Protective Effect of Ambroxol against Paraquat-induced Pulmonary Fibrosis in Rats

Qiao-Ming Zhi¹, Li-tao Yang² and Hai-chen Sun¹

Abstract

Objective To evaluate the possible therapeutic effect of ambroxol on pulmonary fibrosis induced by paraquat.

Methods Adult male Sprague-Dawley rats (n =144, 200-250 g) were divided into four groups (Control, Ambroxol, Paraquat, and Paraquat+Ambroxol group) and sacrificed on day 1, 3, 5, 7, 14 and 28. Several significant oxidant stress markers (MDA, SOD and GSH-PX), MPO activity, cytokines (TNF-α, MCP-1, TGF-β1, MMP-2 and TIMP-1), total inflammatory cell count, hydroxyproline content, collagen I and III mRNA were analyzed.

Results In Paraquat group, the MDA, MPO activity, hydroxyproline contents, the mRNA expression of TNF-α, MCP-1, TGF-β1, MMP-2, TIMP-1, collagen I, collagen III and the number of total inflammatory cells were up-regulated in lung tissue, but SOD and GSH-PX activity were down-regulated in lung tissue compared with Control group (p<0.05). In paraquat+ambroxol group, the MDA, MPO activity, hydroxyproline content, the mRNA expression of TNF-α, MCP-1, TGF-β1, MMP-2, TIMP-1 collagen I, collagen III and the number of total inflammatory cells were significantly decreased, while the SOD and GSH-PX activities in lung tissue were increased compared with Paraquat group (p<0.05). Histological examination of paraquat-treated rats showed lung injury with interstitial edema and widespread inflammatory cell infiltration in the alveolar space and septum, as well as pulmonary fibrosis. Ambroxol could markedly reduce such damage in lung tissue and prevent pulmonary fibrosis.

Conclusion The results of this study indicated that ambroxol could reduce lung damage and prevent pulmonary fibrosis induced by paraquat.

Key words: ambroxol, paraquat, intoxication, lung, pulmonary fibrosis


Introduction

Paraquat (1,1’-dimethyl-4,4’-bipyridinium dichloride) is one of the most widely used non-selective herbicides for broadleaf weed control. Under normal conditions of the manufacture and use, paraquat has been proven to be safe in practice. However, intentional and/or accidental skin contact or ingestion of commercial liquid formulations of paraquat has caused a large number of human fatalities; many cases of acute poisoning and deaths have been reported over the past few decades (1). It is one of the most toxic poisons involved in suicide attempts in China (2).

Affected organs include the liver, kidney, stomach, heart, and thymus, but the lung is the primary target organ for paraquat toxicity, where paraquat is accumulated through the process of active polyamine transport in the alveolar type I and II epithelial cells (3). There are two distinct stages in the development of pulmonary fibrosis caused by paraquat: an early destructive stage characterized by alveolitis, pulmonary edema, and infiltration of inflammatory cells, followed by a final fibrotic stage where fibroblasts proliferate, collagen is deposited, and massive pulmonary fibrosis occurs (4). Although the definite and detailed molecular mechanism...
of paraquat toxicity has not been fully explained, it is generally understood that paraquat toxicity involves the generation of considerable oxygen free radicals that interact with membrane lipids leading to cell death and lung tissue damage (5).

Moreover, the inflammatory cells associated with alveolitis (macrophage, lymphocytes, neutrophils etc.) may produce abundant inflammatory cytokines, chemokines or growth factors (such as TNF-α, MCP-1, TGF-β1, MMP-2 and TIMP-1), which also play a significant role in epithelial cell injury and pulmonary fibrosis (6, 7).

Ambroxol-HCl (trans-4-[2-amino-3.5-dibrombenzylamino]-cyclohexanhydrochloride), which stimulates the release of surfactant production by type II pneumocytes and reduces bronchial hyper-reactivity, is used clinically as a mucolytic agent or expectorant in the treatment of chronic bronchitis (8). But recent research has demonstrated that ambroxol, efficiently scavenges reactive oxygen species and that it has anti-inflammatory properties (9, 10). Regarding paraquat intoxication, there are a few reports on ambroxol which refer to its antioxidation (11), inhibition of neutrophil chemotaxis and inflammatory cells (12) and induction of surfactant synthesis (13, 14). However, in all of the studies focused on the lung injury induced by paraquat at the early stage, few studies have discussed ambroxol’s possible depressant effect on paraquat-induced pulmonary fibrosis at the late stage.

In the present study, we observed paraquat-intoxicated rats continually for four weeks which was long enough for pulmonary fibrosis to become evident. Several significant oxidant stress markers (MDA, SOD and GSH-PX), MPO activity, cytokines (TNF-α, MCP-1, TGF-β1, MMP-2 and TIMP-1), total inflammatory cell count and fibrosis markers (hydroxyproline, collagen I, collagen III and fibrosis score) were systematically analyzed to evaluate ambroxol’s possible therapeutic effect on pulmonary fibrosis induced by paraquat.

**Materials and Methods**

**Chemicals and drugs**

Paraquat dichloride (1,1’-dimethyl-4,4’-bipyridinium dichloride) was produced and supplied by Syngenta Co., Ltd. Ambroxol-HCl (trans-[2-amino-3.5-dibrombenzylamino]-cyclohexanhydrochloride) was supplied by Boehringer Ingelheim Pharma KG (Germany).

**Animal grouping and surgical procedures**

A total of 144 adult male Sprague-Dawley rats weighing 200-250 g were purchased from the Animal Center of Jinling Hospital (Nanjing, China). The animals were randomized into four groups and were given doses based on body weight: (i) Control group, n=36: animals were treated with sterile saline (volume equal to Paraquat) followed by daily ip of sterile saline (volume equal to ambroxol) for 28 consecutive days. (ii) Ambroxol group, n=36: animals were treated with sterile saline (volume equal to Paraquat) followed by daily ip of ambroxol (35 mg/kg) for 28 consecutive days. (iii) Paraquat group, n=36: animals intoxicated with paraquat (18 mg/kg i.p.) followed by daily ip of sterile saline (volume equal to ambroxol) for 28 consecutive days. (iv) Paraquat + Ambroxol group, n=36: animals intoxicated with paraquat (18 mg/kg i.p.) and then given a daily ambroxol injection (35 mg/kg i.p.) for 28 consecutive days. On days 1, 3, 5, 7, 14 and 28 after paraquat intoxication, rats were sacrificed and lung tissue samples were immediately harvested. The superior lobe of the left lung was kept in 10% formaldehyde for histology under a light microscope. The remaining lung tissues were frozen in liquid nitrogen for biochemical measures. It should be emphasized that the dose (35 mg/kg) of ambroxol used in our study was much higher than the clinical dose (45 mg/body) used as a mucolytic agent for respiratory diseases. This dose (35 mg/kg) of ambroxol has been confirmed to have protective effects on lung injury in paraquat-intoxicated rats (11, 14).

**Measurement of MDA, SOD, GSH-PX and MPO**

MDA content in lung tissue was spectrophotometrically determined at 532 nm by the thin barbituric acid (TBA) method according to the MDA detection kit instruction (Nanjing Jiancheng Bioengineering Institute, China). SOD activity in lung tissue and BALF were determined by the xanthine oxidase (XO) method according to the SOD detection kit instruction (Nanjing Jiancheng Bioengineering Institute). GSH-PX activity was spectrophotometrically determined at 412 nm by the DTNB [5,5’-dithiobis(2-nitrobenzoic acid)] method according to the GSH-PX Detection Kit instruction (Nanjing Jiancheng Bioengineering Institute). MPO activity was measured according to the MPO Detection Kit instruction (Nanjing Jiancheng Bioengineering Institute, China).

**Measurement of TNF-α, MCP-1, TGF-β1, MMP-2, TIMP-1 mRNA expression**

Total RNA was extracted from lung samples using the TRizol Reagent (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer’s instructions. The quantity and quality of isolated RNAs were evaluated using absorbance measurements at 260 and 280 nm wavelengths. cDNA was synthesized with a First Strand cDNA Synthesis Kit for RT-PCR according to the manufacturer’s instructions (Fermentas Life Science Co., Vilnius, Lithuania). All of the realtime PCR primers used in this study are listed in Table 1.

Real-time PCR using the SybGreen I dye was performed with QuantiTest SYBR GREEN I PCR Master Mix (Generay Bio Co., Shanghai, China). Real-time PCR was performed with the following program: initial denaturation at 94°C for 5 minutes, followed by 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s. Each lung tissue RNA sample was measured with three repeats, and triple reactions in one repeat.
Table 1. Oligonucleotide Primers Used for Gene Expression Analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'--3')</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Sense</td>
<td>CCCACGTCGTAGCAAACCAAC</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ACGTAGTCGGGGCAGCCTTG</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>Sense</td>
<td>GCCAGCCAGAAAACGCCAAGC</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GGGCATTAACCTGACCTGCTG</td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Sense</td>
<td>GCTCGGTGTTCACACAGCA</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GAGTTCTACGTTGCTTCCA</td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>Sense</td>
<td>CTGGGCAACAAATGTAGAGAG</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GTGTAAGTGATAGATGAGGCG</td>
<td></td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Sense</td>
<td>GCCCTCGAGATCTCTTGG</td>
<td>286</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TCGGTTCTGGGACTTGGT</td>
<td></td>
</tr>
<tr>
<td>Collagen I</td>
<td>Sense</td>
<td>CCTGGTCCTCGAGGTCGCA</td>
<td>284</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ATGTGCGGGCGGGGTCTTG</td>
<td></td>
</tr>
<tr>
<td>Collagen III</td>
<td>Sense</td>
<td>GTGGGACCTGGTTCTTCTACCT</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GGTGGGACCTTGCTAGTGCGCT</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Sense</td>
<td>TGCTGAGTGATGCTGGAGT</td>
<td>268</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TGCTGAGTGATGCTGGAGT</td>
<td></td>
</tr>
</tbody>
</table>

Measurement of hydroxyproline content

The collagen content in the lung tissue was determined by analysis of hydroxyproline. According to the detection kit instruction (Nanjing Jiancheng Bioengineering Institute, China), absorbance was measured at 550 nm to determine hydroxyproline content.

Measurement of Collagen I and Collagen III mRNA expression

Similar to the above qRT-PCR procedures, Collagen I and Collagen III mRNA expressions were detected; initial denaturation was carried out at 94°C for 5 minutes, followed by 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s. The real-time PCR primers are also listed in Table 1.

Histological examinations of the lungs

The lung tissue fixed in 10% neutral buffered formalin was processed through graded alcohol, xylene, and paraffin by an automatic tissue processor and blocked in paraffin. Sections of 4 μm thickness were obtained and stained by Hematoxylin and Eosin (H&E). The slides were examined by light microscopy and photographed. The severity of fibrosis on day 28 in each lung section was semi-quantitatively assessed on a scale from 0 (normal lung) to 8 (total fibrotic obliteration of fields) according to the method proposed by Ashcroft et al (15). At least 10 high-power fields (400×) in each lung section were evaluated and scored independently by two investigators in a blinded manner. Meanwhile, total inflammatory cells on days 1, 7 and 28 in each lung section were also counted and calculated in at least 10 high-power fields (400×) by our two investigators.

Statistical analysis

Data are presented as the mean ± SEM. Comparisons between control group and paraquat group, comparisons between paraquat group and paraquat + ambroxol group at each time point were performed using one-way analysis of variance (ANOVA) followed by Student Newman-Keuls test. In all cases p<0.05 was considered significant.

Results

Analysis of MDA, SOD, GSH-PX and MPO

As shown in Fig. 1A, paraquat intoxication caused a significant increase of MDA contents in lung tissue when compared to control group (p<0.05), and ambroxol treatment decreased MDA contents in the lung tissue on days 1, 3, 5, 7, and 14 (p<0.05). Meanwhile, SOD and GSH-PX activities were significantly decreased in lung tissue after paraquat intoxication when compared to the control group (p<0.05). On the contrary, ambroxol treatment increased SOD activities in lung tissue on days 1, 3, 5 and 7 (p<0.05) (Fig. 1B), and GSH-PX on days 1, 3, 5, 7, 14 and 28 (p<0.05) (Fig. 1C). For MPO activity assay, paraquat changed MPO levels on days 1, 3, 5, 7, and 14, respectively, compared to the data of control group (p<0.05). When ambroxol was given, the MPO levels in lung tissue were also down-regulated to a statistical difference on days 1, 3, 5, 7, and 14 (p<0.05).
Figure 1. Effect of treatment with ambroxol on paraquat-induced changes in MDA, SOD, GSH-PX and MPO. (A) MDA contents in lung tissue, (B) SOD activities in lung tissue, (C) GSH-PX activities in lung tissue, (D) MPO activities in lung tissue. *(p<0.05), significantly different for paraquat group compared with controls; †(p<0.05), significantly different for paraquat+ambroxol group compared with paraquat group.

**RT-qPCR analysis of TNF-α, MCP-1, TGF-β1, MMP-2, and TIMP-1 mRNA expression**

In the lung samples analysis, the CT value of each sample was determined with the same threshold, and the expression analysis of each sample was calculated using relative quantitative $2^{-\Delta\Delta CT}$ method. As shown in Fig. 2A, lung TNF-α reached a peak on day 1 followed by a time-dependent decrease (p<0.05). Treatment with ambroxol significantly decreased TNF-α level on days 1, 3, 5, 7, 14 and 28 (p<0.05). Lung MCP-1 of paraquat group also increased when compared to controls (p<0.05) and ambroxol could reduce MCP-1 mRNA on days 3, 5, 7, 14 and 28 (p<0.05) (Fig. 2B). Lung TGF-β1 mRNA expression progressively increased after paraquat intoxication, peaking on day 7 at 2.58 fold controls, and then deceased gradually, but it was still higher than the control group on day 28 (p<0.05). Ambroxol treatment decreased lung TGF-β1 mRNA levels on days 3, 5, 7, 14 and 28 (p<0.05) (Fig. 2C). Lung MMP-2 mRNA expression significantly increased from day 1, and also reached the peak on day 7, which was about 2.83 fold the control group, and then decreased slowly afterwards, but it was still higher than the control group on day 28 (p<0.05). The expression of TIMP-1 mRNA in lung tissue after paraquat intoxication was also higher than the control group on day 1, then increased gradually, reached the peak on day 14, at about 7.28 fold the control group, and decreased from day 14, but it also was higher than the control group on day 28 (p<0.05). After ambroxol treatment, we observed that the lung MMP-2 mRNA expression had statistical significance on days 3, 5, 7, 14 and 28 compared with paraquat group (p<0.05). Meanwhile, lung TIMP-1 mRNA expression also significantly decreased on days 3, 5, 7, 14 and 28 (p<0.05) compared with the paraquat group (Fig. 2D, E).

**Analysis of hydroxyproline content**

In order to evaluate the collagen content in the lung tissue, we tested the hydroxyproline content in the lung tissue on days 1, 7 and 28. As shown in Fig. 3, Paraquat intoxication caused an increase of hydroxyproline contents in the lung. On day 28, the hydroxyproline content in the paraquat intoxication group was up to 13.54 ± 1.21 mg/g, which was significantly higher than the control group (p<0.05). But after the ambroxol treatment, the hydroxyproline content on day 28 was significantly decreased, at only 6.02±1.02 mg/g (p<0.05).

**RT-qPCR analysis of collagen I and III mRNA expression**

We wanted to further evaluate the collagen levels, so we detected collagen I and III mRNA expression on days 1, 7 and 28, respectively, using RT-qPCR. As anticipated, the data were consistent with the results of hydroxyproline content and observed photographs under light microscope; collagen I and III mRNA expressions increased in the process of pulmonary fibrosis. When treated with ambroxol, collagen I and III gene expressions were both significantly de-
Figure 2. Effect of treatment with ambroxol on paraquat-induced changes in (A) TNF-α, (B) MCP-1, (C) TGF-β1, (D) MMP-2 and (E) TIMP-1mRNA expressions in lung tissue. *(p<0.05), significantly different for paraquat group compared with controls; *(p<0.05), significantly different for paraquat+ambroxol group compared with paraquat group.

Figure 3. Effect of treatment with ambroxol on paraquat-induced changes in hydroxyproline content in lung tissue. *(p<0.05), significantly different for paraquat group compared with controls; *(p<0.05), significantly different for paraquat+ambroxol group compared with paraquat group.

Assessment of pulmonary fibrosis

Histological comparisons of rat lung inflammatory response and collagen deposition of control group, paraquat intoxication group or paraquat+ambroxol treatment group with Hematoxylin and Eosin staining (H&E ×400) are seen in Fig. 5. Figure 5 (A) represents the control lung tissue. (B) Paraquat intoxicated rat lung on day 7 showed acute injury with widespread inflammatory cell infiltration in the alveolar space and septum. (C) Paraquat+ambroxol treated rat lung on day 7 showed interstitial edema but less inflammatory cell infiltration. (D) Paraquat treated rat lung on day 28 showed widespread inflammation in alveolar spaces and septum and evident fibrosis was seen (arrows represented deposited collagen. Quantified results of hydroxyproline content, collagen I and III mRNA expression are shown in Fig. 3, 4, respectively). (E) Paraquat+ambroxol treated rat lung on day 28 showed that only focal inflammation in alveolar spaces and septum was seen, interstitial edema was alleviated and inflammatory cell infiltration was lessened obviously. What was more important was that no fibrosis was evident.

Assessment of pulmonary fibrosis score

The severity of fibrosis was assessed using the Ashcroft score as seen in Fig. 6A. On day 28, the pulmonary fibrosis score of paraquat group was significantly higher than the control group (p<0.05). In paraquat + ambroxol group, the fibrosis score was significantly reduced on day 28 when compared to the paraquat group (p<0.05).
SOD that can catalyze the dismutation of the highly reactive superoxide anion to O$_2$ and to the less reactive species H$_2$O$_2$ as well as GSH-PX that catalyzes the reduction of hydroperoxides using GSH (18). Thereby, SOD and GSH-PX could protect mammalian cells against oxidative damage and their activities could also indirectly reflect the levels of lipid peroxidation. Here, we used ambroxol as an antioxidant to inhibit lung injury induced by oxygen radicals and used MDA, SOD and GSH-PX as representative oxidant stress markers. Our data in the oxidant stress study suggested that paraquat caused increased contents of MDA and decreased activities of SOD and GSH-PX. This phenomenon indicated that paraquat intoxication (18 mg/kg i.p.) caused abundant oxygen free radicals and led to excessive lipid peroxidation which indeed brought about severe lung injury. After ambroxol treatment, the MDA contents were reduced and the SOD and GSH-PX activities increased. The above results might suggest that the protective effect of ambroxol against paraquat-induced lung fibrosis may be due to its free radical scavenging and antioxidant activity. Reduction of lipid peroxidation may contribute to its protective effect in lung tissue. Some studies aiming at using antioxidants such as melatonin, enalapril and captopril have demonstrated that these antioxidants could provide some prevention of the fibrogenic effect of paraquat (19, 20). Our investigation of ambroxol is consistent with their conclusions.

Discussion

In the present study we evaluated the effect of ambroxol on the evolution of paraquat-induced lung injury and pulmonary fibrosis. The histopathological photographs and results of hydroxyproline contents, collagen I and III mRNA expressions as well as pulmonary fibrosis scores, all demonstrated that ambroxol might alleviate inflammatory reaction at the early stage and prevent the development of pulmonary fibrosis at the late stage.

The pathogenesis of pulmonary fibrosis has not been precisely explained, but abundant oxygen free radicals such as superoxide (O$_2^{-}$), hydroxyl (OH$^-$) and hydrogen peroxide (H$_2$O$_2$) have been proven to participate in this complex process. Oxygen free radicals can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in the plasmalemma or intracellular organelles (5, 16, 17). Lipid peroxidation is generally monitored by measuring MDA which results from free radical damage to membrane components of the cells and it is one of the production of reactive oxygen species. Through selective pressure and evolution of oxidant stress, numerous defense mechanisms, both enzymic and nonenzymic, have emerged to protect cells against oxidative injury. Among the latter are SOD that can catalyze the dismutation of the highly reactive superoxide anion to O$_2$ and to the less reactive species H$_2$O$_2$.
Figure 5. Histological comparison of rat lung inflammatory response and collagen deposition of control, paraquat or paraquat+ambroxol with Hematoxylin and Eosin staining (H&E ×400). (A) Image of control lung tissue. (B) Image of paraquat treated rat lung on day 7. (C) Image of paraquat+ambroxol treated rat lung on day 7. (D) Image of paraquat treated rat lung on day 28. (arrows represent deposited collagen). (E) Image of paraquat+ambroxol treated rat lung on day 28.

Figure 6. Effect of treatment with ambroxol on paraquat-induced changes in (A) the number of total inflammatory cells, (B) pulmonary fibrosis scores in lung tissue. *(p<0.05), significantly different for paraquat group compared with controls; #*(p<0.05), significantly different for paraquat+ambroxol group compared with paraquat group.
characterized by an excessive deposition of extracellular matrix (ECM) in the interstitium, resulting in respiratory failure associated with inflammation and neutrophil recruitment and the turnover of the extracellular matrix is partially regulated by proteases such as MMP-2 and TIMP-1 (24, 25). In the present study, we detected MPO activity and also measured TNF-α, MCP-1, TGF-β1, MMP-2 and TIMP-1 mRNA expressions by quantitative real-time PCR, we found that paraquat intoxication caused an obvious increase of MPO activity and cytokine levels, which may cause the severe lung injury and eventually induce the formation of pulmonary fibrosis. After treatment with the drug, Ambroxol efficiently decreased the MPO activity and blocked the induction of inflammatory mediators such as TNF-α, MCP-1, TGF-β1, MMP-2 and TIMP-1. Furthermore, our data on inflammatory cell counting in lung tissue showed that paraquat intoxication could lead to abundant inflammatory cells in lung tissue, while treated with ambroxol; inflammatory cell infiltration was significantly alleviated. It could be explained that the observed ambroxol protective effects against paraquat-induced intoxication might be the result of less infiltration by inflammatory cells. Fewer inflammatory cells and less inhibition of these significant cytokines may lead to failure in the formation of pulmonary fibrosis, correct the ECM deposition and prevent the development of pulmonary fibrosis.

Recently, many researchers have found that an abundance of oxygen free radicals leads to severe inflammatory response. Meanwhile, the use of antioxidants or scavengers of reactive oxygen radicals may reduce the levels of some inflammatory mediators, chemokines or growth factors (26, 27). The present study may support this hypothesis. Ambroxol decreased the contents of reactive oxygen radicals and elevated some defense enzymes’ activities. Less reactive oxygen radicals and more active defense enzymes alleviated cellular injuries, which might possibly decrease the infiltration of inflammatory cells and reduce the levels of cytokines that indirectly participated in the process of pulmonary fibrosis. Some studies have also reported that ambroxol could relieve the paraquat-induced lung tissue injury by influencing synthesis and the secretion of pulmonary surfactant (14, 28). Whether stimulation of surfactant synthesis or secretion by ambroxol is related to the process of pulmonary fibrosis, needs further investigation for confirmation in the future.

In conclusion, we have demonstrated a possible protective effect of ambroxol in paraquat-induced pulmonary fibrosis. This effect may be related to the elimination of free oxygen radicals and elevation of enzymes or inhibition of inflammatory cytokines by ambroxol (Fig. 7). The present study indicated that ambroxol might be a new therapeutic agent for the prevention of pulmonary fibrosis induced by paraquat intoxication. However, the exact mechanism of ambroxol’s protective effect against paraquat-induced pulmonary fibrosis should be verified with further animal and human studies.
The authors state that they have no Conflict of Interest (COI).

Acknowledgement
We greatly thank for Prof. Xinhua Zhang, Department of Pathology, Nanjing Jinling Hospital for histological examinations in this study.

References