated for 30 min with secondary antibodies labelled with horseradish peroxidase (HRP), at room temperature. Controlled color development was performed using 3,3-diaminobenzidine (DAB) stain, followed by counterstaining with hematoxylin, and the sheets were then sealed with neutral resins following hydrochloric acid/alcohol differentiation. For the negative control, the primary antibodies were replaced with PBS.

Results analysis

Concerning the immunohistochemical results, claybank coloring in lung tissues was indicative of positive color development, and the expression levels of CTGF and type I collagen were quantitatively analyzed using the professional image analysis soft system, Image-Pro® Plus (Media Cybernetics, Inc., Rockville, MD, USA). Five views were selected randomly from each slice (magnification, $\times 400$), and the integral optical density (IOD) was considered to represent the expression level of CTGF. Similarly, 5 views were selected randomly from each slice

(magnification, $\times 100$), and the IOD was considered to represent the expression level of type I collagen.

Statistical analysis

SPSS version 18.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used to process all the data, which are presented as the mean \pm standard deviation. One-way ANOVA was applied for comparisons among groups, whereas the Student-Newman-Keuls *q* test was used for pairwise comparisons. *P* < 0.05 was considered to indicate a statistically significant difference.

Results

Pathological changes in the lung tissue

Comparisons between the samples were made to assess the extent of pulmonary alveolitis. In group N, the alveolar walls were revealed to be thin, with a normal structure and without special changes. In group B, a notable inflammatory reaction was observed in the alveolar septum and alveoli 7 days after intratracheal instillation of bleomycin, as well as massive inflammatory cell infiltration, which was markedly greater compared with that in group N (*P* < 0.01). Subsequently, the numbers of inflammatory cells decreased over time, and the affected areas gradually came to be dominated by fibrosis. Additionally, a broadening alveolar septum, fibroblast hyperplasia, and multi-focal fibrosis regions of various sizes were observed. In the 3 simulated aspiration groups, alveolitis peaked within the first 2 weeks, and then remained relatively high, reaching or surpassing the level in group B from the 28th day onwards. The degree of alveolitis for each experimental group is shown in Figure 1.

Assessment of the extent of fibrosis

Masson trichrome staining was used to determine the extent of fibrosis in lung tissues. A small amount of collagenous fibers, which is the main component of the extracellular matrix (ECM), was observed in the lung tissues of rats in group N, and no special abnormal changes were evident. Pulmonary interstitial fibrosis occurred at an early stage in rats in group B; the collagenous fibers increased gradually at the late stage, and pulmonary interstitial fibrosis became progressively aggravated over time, peaking on the 28th day and then tending to decline. The simulated aspiration groups (groups A, P, and P+G) exhibited a low degree of fibrosis at the early stage, and the fibrosis degree subsequently increased over time and with increasing hydrochloric acid instillation time, although it did not reach the same level of fibrosis as in group B, but it was substantially greater compared with that in group N (*P* < 0.01). No distinct differences in the degree of fibrosis were identified

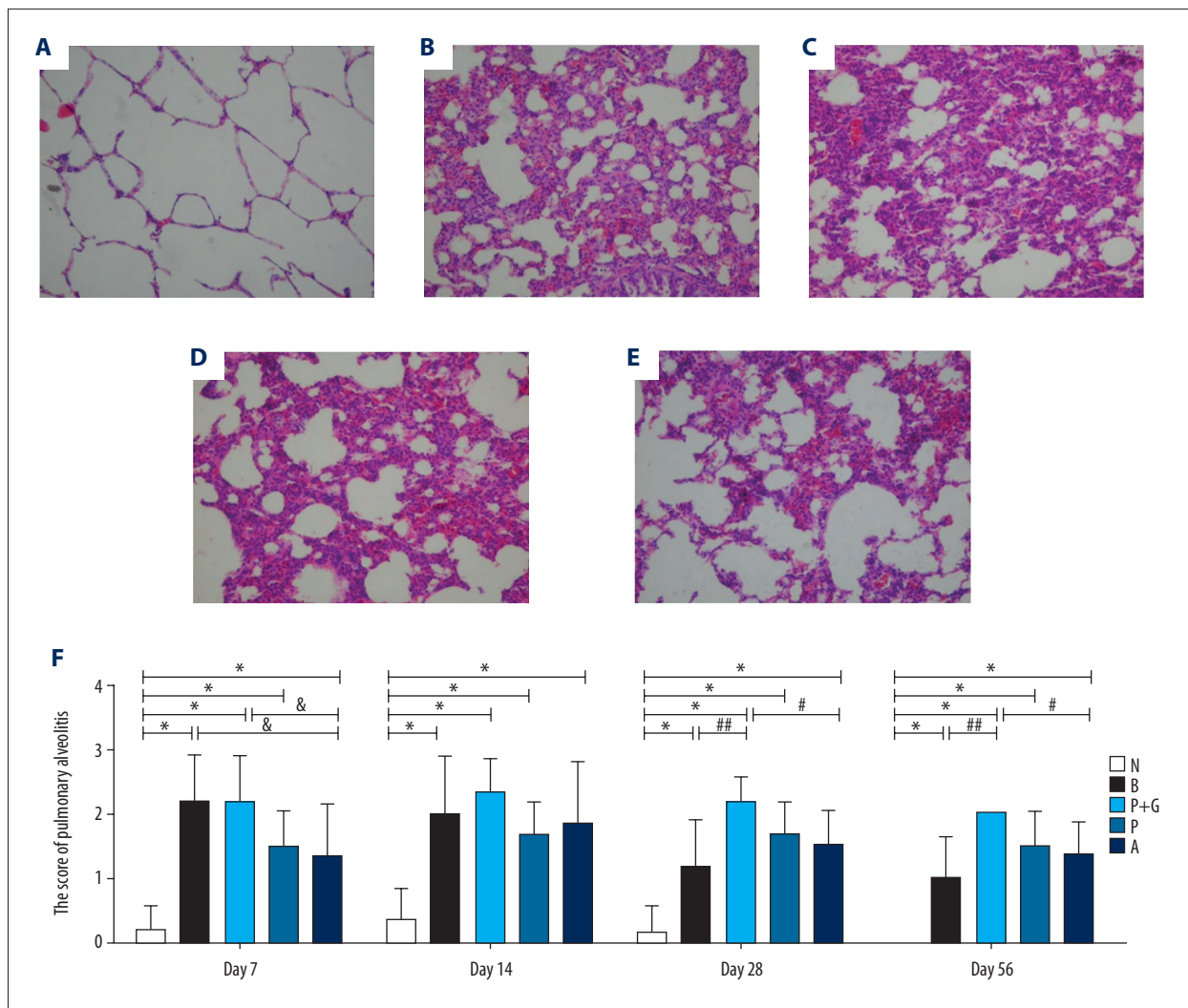


Figure 1. Comparison of pulmonary alveolitis degree. (A–E) Pulmonary alveolar inflammation in each group (7d) (hematoxylin and eosin staining $\times 100$) (Scale bar: 50 μm). (A) The negative control group (group N), (B) the bleomycin group (group B), (C) the pepsin combined with gastric fluid group (group P+G), (D) the pepsin group (group P), and (E) the hydrochloric acid group (group A). (F) Results of pulmonary alveolitis scores of rats in each group: data are presented as the means \pm SEM of 3 replicates. * $P < 0.01$ compared with group N; & $P < 0.05$ compared with group A; # $P < 0.05$; ## $P < 0.01$ compared with group P+G.

among the P+G, P, and B groups on the 56th day ($P > 0.05$). The extent of fibrosis in group A was lower compared with that in group P+G from the 28th day ($P < 0.05$). Finally, the degree of fibrosis in the 3 simulated aspiration groups did not exceed that in group B. These results are illustrated in Figure 2.

TGF- $\beta 1$ mRNA expression in lung tissue

The mRNA expression level of TGF- $\beta 1$ on the 7th and the 14th days was markedly higher in group B compared with that in the other groups ($P < 0.05$); however, the expression level also exhibited a trend of gradually declining after that point, such that there was no significant difference observed between groups B and N on the 56th day ($P > 0.05$). The expression level

of TGF- $\beta 1$ mRNA in group P+G always remained higher than in group N ($P < 0.05$), and the levels in groups P and A were higher compared with that in group N from the 14th day onwards. The expression levels of TGF- $\beta 1$ mRNA on the 7th and the 14th days in the 3 simulated aspiration groups were notably lower compared with those in group B ($P < 0.05$), and no distinct difference was identified between the simulated aspiration groups and group B on the 28th day ($P > 0.05$). Lastly, the expression levels of TGF- $\beta 1$ mRNA were observed to be higher in the 3 simulated aspiration groups compared with that in group B on the 56th day ($P < 0.05$). These results are shown in Figure 3.

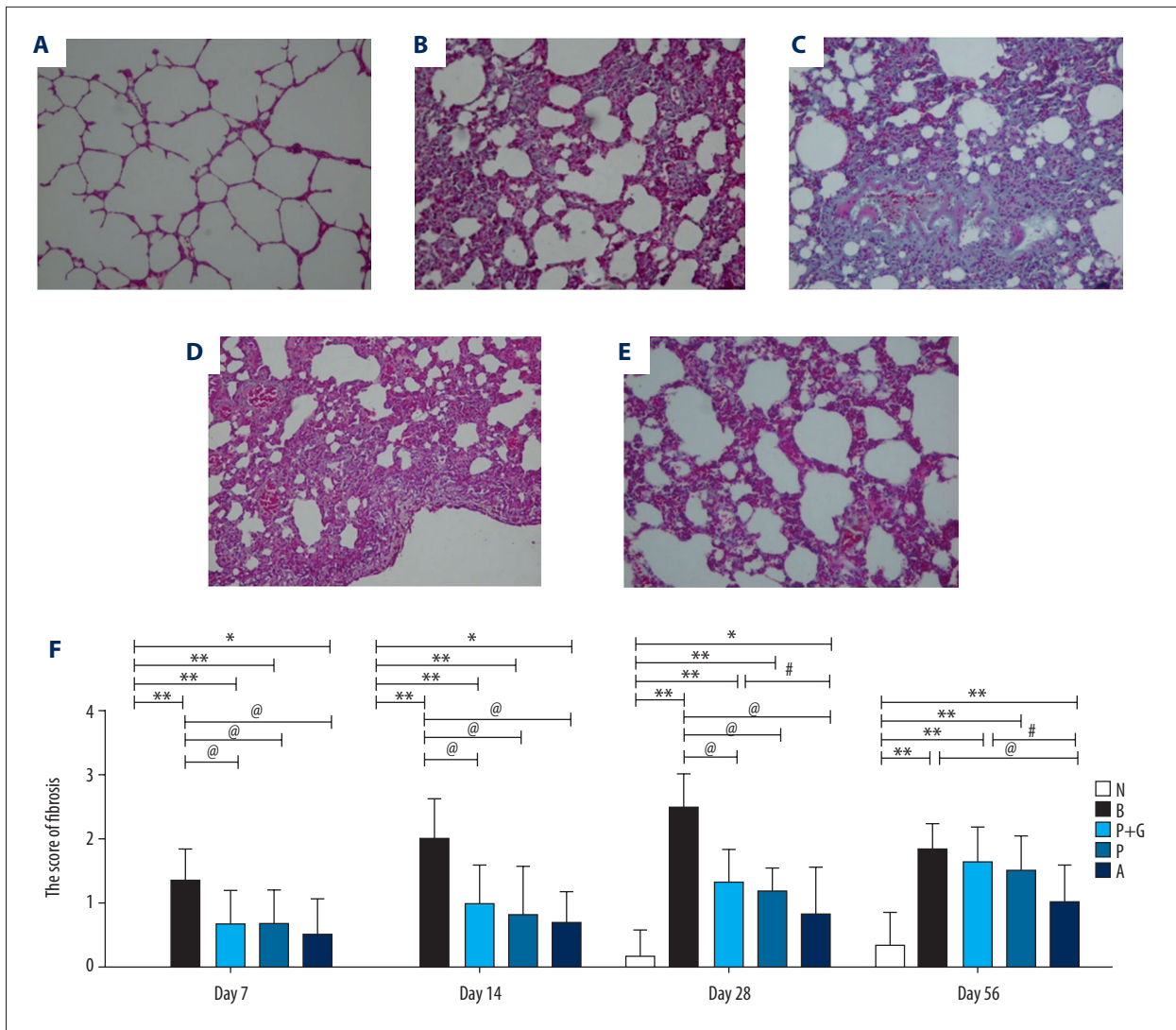


Figure 2. Comparison of fibrosis degree. (A–E) Pulmonary fibrosis in each group (14d) (Masson trichrome staining $\times 100$) (Scale bar: 50 μm). (A) The negative control group (group N), (B) the bleomycin group (group B), (C) the pepsin combined with gastric fluid group (group P+G), (D) the pepsin group (group P), and (E) the hydrochloric acid group (A group). (F) Results of pulmonary fibrosis scores of rats in each group: data are presented as the means \pm SEM of 3 replicates. * $P < 0.01$, ** $P < 0.01$ compared with group N; @ $P < 0.05$ compared with group B; # $P < 0.05$ compared with group P+G.

CTGF expression in the lung tissue

CTGF, the host factor that regulates fibroblast proliferation and ECM synthesis, functions as the downstream mediator of fibrosis, promoting the effects of TGF- $\beta 1$ and exerting a vital role during pulmonary fibrosis development. Its major biological functions are to stimulate the proliferation and differentiation of fibroblasts, mediate cell adhesion, and promote ECM deposition. As reported in the literature, CTGF protein expression is clearly increased in pulmonary fibrosis, and this increase is positively correlated with the expression levels of TGF- $\beta 1$ and type I collagen, further confirming that CTGF expression is closely associated with the degree of pulmonary

interstitial fibrosis. This experiment demonstrated that CTGF was poorly expressed, or was not expressed, in lung tissues in group N, a finding which is consistent with previously published studies, and it was predominantly expressed in the bronchus, bronchial epithelial cells, and type II alveolar epithelial cells. CTGF expression in group B began to increase from the 7th day onwards, peaking on the 14th day, and remaining high until the 28th day. Thereafter, it exhibited a trend towards decline, although it continued to remain substantially higher than in group N on the 56th day, and the difference between the 2 groups was statistically significant ($P < 0.05$). CTGF expression levels in groups P+G and group Ps were higher compared with that in group N on the 14th day and the 28th days,

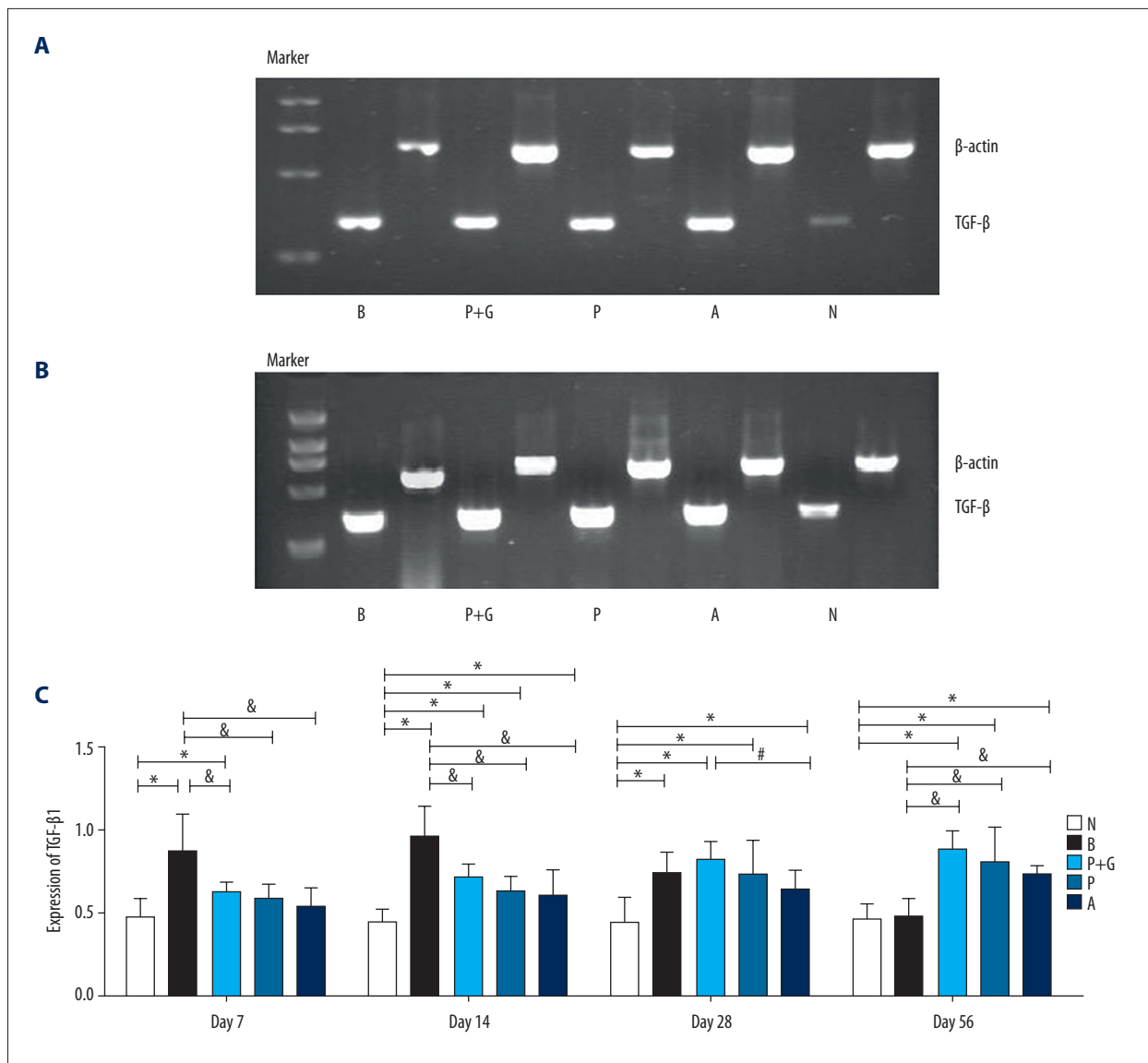


Figure 3. Expression of TGF-β1mRNA in lung tissue. (A) Expression of TGF-β1mRNA in lung tissue in 28d (B) Expression of TGF-β1mRNA in lung tissue in 56d N: the negative control group (group N), B: the bleomycin group (group B), P+G: the pepsin combined with gastric fluid group (group P+G), P: the pepsin group (group P), A: the hydrochloric acid group (group A); (C) The expression of TGFβ1mRNA in lung tissue of rats in each group: The values were presented as the means ±SEM of 3 replicates. * P<0.05 compared with N group; & P<0.05 compared with group B; # P<0.05 compared with P+G group.

respectively ($P<0.05$), and increased over time, although they were not significantly different from that in group B from the 56th day ($P>0.05$). Finally, CTGF protein expression in group A on the 56th day was higher compared with that in group N, although it was not apparently different from that in group B ($P>0.05$); however, CTGF protein expression in group A was clearly lower compared with that in the group P+G ($P<0.05$). These results are illustrated in Figure 4.

Type I collagen expression in lung tissue

Overexpression of TGF-β1 and CTGF in fibrotic lung tissue results in type I and type III collagen hyperplasia, and collagen deposition in the pulmonary interstitium leads to matrix structure disorders of lung tissue, giving rise to fibrosis. The results of the present experiment indicated that the expression of type I collagen in lung tissue was clearly higher in the rats in group B at all time points compared with the rats in group N ($P<0.05$), and massive claybank type I collagen fibers were identified in the vessel walls, bronchial walls, and alveolar

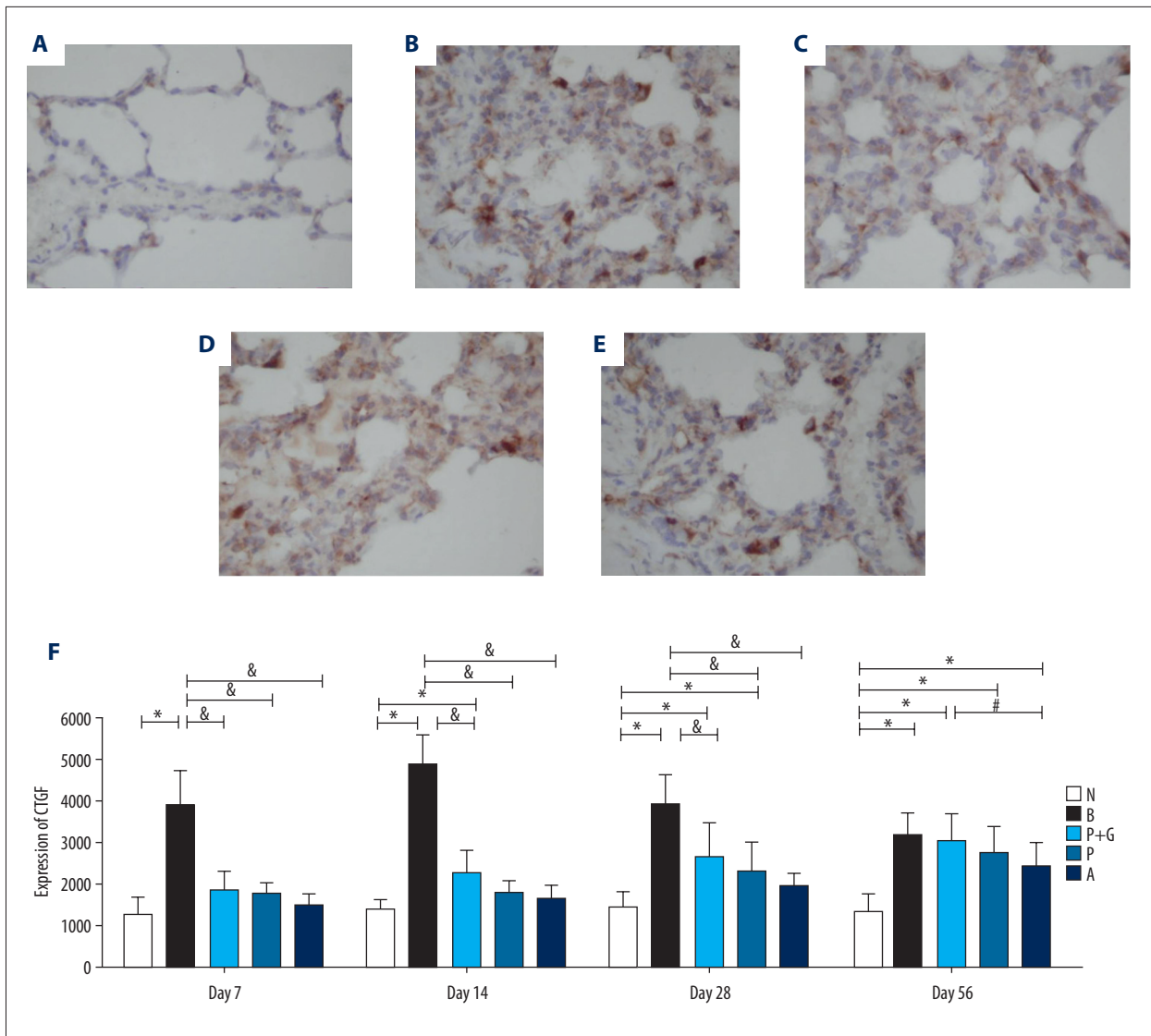


Figure 4. Expression of CTGF in lung tissue. (A–E) Expression of CTGF in each group (28d) (immunohistochemical staining $\times 100$) (scale bar: 50 μm). (A) The negative control group (group N), (B) the bleomycin group (group B), (C) the pepsin combined with gastric fluid group (group P+G), (D) the pepsin group (group P), (E) the hydrochloric acid group (group A). (F) Expression of CTGF in lung tissue of rats in each group: The IOD values were presented as the means \pm SEM of 3 replicates. * $P < 0.05$ compared with group N; & $P < 0.05$ compared with group B; # $P < 0.05$ compared with group P+G.

septum, which are observations consistent with the pathological and morphological changes noted in lung tissues. The expression level of type I collagen in the 3 simulated aspiration groups (groups A, P, and P+G) at all time points was lower compared with that in group B ($P < 0.05$). The expression of type I collagen fibers was higher in the group P+G than that in group N from the 14th day ($P < 0.05$), and it was always higher compared with that in group A ($P < 0.05$). Finally, the expression of type I collagenous fibers was higher in group P compared with that in group N from the 28th day ($P < 0.05$), although it did not reach the expression levels identified in group P+G or group B. These results are shown in Figure 5.

Discussion

Repeated aspiration of gastric contents is known to cause inflammatory cell infiltration, bronchiolitis obliterans, or even pulmonary fibrosis in the rat lung. The present study was mainly focused on aspiration of gastric acid (which predominantly comprises hydrochloric acid); the effects of pepsin have yet to be fully elucidated. Previous research has indicated that inhalation of pepsin may also result in chronic lung injury, whereas repeated chronic lung injury provoked pulmonary fibrosis [17]. The present study simulated the minor aspiration induced by gastroesophageal reflux through repeated injections

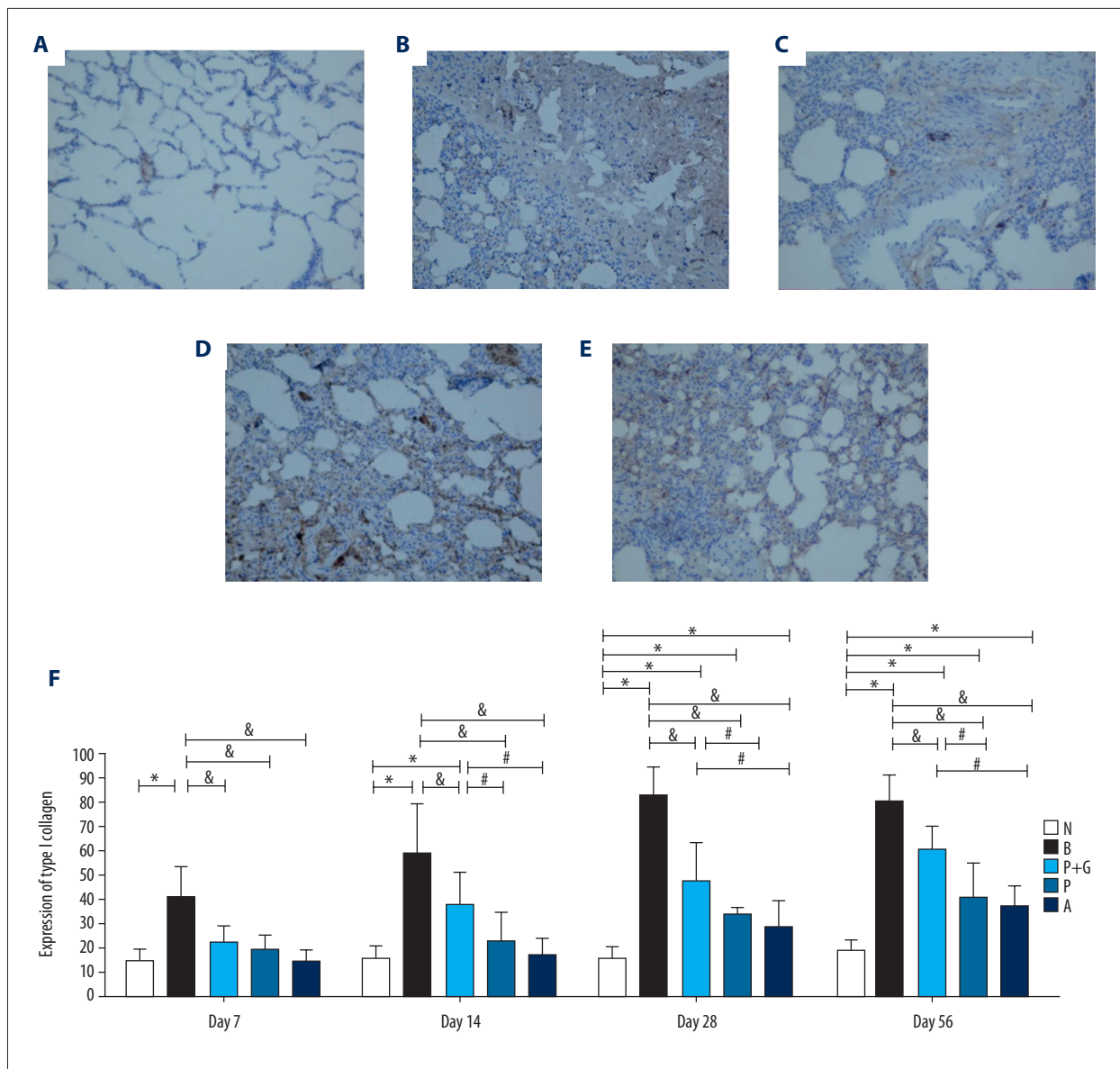


Figure 5. Expression of type I collagen in lung tissue. (A–E) Expression of type I collagen in each group (56d) (immunohistochemical staining $\times 100$) (scale bar: 50 μm). (A) The negative control group (group N), (B) the bleomycin group (group B), (C) the pepsin combined with gastric fluid group (group P+G), (D) the pepsin group (group P), (E) the hydrochloric acid group (group A). (F) The expression of I collagen in lung tissue of rats in each group: IOD values are presented as the means \pm SEM of 3 replicates. * $P < 0.05$ compared with group N; & $P < 0.05$ compared with group B; # $P < 0.05$ compared with group P+G.

of hydrochloric acid, gastric fluid, and pepsin into the lung to observe the roles of these agents in the genesis and development of pulmonary fibrosis. The actual effects of inhalation of gastric fluid, hydrochloric acid, and pepsin on lung tissues were determined by observing pathological changes in lung tissues on the 7th, 14th, 28th, and 56th days, by measuring the mRNA expression levels of TGF- β_1 and the factor downstream of TGF- β , CTGF, and by quantifying type I collagen fibers.

Although the bleomycin-induced pulmonary fibrosis model cannot completely simulate the chronic fibrosis progression process of human IPF [26], it is recognized as the animal model that is most similar to human pulmonary interstitial fibrosis, since no better animal model is currently available. Therefore, these experiments were designed such that group B was selected as the positive control group and group N was the negative control group, in order to compare results with those identified in the 3 simulated aspiration groups (groups A, P, and P+G) and to analyze pathological and physiological processes,

as well as dynamic morphological changes, in lung injury and pulmonary fibrosis induced by experimental aspiration of hydrochloric acid, pepsin, and gastric fluid.

The pathological results of the lung tissues indicated that the alveolar structure was normal in group N. In group B, a small amount of macrophage and lymphocyte infiltration was evident in the alveolar septum and alveoli, as well as fibroblast hyperplasia, clear thickening of the alveolar wall, and extensive collagen fiber hyperplasia in the alveolar septum. Pulmonary interstitial fibrosis occurred at the early stage, and gradually became aggravated at the late stage over time, subsequently exhibiting a trend towards decline until the 56th day, which is generally consistent with findings reported in the literature [1113,16,22].

Alveolitis in groups A, P, and P+G tended to gradually increase, and the severity exceeded that observed in group B from the 28th day, indicating that continuous stimulation by pepsin, gastric fluid, and hydrochloric acid aggravated the range and extent of alveolitis. The degree of fibrosis was low in the early stage in the 3 simulated aspiration groups and gradually increased over time and with increasing hydrochloric acid instillation time, and, although it did not reach the same degree of fibrosis as identified in group B, it was substantially greater compared with that in group N. No distinct differences in the degree of fibrosis were identified among groups P+G, P, and B until the 56th day.

Among all the cytokines involved in pulmonary fibrosis, TGF- β has the most critical role, and TGF- β_1 also has the closest association with pulmonary fibrosis [27]. The fibrosis-promoting effects of TGF- β_1 in the lung include stimulation of the synthesis and deposition of all ECM components, acting on multiple steps of these processes, including upregulation of the transcription and translation of matrix components, and stimulation of the synthesis and deposition of all types of ECM. Therefore, TGF- β_1 accelerates abnormal epithelial cell hyperplasia and abnormal repair of re-epithelialization, promotes ECM deposition and the genesis and development of pulmonary fibrosis, and serves an important role in the pathogenesis of pulmonary fibrosis [4,27]. In the present study, the expression level of TGF- β_1 mRNA was found to increase, prior to falling back towards the original level in group B. The mRNA expression level of TGF- β_1 was markedly higher in lung tissues in the 3 simulated aspiration groups compared with that in group N from the 14th day onwards, and tended to gradually increase, although it did not significantly differ from that in group B from the 28th day. The above results revealed that hydrochloric acid and pepsin exerted certain promoting effects on fibrosis formation, and were able to upregulate TGF- β_1 expression in lung tissues at the genetic level and promote ECM deposition, thereby facilitating the genesis and development of pulmonary fibrosis.

CTGF, the host factor that regulates fibroblast proliferation and ECM synthesis, is the downstream mediator of fibrosis and promotes the effect of TGF- β_1 . As demonstrated in a previous study, CTGF protein expression is markedly increased in pulmonary fibrosis [28] and this is positively correlated with the expression levels of TGF- β_1 and type I collagen, further confirming that CTGF expression is closely correlated with the degree of pulmonary interstitial fibrosis. The present study demonstrated that CTGF was poorly expressed, or was not expressed at all, in lung tissues in group N, and was predominantly expressed in the bronchus, bronchial epithelial cells, and type II alveolar epithelial cells. CTGF expression in group B began to increase from the 7th day, peaked on the 14th day, and remained high until the 28th day. Subsequently, it tended to decline, but remained substantially higher than in group N. CTGF expression levels in group P+G and in group P were higher compared with that in group N from the 14th day, and increased over time, although they did not significantly differ from that in group B based on measurements taken on the 56th day.

Over-deposition of the ECM is the outstanding feature of interstitial pulmonary fibrosis, and collagen is the main component of the ECM. Collagen metabolism disorders occur in rat and human pulmonary interstitial fibrosis, and are characterized by an increased content of type I and type III collagen. Increased TGF- β_1 and CTGF expression results in type I collagen hyperplasia [29], and collagen deposition in the pulmonary interstitium leads to matrix structure disorders of the lung tissue, giving rise to fibrosis [30]. The results of the present study indicated that type I collagen expression in lung tissue was clearly higher in the rats in group B at all time points compared with that in group N, which was consistent with the observed pathological and morphological changes. Type I collagen expression in the 3 simulated aspiration groups at all time points was lower compared with that in group B. The expression of type I collagenous fibers was higher in group P+G than in group N from the 14th day. The expression of type I collagen was higher in group A and group P compared with that in the negative control group from the 28th day onwards, reflecting the trend of repeated aspiration-induced fibrosis.

The early-stage pathological change that predominantly occurred in group B was alveolitis. At the late stage, inflammation tended to gradually subside and extensive fibrosis developed, peaking on the 28th day, and then tending to progressively decrease. Compared with group B, the results in the present study indicated that repeated minor aspiration of gastric fluid and pepsin resulted in a slow rate of fibrosis progression; however, as simulated aspiration through repeated injections of hydrochloric acid and pepsin into the trachea caused abnormal and excessive repair, the fibrosis process exhibited a trend towards progressive aggravation. Although the simulated aspiration groups did not exhibit the same degree of

fibrosis induced by bleomycin, these models more accurately simulate the course of pulmonary fibrosis compared with bleomycin, and are more consistent with the biological characteristics, as well as the genesis and developmental processes, of pulmonary fibrosis.

Although the degrees of fibrosis in groups P+G, P, and A were determined, these experiments were not necessarily able to completely reflect the overall effects of gastric content aspiration on pulmonary fibrosis due to restrictions associated with the experimental conditions and the experimental cycle [31–33]. For example, differences existed in both the components and timing of simulated aspiration compared with those of gastroesophageal reflux in GERD. In addition, gastric contents have more complex components in addition to the gastric fluid, pepsin and bile salt, and these must also be comprehensively considered. Furthermore, the pathogenesis of pulmonary fibrosis is complicated, involving interactions among multiple factors. Therefore, multiple methods are required to monitor additional relevant factors, and to observe the precise effects of acid on the rat lung, as well as their associations with fibrosis.

The repeated aspiration results in the animal models presented in this study demonstrated that, compared with the aspiration of hydrochloric acid or pepsin alone, mixed aspiration of pepsin and gastric fluid caused more severe lung injury, which may be associated with the activation of pepsin by gastric

acid. However, these results were obtained from animal experiments, and do not necessarily reflect the situation in humans. As we all know, 56 days for rats could be the equivalent of 3 to 5 years for humans. For example, the frequency of aspiration simulation in the experimental animals may be substantially lower than the actual aspiration frequency in humans. It was not possible to increase the simulated aspiration frequency in animals, as additional increases in anesthesia, tracheal intubation, and intratracheal injections may have resulted in increased mortality among the experimental animals, and therefore have affected the experiment results.

Conclusions

In the present study, aspiration of gastric contents was revealed to cause pulmonary fibrosis, and mixed aspiration of pepsin and gastric fluid was shown to accelerate the occurrence of this process compared with aspiration of hydrochloric acid alone. These experiments have demonstrated the association between the aspiration of gastric contents induced by chronic gastroesophageal reflux and pulmonary fibrosis from a single perspective. Although the actual effects of gastric content inhalation induced by gastroesophageal reflux on the lung cannot be precisely simulated, simulation models are able to provide strong experimental evidence to elucidate the association between human GERD and IPF.

References:

1. Kim DS: Acute exacerbations in patients with idiopathic pulmonary fibrosis. *Respir Res*, 2013; 14: 86
2. Puglisi S, Torrisi SE, Vindigni V et al: New perspectives on management of idiopathic pulmonary fibrosis. *Ther Adv Chronic Dis*, 2016; 7(2): 108–20
3. Pillai M, Olson AL, Huie TJ et al: Obstructive sleep apnea does not promote esophageal reflux in fibrosing interstitial lung disease. *Respir Med*, 2012; 106(7): 1033–39
4. Vancheri C: Gastro-oesophageal reflux and idiopathic pulmonary fibrosis. *Sarcoidosis Vasc Diffuse Lung Dis*, 2013; 30(Suppl. 1): 37–39
5. Fahim A, Crooks M, Hart SP: Gastroesophageal reflux and idiopathic pulmonary fibrosis: A review. *Pulm Med*, 2011; 2011: 634613
6. Hoppo T, Komatsu Y, Jobe BA: Gastroesophageal reflux disease and patterns of reflux in patients with idiopathic pulmonary fibrosis using hypopharyngeal multichannel intraluminal impedance. *Dis Esophagus*, 2014; 27(6): 530–37
7. Havemann BD, Henderson CA, El-Serag HB: The association between gastro-oesophageal reflux disease and asthma: A systematic review. *Gut*, 2007; 56: 1654–64
8. Ghebre YT, Raghu G: Idiopathic pulmonary fibrosis: Novel concepts of proton pump inhibitors as antifibrotic drugs. *Am J Respir Crit Care Med*, 2016; 193(12): 1345–52
9. Tobin RW, Pope CE, Pellegrini CA et al: Increased prevalence of gastroesophageal reflux in patients with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*, 1998; 158(6): 1804–8
10. Salvioli B, Belmonte G, Stanghellini V et al: Gastro-oesophageal reflux and interstitial lung disease. *Dig Liver Dis*, 2006; 38(12): 879–84
11. Savarino E, Carbone R, Marabotto E et al: Gastro-oesophageal reflux and gastric aspiration in idiopathic pulmonary fibrosis patients. *Eur Respir J*, 2013; 42(5): 1322–31
12. Lee JS, Collard HR, Raghu G et al: Dose chronic micro aspiration cause idiopathic pulmonary fibrosis? *Am J Med*, 2010; 123(4): 304–11
13. Mays EE, Dubois JJ, Hamilton GB: Pulmonary fibrosis associated with tracheobronchial aspiration: A study of the frequency of hiatal hernia and gastroesophageal reflux in interstitial pulmonary fibrosis of obscure etiology. *Chest*, 1976; 69(4): 512–15
14. Raghu G, Freudenberger TD, Yang S et al: High prevalence of abnormal acid gastro-oesophageal reflux in idiopathic pulmonary fibrosis. *Eur Respir J*, 2006; 27(1): 136–42
15. Gao F, Hobson AR, Shang ZM et al: The prevalence of gastro-oesophageal reflux disease and esophageal dysmotility in Chinese patients with idiopathic pulmonary fibrosis. *BMC Gastroenterol*, 2015; 15: 26
16. Popper H, Juettner F, Pinter J: The gastric juice aspiration syndrome (Mendelson syndrome). Aspects of pathogenesis and treatment in the pig. *Virchows Arch A Pathol Anat Histopathol*, 1986; 409: 105–17
17. Davis CS, Mendez BM, Flint DV et al: Pepsin concentrations are elevated in the bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis after lung transplantation. *J Surg Res*, 2013; 185(2): e101–8
18. Hoppo T, Komatsu Y, Jobe BA: Gastroesophageal reflux disease and patterns of reflux in patients with idiopathic pulmonary fibrosis using hypopharyngeal multichannel intraluminal impedance. *Dis Esophagus*, 2014; 27(6): 530–37
19. Raghu G, Meyer KC: Silent gastro-oesophageal reflux and microaspiration in IPF: Mounting evidence for anti-reflux therapy? *Eur Respir J*, 2012; 39(2): 242–45
20. Lee JS, Collard HR, Anstrom KJ et al: Anti-acid treatment and disease progression in idiopathic pulmonary fibrosis: An analysis of data from three randomised controlled trials. *Lancet Respir Med*, 2013; 1(5): 369–76
21. Lee JS, Ryu JH, Elicker BM et al: Gastroesophageal reflux therapy is associated with longer survival in patients with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*, 2011; 184(12): 1390–94

22. Samareh Fekri M, Poursalehi HR, Najafipour H et al: Pulmonary complications of gastric fluid and bile salts aspiration, an experimental study in rat. *Iran J Basic Med Sci*, 2013; 16(6): 790–96
23. Appel JZ, Lee SM, Hartwig MG et al: Characterization of the innate immune response to chronic aspiration in a novel rodent model. *Respir Res*, 2007; 8: 87
24. Downing TE, Sporn TA, Bollinger RR et al: Pulmonary histopathology in an experimental model of chronic aspiration is independent of acidity. *Exp Biol Med*, 2008; 233(10): 1202–12
25. Szapiel SV, Elson NA, Fulmer JD et al: Bleomycin-induced interstitial pulmonary disease in the nude, athymic mouse. *Am Rev Respir Dis*, 1979; 120(4): 893–99
26. Moeller A, Carlos RL, Wang L et al: Models of pulmonary fibrosis. *Drug Discovery Today: Disease Models*, 2006; 3: 243–49
27. Lasky JA, Brody AR: Interstitial fibrosis and growth factors. *Environ Health Peep*, 2000; 108: 751–62
28. Zhu B, Ma AQ, Yang L et al: Atorvastatin attenuates bleomycin-induced pulmonary fibrosis via suppressing iNOS expression and the CTGF (CCN2)/ERK signaling pathway. *Int J Mol Sci*, 2013; 14(12): 24476–91
29. Thannickal VJ, Toews GB, White ES et al: Mechanisms of pulmonary fibrosis. *Annu Rev Med*, 2004; 55: 395–417
30. Xu L, Li LC, Zhao P et al: Total polysaccharide of Yupingfeng protects against bleomycin-induced pulmonary fibrosis via inhibiting transforming growth factor- β 1-mediated type I collagen abnormal deposition in rats. *J Pharm Pharmacol*, 2014; 66(12): 1786–95
31. Hershcovici T, Jha LK, Johnson T et al: Systematic review: The relationship between interstitial lung diseases and gastro-oesophageal reflux disease. *Aliment Pharmacol Ther*, 2011; 34(11–12): 1295–305
32. Sekine Y, Ko E: The influence of intraoperative oxygen inhalation on patients with idiopathic pulmonary fibrosis. *Masui*, 2011; 60(3): 307–13
33. Tcherakian C, Cottin V, Brillet PY et al: Progression of idiopathic pulmonary fibrosis: lessons from asymmetrical disease. *Thorax*, 2011; 66(3): 226–31