

Effect of Pirfenidone on Bleomycin Induced Pulmonary Alveolar Fibrosis in Adult Male Rats (Histological, Immunohistochemical, Morphometrical and Biochemical Study)

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Abstract: *Introduction:* Bleomycin is a chemotherapeutic agent commonly used to treat curable diseases such as Hodgkin's lymphoma. The major limitation of bleomycin therapy is the pulmonary toxicity. Pirfenidone is a modified phenyl pyridine that has an antioxidant, anti-transforming growth factor and anti-platelet derived growth factor effects. *Aim of the study:* to evaluate the histological, immunohistochemical and biochemical changes in the pulmonary alveoli of adult male albino rats after intake of bleomycin and the possible role of pirfenidone in minimizing these changes. *Material and Methods:* Forty adult male albino rats were used in this study. They were divided equally into 4 equal groups; the first group (control), the second group that received bleomycin for 10 days, the third group that received pirfenidone for 10 days and the fourth group that received pirfenidone & bleomycin for 10 days. The lungs were dissected out, processed and lung sections were stained with Hx&E, Masson's trichrome and immunohistochemically. Then they were examined by light microscope for histological and immunohistochemical study to evaluate the structure of pulmonary alveoli. Biochemical measurement of malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and tumor necrosis factor- α (TNF- α) were also performed. *Results:* Bleomycin treatment in the second group induced alveolar inflammation, interstitial pulmonary inflammation and pulmonary alveolar fibrosis, while pirfenidone significantly reduced these induced lung injuries in the fourth group rats that treated with pirfenidone and bleomycin. These protective effects were associated with a significant ($P<0.05$) reduction in the levels of MDA, and TNF- α associated with a significant ($P<0.05$) increase in the levels of GSH-P in the homogenate of lung tissue compared with the second group. *Conclusion:* The present study showed a protective effect of pirfenidone on the structure of pulmonary alveoli subjected to bleomycin intake. So intake of pirfenidone with bleomycin is advised for treatment of pulmonary alveolar toxicity.

Keywords: Bleomycin, Pirfenidone, Pulmonary Fibrosis, Inflammatory Cytokines

1. Introduction

Pulmonary fibrosis is a chronic and serious lung disease, of unknown etiology limited to the lungs that can be developed as a complication of many respiratory and systemic diseases.[1] It causes replacement of normal lung tissue with scar tissue or excess fibrous connective tissue. It is also characterized by alveolar epithelial cell injury, interstitial inflammation, fibroblast proliferation and impairment of lung

function.[2]

Bleomycin is the most widely used experimental model of lung fibrosis, because the pathology in rats is very similar to human.¹ It is a chemotherapeutic antibiotic, produced by the bacterium "Streptomyces Verticillus" that is used as an anticancer drug mainly in treatment of Hodgkin, non-Hodgkin lymphomas and testicular carcinoma.[3] Bleomycin reduces molecular oxygen to superoxide and hydroxyl radicals which cause DNA strand cleavage or breakdown.[4]

Currently, there are no approved medical antifibrotic therapies for pulmonary fibrosis.[5] Pirfenidone is an orally active small molecule comprising a modified phenyl pyridine that is able to move through cell membranes without requiring a receptor. It is easily absorbed from the gastrointestinal tract after oral administration with a peak blood level after 1–2 h. It crosses the blood-brain barrier and is eliminated in urine within 6 hours. Modulation of fibrogenesis by pirfenidone is still unclear in detail, but its effects are probably multi-targeted because it has antioxidant, anti-transforming growth factor (anti-TGF) and anti-platelet derived growth factor effects.[6] The most common adverse effects of pirfenidone include gastrointestinal symptoms (nausea, dyspepsia, diarrhea, abdominal discomfort, and vomiting), anorexia, fatigue, sedation and photosensitivity rash. Overall, pirfenidone appears to be reasonably safe in various patient populations with chronic fibrotic disorders, multiple sclerosis, chronic hepatitis C and chronic allograft rejection.[7]

The aim of the present study was to evaluate the possible protective effect of pirfenidone on bleomycin induced pulmonary fibrosis in the lung of adult male albino rats.

2. Materials and Methods

In this study, forty adult male albino rats weighing 150–250 gm were used. The animals were obtained and housed at the animal laboratory house, Moshtohor faculty of Veterinary Medicine, Benha University, Egypt. Strict care and cleaning measures were utilized to keep the animals in a normal healthy state. The animals were housed in animal cages at room temperature ($25\pm 1^\circ\text{C}$), relative humidity (55 ± 5) with 12h light/12h dark cycle, fed standard balanced diet and water ad-libitum. All ethical protocols for animal treatment were followed and the experimental protocol was approved by the Ethical Committee of Benha Faculty of Medicine.

Used drugs: Bleomycin hydrochloride (Nippon Kayaku, Japan), 15 mg powder per vial was dissolved in normal sterile saline as a vehicle. Pirfenidone (Licheng Chemical Co. Ltd. Shanghai, China) was dissolved in a 0.5% carboxymethylcellulose solution as a vehicle.

Experimental design: Rats were divided into four equal groups (10 rats for each).

Group 1 (Control group= G1): The animals of this group were further subdivided into 2 subgroups each one included 5 rats.

Subgroup 1A: Rats were injected intraperitoneally (IP) with saline as a vehicle once daily for 10 days, and then sacrificed at the same time as the corresponding experimental groups.

Subgroup 1B: Rats were received 0.5% carboxymethylcellulose solution as a vehicle orally by gastric tube for 10 days, and then sacrificed at the same time as the corresponding experimental groups.

Group 2 (Bleomycin group= G2): Rats were received bleomycin hydrochloride IP in a dose of (10 mg/kg of body weight/ once daily for 10 days) to induce lung fibrosis.[8]

Group 3 (Pirfenidone group= G3): Rats were received

pirfenidone orally by a gastric tube in a dose of (500 mg/kg of body weight once daily for 10 days). [9]

Group 4 (Bleomycin and Pirfenidone group= G4): Rats were received pirfenidone and bleomycin as in the previous groups for 10 days.

At the end of the experiment, the rats were anaesthetized by inhalation of ether, sacrificed and then the lungs were exposed and excised. The lung biopsies were divided and fixed immediately in 10 % neutral buffered formalin. Paraffin sections were prepared and stained with hematoxylin and eosin (Hx&E) to verify the general histological details of the lungs and Masson's trichrome to assess the sub-epithelial collagen deposition.[10]

Immunohistochemical study: was performed to detect α -smooth muscle actin (α -SMA) as a marker of myofibroblast differentiation and fibrosis. Anti α -SMA immunohistochemical staining was done as follow: Paraffin sections were deparaffinized in xylene for 1-2 minutes, rehydrated in descending grades of ethanol and then brought to distilled water for 5 minutes. Sections were incubated in hydrogen peroxide for 30 minutes then rinsed in PBS (3 times, 2 minutes each). Each section was incubated for 60 minutes with 2 drops (100 μl) of the primary antibody (α -SMA mouse monoclonal antibody, (Lab. Vision Corporation laboratories, CA 94538, USA, catalogue number MS-113-R7). Slides were rinsed well in PBS (3 times, 2 minutes each), incubated for 20 minutes with 2 drops of biotinylated secondary antibody for each section then rinsed well with PBS. Each section was incubated with 2 drops (100 μl) of enzyme conjugate "Streptavidin-Horseradish peroxidase" for 10 minutes at room temperature then washed in PBS. Two drops of the substrate-chromogen mixture diaminobenzidine (DAB) were applied to each section and incubated at room temperature for 5-10 minutes then rinsed well with distilled water. The sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich Co., St Louis, MO, USA) then dehydrated and mounted. α -SMA +ve cells showed brown cytoplasmic deposits and the primary antibody was omitted for negative control sections.[11]

Biochemical measurements: Portions of lung tissues were homogenized in a saline solution (0.9%), centrifuged at 3000 rpm for 15 min, and the supernatant was stored at -20°C until they were analyzed for:

1. Malondialdehyde (MDA) which is the breakdown product of lipid peroxidation that was analyzed to determine lipid peroxidation.[12]
2. Glutathione peroxidase (GSH-Px) which is a lung content that was determined by using a commercial kit (Biodiagnostic, Egypt).[13]
3. Tumor necrosis factor- α (TNF- α) which is a lung proinflammatory cytokine that was measured by using the commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits for rats according to manufacturer's instructions (Sigma-Aldrich Co., St Louis, MO, USA) The results were expressed as picograms per milligram of tissue protein (pg/mg).[14]

Morphometric analysis: The Image-Pro Plus program

version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA) was used to determine the following:

1- The mean area % of the stained collagen fibers in the lungs of different experimental groups.

2- The mean area % of α -SMA immunohistochemical expression in the lungs of different experimental groups.

Statistical analysis: The histological and immunohistochemical data were analyzed by using the statistical package SPSS version 20 (SPSS Inc., Chicago, Illinois, USA). Data were expressed as mean \pm SD. The statistical significance in differences between groups was analyzed by using one-way analysis of variance (ANOVA) test, followed by the post-hoc test of Tukey's to compare the mean area % of collagen fibers and α -SMA immunohistochemical expression in the lungs of different experimental groups.

P values < 0.01 were considered a highly significant and < 0.05 were considered significant.

3. Results

1. Histological results:

A. Hematoxylin and eosin:

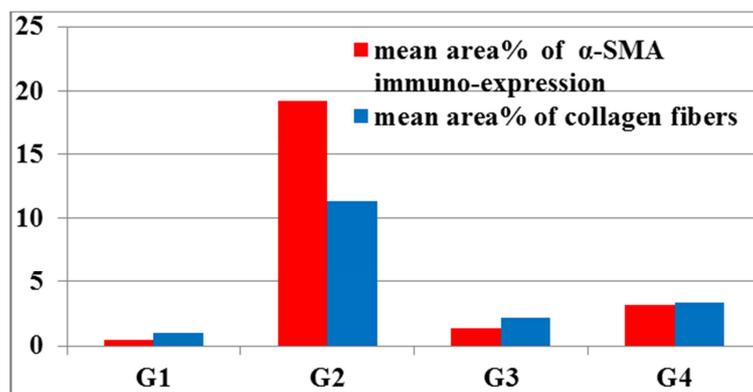
Sections of G1 showed normal histological architecture of the lung with many alveoli, alveolar sacs, alveolar ducts, bronchioles and small blood vessels (Fig.1). While G2 showed a various histological changes in the form of many collapsed alveoli, dilated or ruptured alveoli and extravasated RBCs with a multiple thick interalveolar septa between the alveoli (Fig. 2). Other sections from G2 revealed multiple thick interalveolar septa between collapsed alveoli and were studded with mononuclear cellular infiltration (Fig. 3).

On the other hand G3 showed a histological picture

Table 1. Showing the mean area % of collagen fibers \pm SD and the mean area % of smooth muscle actin (α -SMA) immuno-expression \pm SD in all experimental groups.

Groups		G1	G2	G3	G4
The mean area% of collagen fibers	Mean \pm SD	1.02 \pm 0.89	11.35 \pm 2.37	2.21 \pm 1.42	3.38 \pm 1.79
	P value		0.00**	0.17*	0.14*
The mean area% of α -SMA immuno-expression	Mean \pm SD	0.47 \pm 0.17	19.22 \pm 0.26	1.40 \pm 0.27	3.13 \pm 0.35
	P value		0.00**	0.17*	0.21*

SD = Standard deviation, highly significant** ($P < 0.01$) for G2 compared with G1, and significant* ($P < 0.05$) for G3 and G4 compared with G2. (ANOVA test).



Histogram 1. Showing the mean area % of collagen fibers and the mean area % of α -SMA immuno-expression in all experimental groups.

nearly similar to the normal histological architecture of the lung (Fig. 4) while, G4 rats showed a picture more or less similar to that of G1 that had many alveoli with apparently thin interalveolar septa, while few interalveolar septa were thick and studded with mononuclear cellular infiltration (Fig. 5).

B. Masson's trichrome stain:

Sections of G1 revealed a minimal amount of collagen fibers around the alveoli or within the interalveolar septa with small blood vessels (Fig.6). However, G2 rats showed an extensive accumulation of collagen fibers around alveoli, bronchioles and small blood vessels or within the interalveolar septa (Fig.7). On the other hand G3 sections showed a minimal amount of collagen fibers (Fig. 8) while, G4 rats showed a moderate amount of collagen fibers around the alveoli and small blood vessels or within the interalveolar septa (Fig. 9).

1. Immunohistochemical results: Sections of G1 revealed absence of α -SMA immuno-reactivity (Fig.10) while, G2 rats showed a positive immuno-reactivity of α -SMA within the cytoplasm of cells lining the alveoli and interalveolar septa (Fig.11). On the other hand G3 sections showed absence of α -SMA immunoreactivity (Fig.12) while, G4 rats showed a weak immunoreactivity of α -SMA within the cytoplasm of cells lining the alveoli and interalveolar septa (Fig. 13).

2. Morphometric results: The mean area % of collagen fibers and α -SMA immunoexpression for all groups were presented in table 1 and histogram1. The mean area % of collagen fibers and α -SMA immunoreactivity showed a highly significant increase in G2 compared with G1 ($P < 0.01$) while, they were significantly decreased in G3 and G4 compared with G2 ($P < 0.05$).

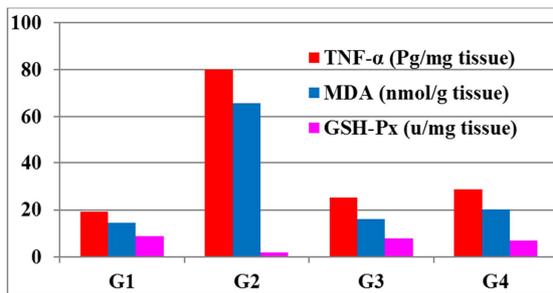
3. Biochemical Results: As shown in table 2 and histogram 2, the MDA and TNF- α showed a highly significant increase ($P < 0.01$) in G2 compared to G1, while they were significantly decreased in G3 and G4 compared to

G2 ($P < 0.05$). On the other hand, GSH-Px activity showed a highly significant decrease ($P < 0.01$) in G2 compared to G1, while they were significantly increased in G3 and G4 compared to G2 ($P < 0.05$).

Table 2. Showing changes in MDA, GSH-Px and TNF- α levels in all experimental groups.

Groups		G1	G2	G3	G4
MDA (nmol/g tissue)	Mean \pm SD	14.6 \pm 1.82	65.76 \pm 5.7**	16.24 \pm 1.06*	20.25 \pm 2.41*
	P value		0.000**	0.212*	0.147*
GSH-Px (u/mg tissue)	Mean \pm SD	8.8 \pm 0.36	1.9 \pm 0.3	7.9 \pm 0.36	6.8 \pm 0.36
	P value		0.000**	0.110*	0.145*
TNF- α (Pg/mg tissue)	Mean \pm SD	19.36 \pm 3.7	80.1 \pm 7.6**	25.4 \pm 4.1*	28.7 \pm 5.6*
	P value		0.000**	0.110*	0.145*

Malondialdehyde (MDA), glutathione peroxidase (GSH-Px), tumor necrosis factor α (TNF- α), SD = standard deviation, highly significant** for G2 compared with G1 and significant* for G3 and G4 compared with G2. (ANOVA test).



Histogram (2). Showing changes in MDA, TNF- α and GSH-Px levels in all experimental groups.

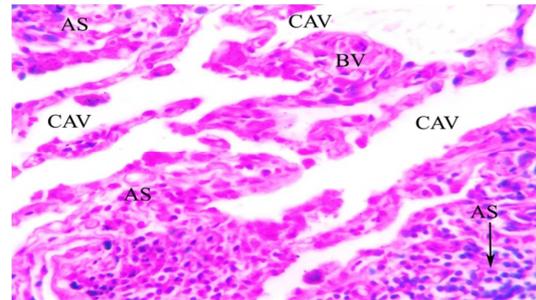


Figure 3. A photomicrograph of a section in the lung of group II rat showing many collapsed alveoli (CAV), thick interalveolar septa (AS), studded with diffuse mononuclear cellular infiltration (arrow \uparrow) and a small blood vessel (BV). [Hx&E, X 640].

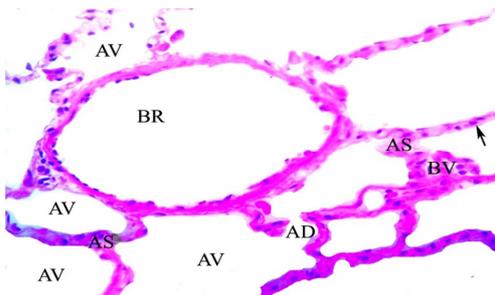


Figure 1. A photomicrograph of a section in the lung of group I rat showing many alveoli (AV), pneumocytes (arrow \uparrow), alveolar ducts (AD), thin interalveolar septa (AS), bronchiole (BR) and a small blood vessel (BV). [Hx&E, X 640].

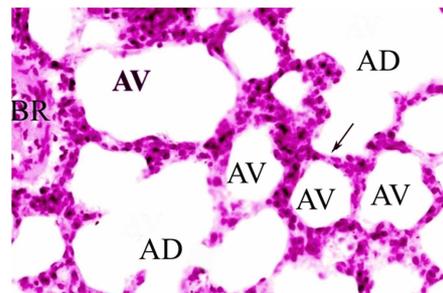


Figure 4. A photomicrograph of a section in the lung of group III rat showing many normal alveoli (AV) of variable sizes, alveolar ducts (AD), thin interalveolar septa (arrow \uparrow) and a bronchiole (BR). [Hx&E, X 640].

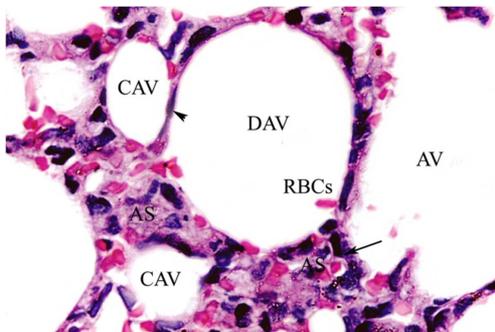


Figure 2. A photomicrograph of a section in lung of group II rat showing many collapsed alveoli (CAV), dilated alveoli (DAV) and ruptured alveoli (AV). Also, there is thick interalveolar septa (As) studded with mononuclear cellular infiltrations (\uparrow), few thin interalveolar septa (arrowhead \blacktriangle) and extravasated RBCs. [Hx&E, X 640].

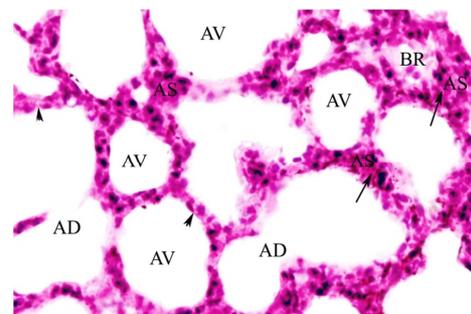


Figure 5. A photomicrograph of a section in lung of group IV rat showing many normal alveoli (AV) of variable sizes and alveolar ducts (AD), few thick interalveolar septa (AS) studded with mononuclear cellular infiltration (arrow \uparrow), many thin interalveolar septa (arrowhead \blacktriangle) and a bronchiole (BR). [Hx&E, X 640].

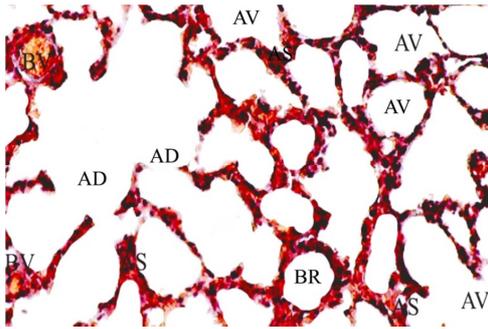


Figure 6. A photomicrograph of a section in the lung of group I rat showing many alveoli (AV) of variable sizes and alveolar ducts (AD) with minimal amount of collagen fibers in the interalveolar septa (AS). A small blood vessel (BV) and a normal bronchiole (BR) are seen. [Masson's trichrome, $\times 400$].

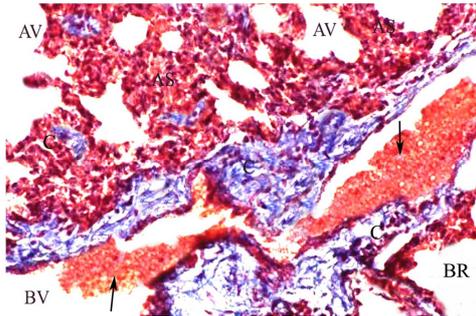


Figure 7. A photomicrograph of a section in the lung of group II rat showing extensive accumulation of collagen fibers (C) around alveoli (AV), within the interalveolar septa (AS) and around bronchiole (BR). Collagen fibers (C) are also noticed around a large congested (arrows) blood vessels (BV). [Masson's trichrome, $\times 400$].

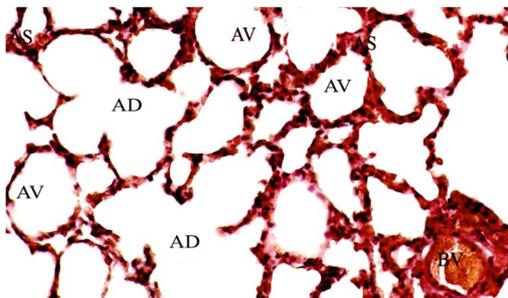


Figure 8. A photomicrograph of a section in lung of group III rat showing many alveoli (AV) with minimal amount of collagen fibers in the interalveolar septa (AS) and around a blood vessel (BV). [Masson's trichrome, $\times 400$].

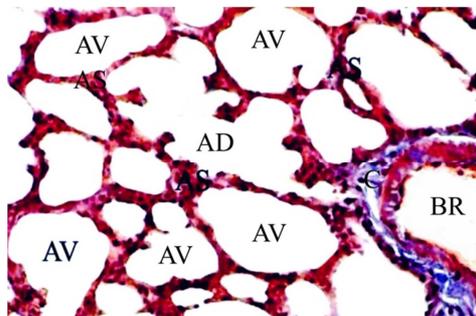


Figure 9. A photomicrograph of a section in lung of group IV rat showing many alveoli (AV) with moderate amount of collagen fibers (C) in the interalveolar septa (AS) and around a bronchiole (BR). [Masson's trichrome, $\times 400$].

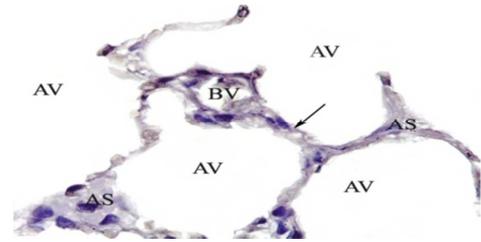


Figure 10. A photomicrograph of a section in the lung of group I rat showing many alveoli (AV), thin interalveolar septa (AS) and a blood vessel (BV). α -SMA immunoreactivity was absent within the cytoplasm of cells lining the alveoli (arrow \uparrow) and interalveolar septa (AS). [Immunostaining for α -SMA $\times 1000$].

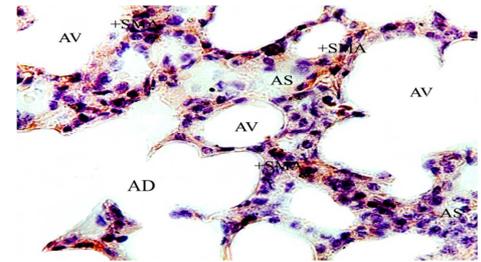


Figure 11. A photomicrograph of a section in lung of group II rat showing alveoli (AV), thick interalveolar septa (AS) and a positive α -SMA immunoreactivity within the cytoplasm of cells lining the alveoli and interalveolar septa (AS). [Immunostaining for α -SMA $\times 1000$].

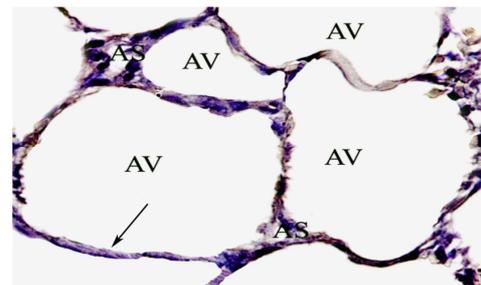


Figure 12. A photomicrograph of a section in the lung of group III rat showing many alveoli (AV) and thin interalveolar septa (AS). α -SMA immunoreactivity is absent within the cytoplasm of cells lining the alveoli (arrow \uparrow) and interalveolar septa (AS). [Immunostaining for α -SMA $\times 1000$].

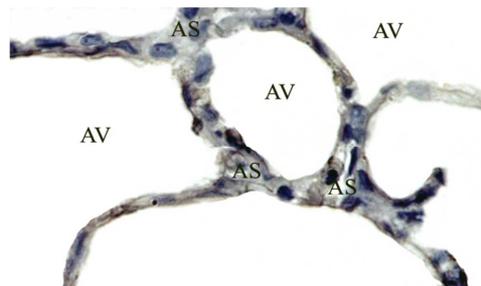


Figure 13. A photomicrograph of a section in the lung of group IV rat showing many alveoli (AV) and interalveolar septa (AS). A weak α -SMA immunoreactivity is observed within the cytoplasm of cells lining the alveoli and interalveolar septa (AS). [Immunostaining for α -SMA $\times 1000$].

4. Discussion

Pulmonary fibrosis is the end stage of a heterogeneous group of disorders which progress to complete loss of lung function and death in affected patients.[15] The histological

examination of lung sections from G2 revealed various changes such as many collapsed alveoli with dilated and ruptured alveoli. Multiple thick interalveolar septa were seen studded with mononuclear cellular infiltration, whereas others were apparently thin. Occasionally extravasated RBCs were seen between the alveoli.

These results were in agreement with a previous histopathological studies which reported that, bleomycin can promote acute cellular inflammation in lung tissue as demonstrated by a strong influx of inflammatory cells such as macrophages and activation of fibroblasts.[16, 17&18]

An apparent increase in collagen fibers around alveoli or within the interalveolar septa was seen in group II of the present study. This was supported with positive α -smooth muscle actin immunoreactivity within the cytoplasm of cells lining the alveoli and interalveolar septa. These findings were in line with previous researchers who found that bleomycin treatment significantly led to upregulation of pro-fibrotic genes such as α -SMA, which is considered as a key marker for myofibroblasts that increase their number.[19]

These results also, were explained by some investigators who mentioned that cytokines such as interleukin-1, macrophage inflammatory protein-1, platelet-derived growth factor (PDGF), and transforming growth factor (TGF) are released from alveolar macrophages in animal models of bleomycin toxicity, resulting in fibrosis.[3] Other investigators noticed that damage and activation of alveolar epithelial cells may result in the release of cytokines and growth factors that stimulate proliferation of myofibroblasts and secretion of a pathologic extracellular matrix, leading to fibrosis.[17]

The pathophysiology of bleomycin toxicity was demonstrated by some researchers who mentioned that the mechanism of action of bleomycin on the lung was mediated through the production of free radicals, reactive oxygen species (ROS) and reactive nitrogen species. Furthermore the chelation of iron ions with oxygen leads to production of DNA-cleaving superoxide and hydroxide free radicals that lead to bleomycin pulmonary toxicity, and lung fibrosis.[20]

On the other hand, some investigators mentioned that Nuclear Factor- κ B (NF- κ B) signaling is thereby playing a major role of epithelial injury. It initially releases proinflammatory cytokines as IL-1, TNF- α , MIP-1, which facilitate the chemotaxis of inflammatory cells as circulating fibroblasts and bone-marrow mesenchymal progenitor cells into the lung. Next it activates transforming growth factor- β 1 (TGF- β 1) expression, the key mediator of pulmonary fibrosis which induces epithelial-mesenchymal transition, generates epithelial-derived fibroblasts, activates fibroblasts and fibroblast-like cells to synthesize excessive collagen and finally induces pulmonary fibrosis.[4] While, other recent studies have demonstrated that fibroblasts can be derived from the lung epithelium through epithelial-mesenchymal transition that may contribute to pulmonary fibrosis.[21]

The biochemical changes in G2 of the present study correlated with the histological and immuno-histochemical changes of the lung tissue, where it revealed a highly

significant increase of MDA and TNF- α with a significant decrease in GSH levels compared to G1. These results were in accordance with a previous investigators who mentioned that, initial elevation in cytokines such as TNF- α after bleomycin administration, is followed by increased expression of the profibrotic cytokine TGF- β that induced a high oxidative stress and inflammation.[22]

On the other hand, bleomycin is known to cause oxidative damage in the lungs that increased lipid peroxidation by ROS which causes a decrease in the efficiency of antioxidant defense mechanism in the inflamed tissue.[23]

Group 4 of the present study showed a marked improvement in the histological and immuno-histochemical changes of the lung tissue, as it revealed a significant decrease in α -SMA immunoreactivity within the cytoplasm of cells lining the alveoli and interalveolar septa compared to G2. On the other hand, the biochemical parameters of G4 revealed a significant decrease of MDA and TNF- α levels with a significant increase in GSH levels compared to G2. These results were in agreement with the findings of some investigators who reported that pirfenidone treatment can reduce pulmonary fibrosis through modulation of cytokines.[24]

Other researchers reported that the protective effect of pirfenidone was due to its anti-inflammatory, antioxidative stress and antiproliferative properties.[25] Furthermore pirfenidone decreases the level of α -SMA, regulates the activity of TGF β and TNF α in vitro, inhibits fibroblast proliferation, inhibits collagen synthesis and reduces cellular markers of lung fibrosis.[26&27]

5. Conclusion

Pirfenidone could significantly prevent bleomycin-induced pulmonary fibrosis in rats as it had a powerful antifibrotic properties through the reduction of oxidative, inflammatory and pro-fibrogenic markers. Thus, pirfenidone could be a promising drug that retards the progression of fibrotic diseases.

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