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Protective efficacy of pirfenidone in rats with pulmonary fibrosis induced by bleomycin

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Abstract. *Background:* Bleomycin causes increased production of reactive oxygen species, leads to pulmonary toxicity, fibroblast activation, and fibrosis. *Objectives*: This study aimed to evaluate the protective effect of pirfenidone on bleomycin-induced lung toxicity in rats. *Methods*: Twenty-eight adult rats were randomly divided into 3 groups; Bleomycin (B group, n=10), Bleomycin and Pirfenidone (B-PND group, n=13), and the control group (n=5). The bleomycin regimen was administered for 9 weeks. Pirfenidone was administered at 100 mg/kg daily. Total antioxidant level (TAS), total oxidant level (TOS), tumor necrosis factor (TNF-α), transforming growth factor (TGF-β1), matrix metalloproteinase-2 (MMP-2), plasminogen activator inhibitor (PAI) levels were studied. Histopathologically, sections were stained with Hematoxylin-eosin and Masson-trichrome for grading-scoring according to the Ashcroft score. *Results:* Stage 3 fibrosis was observed in 50% of the B group rats, stage 3 and higher fibrosis was never detected in the B-PND group and the difference was statistically significant (p=0.003). When evaluating tissue inflammation, the inflammation was higher in the B-PND group than in the other groups (p<0.001). Pleuritis was detected in all rats in group B, while was not observed in B-PND and control group (p<0.001). The TAS level was found to be significantly higher in group B than in group B-PND (p=0.034), while no difference was found between TOS, TNF-α, MMP-2, PAI, TGF-β1. *Conclusions:* Pirfenidone had a statistically significant protective effect in bleomycin-induced lung fibrosis and pleuritis in rats. Despite the presence of inflammation in the tissue, no significant changes were observed in inflammation markers in the peripheral blood. Novel serum biomarkers are needed to indicate the presence of inflammation and fibrosis in the lung.

Key words: bleomycin, pirfenidone, pulmonary fibrosis, pleuritis

Introduction

Pulmonary fibrosis is a pathological process that can occur as a result of many clinical diseases and exposures, affecting respiratory functions and ventilation. It can be idiopathic as well as develop due to interstitial lung diseases, connective tissue diseases, exposure to environmental factors, and drug toxicity. Inflammation that starts with alveolar epithelial cell damage causes irreversible interstitial fibrosis with fibroblast activation (1,2).

Bleomycin is a chemotherapeutic antibiotic derived from Streptomyces verticillus. Bleomycin is used as an anticancer medicine in the treatment of testicular, and ovarian carcinomas, Hodgkin and

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non-Hodgkin lymphomas. Bleomycin converts molecular oxygen to superoxide and hydroxyl radicals by forming a bleomycin-iron complex. This, in turn, has an anti-neoplastic effect by causing strand breaks in DNA and damage to DNA-RNA-protein synthesis (transcription and translation steps) (3). Since the hydrolase enzyme, which inactivates bleomycin, is found at very low levels in lung tissue compared to other tissues, as a side effect, it has the potential to cause interstitial pneumonia and diffuse fibrosis, especially in the lungs. The incidence of lung injury in patients receiving bleomycin varies between 3-40% (4). It is the most widely used experimental model of lung fibrosis because the pathology in rats is very similar to that in humans (5).

Pirfenidone (5-methyl-1-phenyl-2-(1H)-pyridone) (PFD) is a drug with antifibrotic, antioxidant, and anti-inflammatory effects that was approved in 2011 for the treatment of idiopathic pulmonary fibrosis (IPF) (6). Pirfenidone's mechanism of action is not fully known. It is thought that its antifibrotic activity is due to reducing the production of profibrotic cytokines such as TGF-β and fibroblast growth factor (bFGF), collagen synthesis and accumulation, inhibition of extracellular matrix-producing cells and reduction of transformation into myofibroblasts (7). Several previous experimental model studies have reported that pirfenidone may be effective in preventing bleomycin-induced lung, kidney, and liver fibrosis (8-11). There is no study in the literature regarding its use in the prophylaxis of bleomycin-induced pulmonary fibrosis in humans.

This study aimed to evaluate the protective effect of pirfenidone on bleomycin-induced pulmonary fibrosis in adult rats.

Methods

The study was conducted on 28 eight-week-old male Sprague Dawley rats which were obtained from Istanbul Mehmet Akif Ersoy Experimental Research, Development and Training Center (IDEA). Ethical approval of the study was obtained from the local ethics committee (2021/09). During the study, all rats were kept at 18-24 ℃ in daily 12-hour light, 12-hour dark cycle. Besides, food and water were offered to the animals ad libitum.

Animal groups, bleomycin, and pirfenidone treatment procedures

Rats were randomly assigned to 3 groups as Bleomycin alone group (Group B) (n=10), Bleomycin and concomitant Pirfenidone treatment group (Group B-PND) (n=13), and the control group (Group C) (n=5).

Bleomycin application: According to the standard BEP (bleomycin, etoposide and cisplatin) chemotherapy protocol for germ cell tumors such as testicular and ovarian, bleomycin is usually administered on days 1, 8 and 15 of each 21-day cycle for 9 weeks. In our study, similar to this protocol, 0.9 mg/kg bleomycin dissolved in 0.9% saline was administered intraperitoneally 3 times, once every 3 weeks, as 1 mL for 9-week period.

Pirfenidone treatment: In studies investigating the effect of bleomycin on inflammation and fibrosis formation, it was observed that there was a transition from inflammation to fibrosis shortly after bleomycin administration. Because of this short duration, in similar studies, bleomycin and pirfenidone treatment were started either simultaneously or pirfenidone was started a few days before bleomycin treatment (12-14). In line with this, pirfenidone treatment was given concurrently with bleomycin in our study. During the 9-week study, 100 mg/kg daily dissolved in lactated Ringer's solution was given by oral gavage.

In the control group, daily 1 mL 0.9% saline was administered by oral gavage on the first 5 days of the treatment week, and 1 mL intraperitoneally on the second day of the treatment week.

Tissue collection procedure

At the end of 9 weeks, all rats were anesthetized by intraperitoneally administering 90 mg/kg ketamine and 10 mg/kg xylazine. Approximately 3-5 cc blood samples were taken from each animal through the intracardiac route under anesthesia to examine the oxidant and antioxidant capacities. Afterward, a thoracotomy was performed and both lungs were placed in 10% formalin for pathological examination after excision. After these procedures, sacrification was performed by cervical dislocation in rats.

Pathological evaluation

After fixation for 2 days in neutral buffered formalin, lung tissues were taken out and embedded in paraffin wax, cut into 5 μm sections, and stained separately with hematoxylin-eosin (H&E) and Masson-trichrome (MT) stains. The degree of inflammation and tissue damage was noted in H&E stained sections while the degree of fibrosis was graded and

scored as per Ashcroft score from MT stained sections (15,16). Ten fields per section were randomly selected per rat, and a blinded pathologist examined 10 fields per rat using an Olympus microscope (Olympus, Tokyo, Japan). The total score of each section was calculated, and the mean score of each group was determined. The major criteria examined included interstitial thickening of alveolar or bronchiolar walls, collagen deposition, and inflammatory cell infiltration.

Grade of lung fibrosis and histological features

Grade 0 Normal lung
Grade 1 Minimal fibrous thickening of alveolar or bronchiolar walls
Grade 2 Moderate thickening of walls without obvious damage to lung architecture
Grade 3 Increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses
Grade 4 Severe distortion of the structure and large fibrous areas; "honeycomb lung" is placed in this category
Grade 5 Total fibrous obliteration of the field

Grade of inflammatory change; histological features

Grade 1 Minimal inflammatory change
Grade 2 Mild to moderate inflammatory changes (no obvious damage to the lung architecture)
Grade 3 Moderate inflammatory injury (thickening of the alveolar septae)
Grade 4 Moderate to severe inflammatory injury (formation of nodules or areas of pneumonitis that distorted the normal architecture)

Biochemical analysis

For analysis, blood samples were centrifuged at 5000x g for 5 min at 4 °C. The samples prepared for use in the analyses were stored at -80°C until laboratory analyses for determination of biochemical parameters.

Measurement of Total Antioxidant Status (TAS) and Total Oxidant Status (TOS), TAS/TOS assay

TAS and TOS levels of the samples were determined with kits (Rel Assay Diagnostics kit; Mega Tıp, Gaziantep, Turkey). It is a colorimetric method.

In TOS measurement, oxidants in the sample are oxidized to ferric ions. Ferric ions form a colored complex with xylenol orange in an acidic environment. The color intensity associated with the number of oxidants in the sample is measured spectrophotometrically. End-Point measurement is made spectrophotometrically at 560 nm. Results are expressed as μmol H_2O_2 equivalent / L.

In TAS measurement, hydroxyl radical (˙OH) is formed by a Fenton-type reaction. This strong reactive reacts with a colorless molecule at low pH to form yellow-brown dianisidyl radicals. Antioxidants in the samples suppress these oxidation reactions and stop color formation. This reaction is measured spectrophotometrically at 240 nm and results are expressed in mmol Trolox Equivalent / L.

Measurement of cytokines and the other proteins by enzyme-linked immunosorbent assay

The concentrations of TNF-α, TGF-β1, MMP-2, and PAI1 were determined using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience BMS622TWO, İnvitrogen BMS623-3, Abcam ab213910, ab201283). According to the manufacturer's instructions, a colored product is formed in proportion to the number of biochemical parameters present in the sample or standard. The reaction is terminated by the addition of acid and absorbance measured at 450 nm. A standard curve is prepared from standard dilutions for each parameter and sample concentration is determined.

Statistical analysis

The analysis of the data was carried out with the SPSS 25 package program. Frequency and percentage values for qualitative variables and median, minimum and maximum values for quantitative variables are presented. The Chi-square test and Fisher Exact test were used for comparisons between 2 qualitative variables. The Kruskal Wallis H test was used for comparisons between qualitative variables with more than two categories and quantitative variables. In case of significant differences as a result of the Kruskal Wallis H test, the categories were compared in pairs with the Mann-Whitney U test. The type I error rate was considered 0.05 in the study.

Results

Twenty-eight adult rats were included in the study and evaluated in terms of tissue inflammation. In all 3 groups, chronic inflammation accompanied by lymphocyte-dominated plasma cells was observed and more intense involvement was seen in the peribronchial area. There was no difference with regard to inflammatory cells observed in the tissue among all rats. Grade 2, 3, and 4 inflammations were observed in Group B-PND, while only grade 1 and grade 2 inflammation were observed in Group B. Grade 1 inflammation was observed in all rats in Group C (Table 1). The lymphoid proliferation observed in H&E staining in pathological samples from the three groups is shown in Figure 1. The degree of inflammation in Group B-PND was statistically significantly higher when compared to Group B and Group C ($p<0.001$).

When evaluated in terms of fibrosis, 50% of the patients in Group B had stage 3 fibrosis, while stage 3 and more advanced fibrosis was never observed in Group B-PND. Stage 4 and 5 fibrosis was not observed in any of the groups. Grade 1 fibrosis was observed in all rats in Group C (Table 2). In Masson Trichrome staining pathology specimens, severe fibrosis in Group B and mild fibrosis in Group B-PND are presented in Figure 2. The degree of pulmonary fibrosis detected in Group B-PND was statistically significantly lower when compared to Group B ($p<0.05$).

While pleuritis was detected in all rats in Group B, pleuritis was not observed in Group B-PND and Group C (Table 3). The observed pleuritis was seen as lymphoid cell proliferation in the focal area on the pleural surface and was consistent with chronic non-specific pleuritis (Figure 3). The intergroup difference in terms of pleuritis development was statistically significant ($p<0.001$).

While granuloma was detected in 1 rat in group B, it was not detected in the other groups.

In the comparison made in terms of biomarkers measured in serum, the TAS value was found to be significantly lower in Group B-PND than in other groups ($p=0.034$). No difference was observed

Table 1. Intergroup comparison in terms of inflammation.

Inflammation	B-PND* (n:13)	B** (n:10)	Control (n=5)	p-value
Grade 1 % (n)	0	60 (6)	100 (5)	<0,001
Grade 2 % (n)	30.8 (4)	40 (4)	0	
Grade 3 % (n)	46.2 (6)	0	0	
Grade 4 % (n)	23.1 (3)	0	0	

*B-PND: Bleomycin+Pirfenidone, **B: Bleomycin.

Table 2. Intergroup comparison in terms of fibrosis.

Fibrosis	B-PND* (n:13)	B** (n:10)	Control (n=5)	p-value
Grade 1 % (n)	84.6 (11)	20 (2)	100 (5)	0,003
Grade 2 % (n)	15.4 (2)	30 (3)	0	
Grade 3 % (n)	0	50 (5)	0	

*B-PND: Bleomycin+Pirfenidone, **B: Bleomycin.

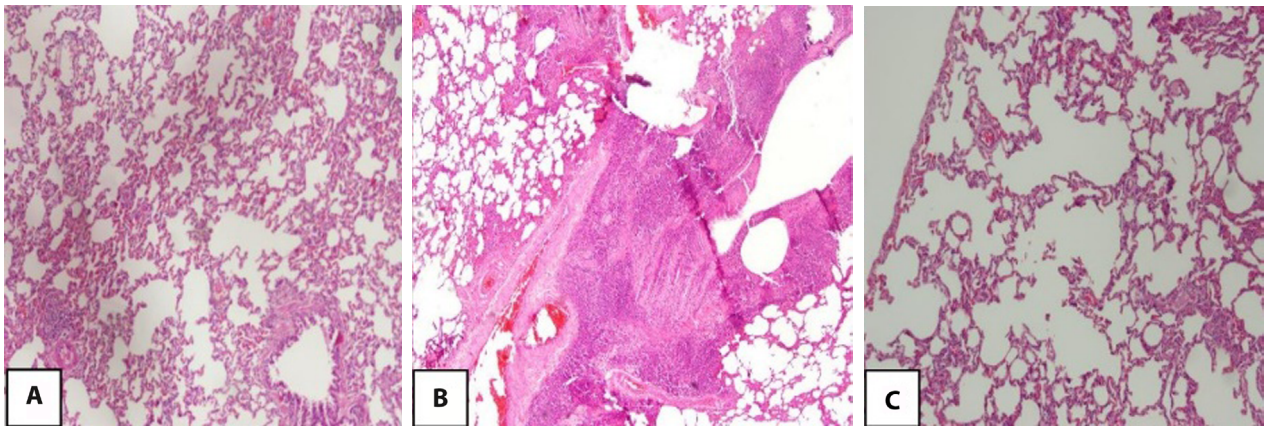

Figure 1. The lymphoid proliferation observed in H&E staining in pathological samples from the three groups A- Moderate lymphoid proliferation, thickening alveolar septa in interstitium, in 4x10 HE, Bleomycin Group (Group B) B- Severe lymphoid proliferation in the interstitium and in the peribroncovascular area, in 4x10 HE, Bleomycin+Pirfenidone (Group B-PND) Group C- Mild-grade lymphoid proliferation in the interstitium, in 3- 4x 10 HE, Control (Group C).

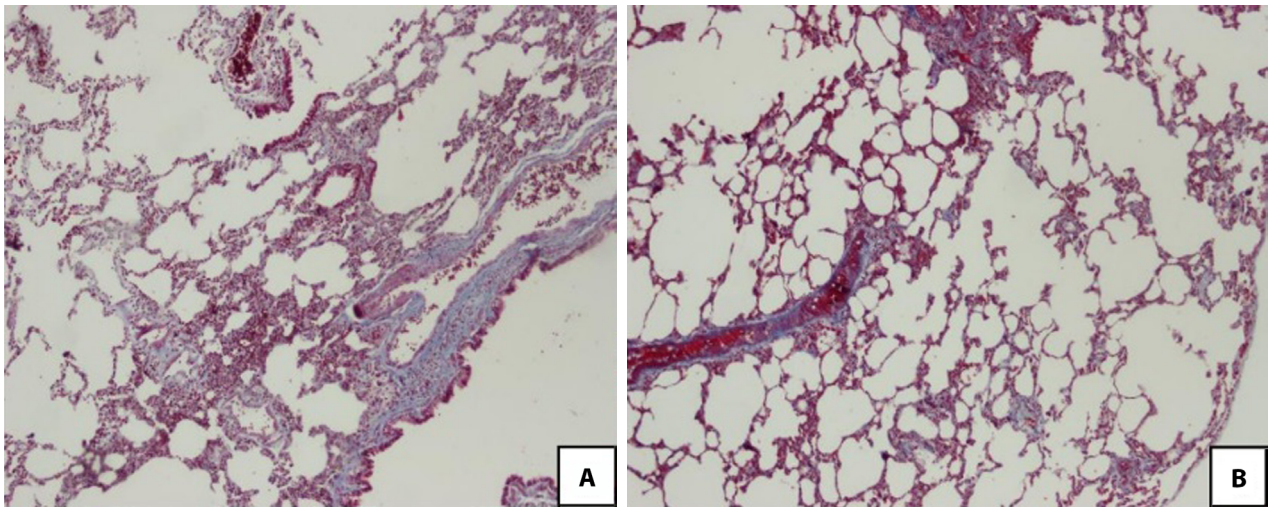

Figure 2. Fibrosis in Masson Trichrome staining in pathological samples A- Severe fibrosis with peribrochiolar, perivascular, interstitial distribution in 4x10 Masson Trichrome, Bleomycin (Group B) Group, B- Middle-grade fibrosis with perivascular and interstitial distribution in 4x10 Masson Trichrome, Bleomisin+Pirfenidon (Group B-PND) Group.

Table 3. Intergroup comparison in terms of pleuritis.

Pleuritis	B-PND* (n:13)	B** (n:10)	Control (n:5)	p-value
No	100% (13)	0	100% (5)	<**0,001**
Yes	0	100% (10)	0	

*B-PND: Bleomycin+Pirfenidone, **B: Bleomycin

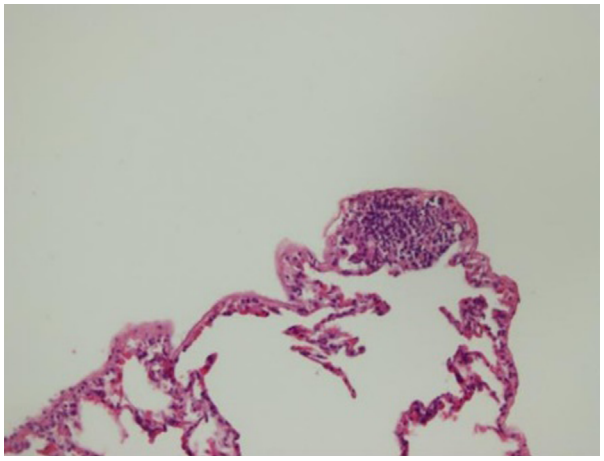

Figure 3. Pleuritis, lymphoid proliferation on the pleural surface in HE X40, Bleomycin (Group B) Group.

between the groups in terms of TOS, TNF-α, PAI-1, MMP-2, and TGF-β levels (Table 4).

Discussion

This study showed that Pirfenidone increased bleomycin-induced pulmonary inflammation and prevented the development of fibrosis and pleuritis in a rat model. Among the inflammation and oxidation markers, TAS was found to be significantly lower in the B-PND group compared to the other groups, while no difference was found between the groups in terms of TOS, TNF-α, PAI-1, MMP-2, and TGF-β levels. Although this study supports the small number of previous literature data showing that pirfenidone reduces the risk of bleomycin-induced pulmonary fibrosis, to the best of our knowledge, the protective effect of pirfenidone in the development of pleuritis due to bleomycin was detected for the first time.

Bleomycin is a cytotoxic agent used in the treatment of many tumors such as germ cell tumors, lymphoma, and Kaposi's sarcoma. Bleomycin causes pneumonia organized in the lungs, eosinophilic hypersensitivity pneumonitis, and interstitial pneumonitis, among which interstitial pneumonitis is observed most frequently up to 46%. As pneumonitis progresses, pulmonary fibrosis develops. Therefore, early treatment with corticosteroids is important (17). After demonstrating the reliability of the use of bleomycin for the development of pulmonary fibrosis in experimental studies, it was used in many studies (17,18). Pirfenidone, on the other hand, is still used in the treatment of IPF in humans with its anti-fibrotic, anti-oxidant, and anti-inflammatory properties.

It was found in previous studies that the mechanism of pulmonary inflammation associated with

Table 4. Intergroup comparison in terms of biomarkers.

Markers	B-PND* (n:13)	B** (n:10)	Control (n:5)	p-value
TOS$^{\beta}$	29,6 (21,5-37,5)	34,65 (21,8-87)	28 (23,5-45,5)	0,195
TAS¶	0,78 (0,7-0,86)	0,87 (0,73-1,13)	0,83(0,78-0,99)	**0,034**
TNFα$^{\phi}$	48,73 (37,36-76,4)	52,86 (45,73-125,4)	43,73(32,81-58,42)	0,206
PAI-1$^{¥}$	2364,8 (1408-3160,4)	2293 (1761,2-3704)	1986 (1507,2-2316,4)	0,207
MMP-2$\wr$	1727,2 (237,8-12204)	1807,5 (719,5-5374)	4942 (699-5914)	0,777
TGFβ$^{\delta}$	335,6 (165,7-430,3)	326,75 (155,3-513,9)	309,2 (256,3-332,7)	0,748

* B-PND:Bleomycin+Pirfenidone; **B:Bleomycin; β:Total oxidant level; ¶:Total antioxidant level; ϕ:Tumor necrosis factor; ¥:Plasminogen activator inhibitor; ≀:Matrix metalloproteinase; δ:Transforming growth factor.

the use of bleomycin is that bleomycin increases the production of DNA-degrading superoxide and hydroxide free radicals and that the overproduction of these reactive oxygen species causes an inflammatory response that causes pulmonary toxicity, activation of fibroblasts, and subsequently fibrosis (12,19,20). Pirfenidone is a well-known antifibrotic agent which has an anti-inflammatory activity by reducing the level of inflammatory markers (21-23). In addition, studies investigating the protective effect of pirfenidone against bleomycin toxicity showed that pirfenidone may have a role in reducing inflammation (24, 25). However, inflammation was observed more in the B-PND group than in the B group in our study. This may be due to the fact that inflammation did not decrease unlike the studies of Iyer and Liu may be that bleomycin was administered intratracheally in these two studies, whereas in our study it was administered intraperitoneally, that is, systemically. Secondarily, the fact that the duration of bleomycin administration was longer (9 weeks) than in other studies may be the reason for the high inflammation. In addition, it can be considered that the dose of pirfenidone used in this process can stop the transition from inflammation to fibrosis, but this dose is too low to suppress inflammation.

In several experimental rat models, pirfenidone has been shown to reduce the development of bleomycin-induced fibrosis. In the study of Song et al., a significant decrease was found in alveolitis and pulmonary fibrosis scores on the 7th, 14th, and 28th days in the rat group given pirfenidone and bleomycin compared to the group given only bleomycin (26). In another study conducted in Egypt, alveolar enlargement, deterioration in its structure, thickening of interalveolar septa, and an increase in mononuclear cell infiltration was found in the rat group that received only bleomycin, while in the group that received bleomycin and pirfenidone, it was shown that the alveolar structures were smoother and stronger, the thickening of the interalveolar septa was minimal, and the mononuclear cell infiltration was less (27). In our study, pirfenidone, which was started together with bleomycin, was shown to be protective against pulmonary fibrosis at the 9th week of treatment and is consistent with the literature.

Two different routes for bleomycin administration were preferred in the studies. Bleomycin was given intratracheally or systemically (intravenously or intraperitoneally). While intratracheal applications result in bronchocentric involvement, systemic applications result in subpleural and parenchymal involvement (28). In our study, the intraperitoneal route was preferred because bleomycin is routinely administered systemically in real life.

Only one study was found in the literature showing that pleural effusion can be detected in the follow-up of cancer patients receiving a bleomycin-containing chemotherapy regimen. In this study conducted by Mertens et al., 12,390 pediatric and adolescent patients were followed, and the rate ratio of the risk of developing pleurisy was shown to be 1.3 (29). The probable cause of the formation of pleural effusion is explained by the fact that toxic reactions initially occur in the subpleural regions rather than deep in the lungs due to poor ventilation due to weak collateral air drifts in the subpleural space. Also, pressure and/or friction on the pleura may play a role in the development of pleural and subpleural changes (30,31). However, there is no data in the literature that pleurisy or pleuritis was observed in rat models given bleomycin. At the same time, no data were found that Pirfenidone prevents the development of pleurisy or pleuritis due to drug toxicity. In

our study, pleuritis developed histopathologically in all rats in the group receiving bleomycin, while pleuritis was not observed in the group receiving pirfenidone prophylaxis. In our research in the literature, the development of pleuritis and the first findings that pirfenidone can protect from this entity were revealed through this study. These findings suggest that pirfenidone may also be protective against the development of bleomycin-induced pleuritis.

There are publications in the literature that bleomycin causes granuloma structures and even cavitating granulomas histopathologically (32,33). In our study, consistent with this information, granuloma was observed in the pathological examination of one rat in the group that received only bleomycin. On the other hand, it was not detected in the group receiving B-PND and the control group. Since we observed granuloma in a single rat model, no comment could be made regarding the protective effect of pirfenidone on the development of bleomycin-associated granuloma. There is no data in the literature on the prevention of granuloma formation by pirfenidone. This issue is still open to research and needs to be supported by larger studies.

TGF-β1 serves a vital role in promoting fibrosis progression, as it can activate various chemokines and cytokines to induce extracellular matrix protein synthesis and suppress collagen degradation, leading to a transition from fibroblasts to myofibroblasts. In the experimental rat model study conducted by Song et al., a significant decrease in TGF-β levels was shown on the 14th and 28th days of the treatment in the group using Pirfenidone+bleomycin compared to the group receiving bleomycin (26). Again, in a study from Japan, it was shown that pirfenidone significantly suppressed TGF-β levels on the 28th day (13). In our study, although higher TGF-β levels were detected in the group receiving only bleomycin, no statistically significant difference was found.

Alveolar epithelial cell (AEC) injury is an early and consistent finding in UIP/IPF (34-36). Early indications that AEC injury may be important in the pathogenesis of progressive pulmonary fibrosis (37,38). Alveolar epithelial cell damage causes alveolar epithelial cell proliferation, which increases the activation of MMP-2 on the cell surface, resulting in increased inflammatory cell migration and ultimately basal membrane damage. Another mechanism that triggers inflammatory cell migration is plasmin, which consists of plasminogen. The defect in PAI-1 triggers fibrosis by causing plasmin activation (39). In the study of Sario et al., in which they investigated the effect of pirfenidone on hepatic fibrosis, a 50-60% decrease was found in PAI-1, MMP-2, TGF-β and tissue inhibitors of metalloproteinase-1 levels in the group receiving pirfenidone at the end of 5 weeks, which resulted in a 70% decrease in collagen accumulation (40). In our study, no significant difference was found between the groups in terms of PAI-1 and MMP-2 levels. Unlike the literature, the reason why there was no difference in inflammatory markers between the two groups in our study may be explained by the fact that pulmonary fibrosis is not a systemic disease but a disease limited to the lung and therefore the presence of inflammation in the tissue is detected but inflammatory markers in peripheral blood are normal.

It was shown that antineoplastic agents cause oxidative stress in patients and accordingly TOS levels increase (41,42). It was reported that pirfenidone has an antioxidant effect by blocking the production of reactive oxygen radicals (43). Consistent with this information, in our study, TOS was found to be lower in the B-PND group compared to the B group, but it was not statistically significant. We believe that significant results can be found if the number of subjects is increased.

Long-term and high-dose use of anti-neoplastic drugs may increase oxidative stress and may also increase total antioxidant levels (44,45). Taherkhani et al. showed a significant decrease in TAS levels after chemotherapy in patients with breast cancer (46). However, in the study of Mohan et al. and Krawczyk et al., it was reported that there was no change in the oxidant-antioxidant balance after chemotherapy in patients diagnosed with lung cancer (47,48). In the study of Xiang et al., it was shown that chemotherapy can increase oxidative stress and antioxidant levels together and that the antioxidant level is higher than the oxidative stress level (49). No prophylactic drugs were used in these four studies. In our study, the TAS level was found to be significantly higher in the group receiving only Bleomycin compared to the B-PND group, and this finding is consistent with the literature findings.

The lack of evaluation of inflammatory markers in lung tissue and BAL was a limitation of our study. Before completing the study, we expected that the inflammation would also decrease with pirfenidone use. Therefore, we did not think that further

techniques showing inflammation in lung tissue would be necessary at the beginning. However, evaluation of inflammatory markers in lung tissue would be a possible explanation for inflammation. Also, we could use more specific techniques to investigate the hypothesis such as mRNA extraction of inflammatory markers in lungs.

In conclusion, it was determined that the use of bleomycin causes pleuritis as well as fibrosis. Pirfenidone used with bleomycin has been shown to be significantly protective against fibrosis and pleuritis. In terms of serum inflammation marker levels, the significant efficacy of pirfenidone could not be demonstrated. Novel serum biomarkers are needed to indicate the presence of inflammation and fibrosis in the lung.

Conflict of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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