RESEARCH

Therapeutic use of fisetin and pirfenidone combination in bleomycin-induced pulmonary fibrosis in adult male albino rats

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Abstract

Pulmonary fibrosis is an important health problem; one of the drugs used in its treatment is pirfenidone (PFD). Fisetin (FST) is a flavonoid with antioxidative, anti-inflammatory, and antifibrotic effects. The aim of this study was to induce PF in rats with bleomycin (BLM) and to investigate the combined effect of PFD and FST in the treatment of fibrosis. In the study, 40 male Wistar rats were divided into five groups ($n=8$). Sham group was administered saline on day 0 and BLM (5 mg/kg, i.t.) was administered to the other groups; BLM + PFD group: PFD (50 mg/kg) was administered every day between the first and 15th days; BLM + FST group: FST (25 mg/kg) was administered between the first and 15th days; BLM + PFD + FST group: PFD (50 mg/kg) and FST (25 mg/kg) were administered by gavage every day between the first and 15th days. At the end of the 15th day, BAL was performed under anaesthesia and lung tissues were removed. Histopathological, biochemical, and RT-PCR analyses were performed in the lung tissue. In our study, the concomitant use of FST and PFD caused downregulation of NF-κB p65, TGF-β1, and α-SMA expressions; downregulation of TIMP-1, MMP-2, and MMP-9 genes; downregulation of HYP, MPO, and MDA activity; decrease in the number of differential cells in BAL; and upregulation of GSH. This shows that FST and PFD have antifibrotic, antioxidative, and anti-inflammatory effects. Our results show that the combined use of PFD and FST in BLM-induced pulmonary fibrosis reduces extracellular matrix accumulation, downregulates the level of gelatinases and their inhibitors, and provides significant improvements in antioxidative defence parameters.

Keywords Bleomycin · Fisetin · Pirfenidone · Pulmonary fibrosis · Matrix metalloproteinases

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Introduction

Pulmonary fibrosis (PF) is an incurable disease characterised by increased connective tissue and inflammation in the lung (Kim et al. 2011). Although the pathogenesis of PF is not fully understood, it is thought to be associated with excessive degradation of the matrix by activation of transforming growth factor-β (TGF-β) and disruption of the alveolar basement membrane, activated matrix metalloproteinase (MMP), and abnormal proliferation of myofibroblasts (Kim et al. 2011). Bleomycin-induced lung fibrosis model is the most important and valid model used to clarify the stages of the disease and lung structure and function (Mouratis and Aidinis 2011). Bleomycin (BLM) is a chemotherapeutic used in some malignancies. The side effect of BLM treatment is lung toxicity (Hosseini et al. 2018). Inflammation and fibrosis are observed in lung damage caused by BLM (Phan and Kunkel 1992). Pirfenidone (PFD) is a drug with analgesic, antipyretic, anti-inflammatory, and antifibrotic effects (Guo et al. 2019). However, it cannot completely treat PF. For this reason, it is also very important to try combined bioactive components together with drugs in PF. Fisetin (FST) is a flavonol, a structurally different chemical substance belonging to the flavonoid group of polyphenols found in many plant fruits and vegetables. In addition, recent studies have shown that fisetin has anticancer (Chou et al. 2013), antioxidant (Prasath and Subramanian 2013), anti-inflammatory (Goh et al. 2012), and antiproliferative (Brenner et al. 2012) effects. It is also reported in some studies that it has antifibrotic effects.

The pathogenesis of PF involves complex mechanisms. Biomarkers that play a role in the diagnosis, prognosis, and pathogenesis of the disease are important in the planning of treatment. One of the factors involved in the pathogenesis of pulmonary fibrosis is abnormal proliferation of myofibroblasts. A marker of myofibroblasts is α-smooth muscle actin (α-SMA) (Brenner et al. 2012; Phan 2008). Collagen synthesis by myofibroblasts is stimulated by TGF-β after fibrosis has occurred (Phan 2008). Hydroxyproline (HYP) level indicates total collagen content and the degree of tissue or organ fibrosis. Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are proteases involved in the degradation and re-organisation of ECM and are involved in the mechanism of IPF (Zuo et al. 2017; Mahalanobish et al. 2020).

Oxidative stress and oxidation/antioxidation imbalance are one of the factors that play an important role in the pathogenesis and progression of IPF. Interaction with TGF-β is extremely important in this process (Liu et al. 2017). TGF-β stimulates the production of ROS (reactive oxygen species) associated with oxidative stress. Thus,

TGF-β stimulation initiates profibrogenic processes. ROS is also effective in nuclear factor kappa-B (NF-κB) activation (Yang et al. 2005). Stimulated immune cells produce cytokines that affect the profibrotic environment. These cytokines are organised by the inflammation-related transcription factor NF-κB (Zhang et al. 2000). Numerous studies have shown that profibrotic cytokines (TGF-β1) are important in triggering increased extracellular matrix synthesis and conversion of fibroblasts to myofibroblasts. Inhibition of these molecules may produce an important antifibrotic effect against pulmonary fibrosis (Chitra et al. 2013). Some of the antioxidants can inhibit the movement of NF-κB to the nucleus. Thus, the binding of NF-κB to DNA can be inhibited (Yang et al. 2005; Suzuki and Packer 1993). TGF-β stimulates oxidative stress, antioxidant defence is reduced, and ROS generation is increased. TGF-β also reduces the production of effective antioxidants such as GSH and SOD (Cheresh et al. 2013).

Analyses of fibrotic markers such as α-SMA, a marker of myofibroblasts, and alteration of anti-fibrotic genes involved in matrix production and degradation (gelatinases (MMP-2, MMP-9), TIMP-1), and HYP content were performed to show which mechanisms are involved in the excessive matrix degradation that occurs during the formation of pulmonary fibrosis. Furthermore, levels of the TGF-β1, NF-κB, and the oxidative stress parameters GSH, MDA, and MPO were analysed to elucidate the role of the combined use of FST and PFD in pulmonary fibrosis. PFD and FST showed promising anti-inflammatory, antifibrotic, and antioxidative effects on these parameters in bleomycin (BLM)-induced pulmonary fibrosis models.

In summary, the aim of this study was to investigate the combined effects of PFD and FST, which have antifibrotic and anti-inflammatory effects in BLM-induced pulmonary fibrosis model in rats, on pulmonary fibrosis by evaluating the above-mentioned fibrotic markers, profibrotic cytokines, gelatinases, and oxidative damage parameters.

Materials and methods

Materials

Bleomycin (Blemisin, Koçak Farma) 15-mg vial powder was purchased. Fisetin (Cat No. HY-N0182) and pirfenidone (Cat No. HY-B0673) were purchased from MedChemExpress (USA). Rat Hydroxyproline ELISA Kit (Cat No. NE01A004003) commercially available from Nepenthe was used for analysis. Antibodies used are as follows: α-SMA polyclonal antibody 120 μL (Elabscience, E-AB-34268); TGF-β1, polyclonal antibody 100 μl (Biorbyt, UK, orb311035), and NF-κB p65 antibody 200 μl (Biorbyt UK,

orb506115). Kits used for immunohistochemistry: UltraVision Polyvalent (Rabbit-Mouse) HRP Kit, Thermo Scientific TP-125-HL, DAB; DAB Chromogen & Substrate System 125 ml, Thermo Scientific.

Animals and study approval

In addition to mice, rats are also used in PF modelling studies. Rats offer some advantages for PF modelling. Rats allow easier manipulation and collection of more tissue samples due to their larger size (Jenkins et al. 2017). Some researchers also consider that rat models have a histopathology more similar to human PF compared to mice. Forty, 8-to-10-week old, 250 ± 50 g Wistar albino male rats were used in the study. The study was carried out at Gaziantep University Experimental Research Centre as the centre. They were kept in well-ventilated plastic cages in a 12-h light and dark environment. Feed and water were given ad libitum. All animals received good care according to standard guidelines. Ethical approval for the study was obtained from Gaziantep University Animal Research Ethics Committee (No. 55/2022; date: 25.10.2022). Ethical regulations were strictly followed according to national and institutional guidelines. Rats were randomly divided into five groups. There were eight rats in each group.

Experimental procedure

Group Sh: A single dose of saline was administered intratracheally* on the first day of the experiment.

Group BLM: On the first day of the experiment, a single dose of BLM (5 mg/kg/day) (0.8 ml/kg) was dissolved in saline and administered intratracheally (Erden et al. 2008).

Group BLM + PFD: A single dose of intratracheal BLM (5 mg/kg/day) was administered on the first day of the experiment, and pirfenidone 50 mg/kg (2 ml/kg) was administered by gavage every day for the next 14 days (Song et al. 2018).

Group BLM + FST: A single dose of intratracheal BLM (5 mg/kg/day) was administered on the first day of the experiment, and 25 mg/kg fisetin (Shahbaz et al. 2021) was given by gavage for the next 14 days.

Group BLM + PFD + FST: A single dose of intratracheal BLM (5 mg/kg/day) was administered on the first day of the experiment, and 25 mg/kg (2 ml/kg) fisetin was administered by gavage for the next 14 days (1 day after the single-dose BLM administration, 50 mg/kg pirfenidone was administered by gavage and 25 mg/kg fisetin by gavage for 14 days. The experiment lasted 15 days). For PFD and FST, 5% DMSO and 95% saline were used as solvents. PFD and FST were given with 1-h interval.

At the end of the experiment, BAL was performed under ketamine (75 mg/kg) + xylazine (10 mg/kg) anaesthesia, and the lung tissues of the rats were removed. The experimental design is shown schematically in Fig. 1.

*The intratracheal administration procedure was as follows: BLM and saline administration was performed under anaesthesia. For anaesthesia, xylazine and ketamine were drawn into the same syringe and administered intraperitoneally. Then, the reflexes of the rats were checked and they were expected to enter anaesthesia. Meanwhile, 15-u vial form of BLM was dissolved in saline. The anterior neck of anaesthetised rats was opened with a midline incision, subcutaneous tissues were carefully excised with the help of a clamp, and the trachea was accessed. BLM was injected into the trachea with a 100-u insulin syringe, the head of the rats was lifted upwards, and wait for 30 s for the BLM to reach the lung completely. Saline was given to the sham group in the same way. After the procedure, the incision site was closed with suture and wound care was performed.
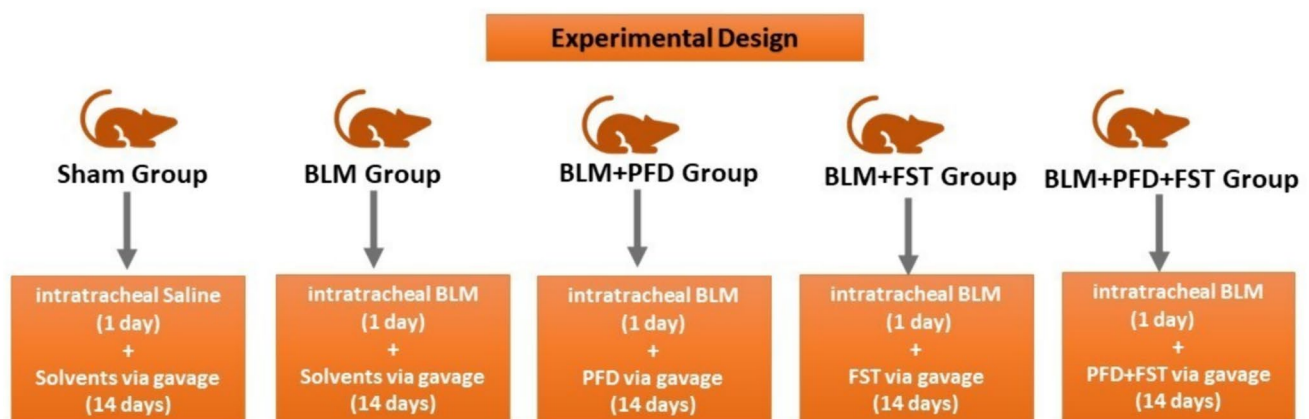
Fig. 1 Summary of experimental design

Histological analysis

Lung tissues removed from the experimental animals were placed in 10% formaldehyde solution. Gradually increasing alcohols were used in the dehydration stage (50%, 70%, 80%, 96%, 3x 100). Xylene was used for transparency and paraffin was used for embedding, and sections were taken on a microtome (5 µm thick). Haematoxylin-eosin (H-E) and Masson trichrome (MT) stains were used to determine histopathological changes. TGF-β1 and α-SMA immunohistochemistry staining was performed to determine fibrotic changes that occur as a result of damage to the tissue.

Haematoxylin-eosine and Masson trichrome

Sections taken from paraffin blocks were kept in an oven at 58 °C and then soaked in xylene for 10 min three times. Then, they were passed through decreasing alcohol series ($2 \times 100\%$, 96%, 80%, 70%, and 50%) and washed with water. After this stage, they were kept in haematoxylin for 10 min and washed with water. This time, it was kept in eosin for 5 min and washed with water. The sections were taken in increasing alcohol series (70%, 96%, 3x 100), kept in xylene after alcohol, and covered with Entellan. MT staining was performed according to the kit protocol (BESLAB, HistoMed, Ankara). Thus, fibrotic areas were highlighted with blue colour (Yildirim and Karabulut 2020).

Immunohistochemistry

TGF-β1, NF-κB, and α-SMA immunohistochemistry staining was performed in lung tissue as a result of BLM-induced pulmonary fibrosis. Avidin-biotin-peroxidase complex (ABC) procedure was used. Sections were taken on polylysine slides and left in an oven at 58 °C overnight. Xylene was used for transparency. It was passed through ethyl alcohol (100%, 96%, 80%, 70%) series. Three percent hydrogen peroxide (H_2O_2) was used for endogenous peroxidase inactivity. Citrate buffer was used for the antigen retrieval step. Washings were performed with phospha-e buffered saline. Other steps were performed according to the kit (TP-125-HL Thermo Fisher Scientific) instructions procedure. DAB (3,3′-diaminobenzidine) was used as the chromogen and then counterstained with Gill I haematoxylin. Finally, the sections were cleared in xylene and sealed with Entellan. Sections stained with TGF-β1, NF-κB p65, and α-SMA were examined under a Nikon Ni-U (Nikon, Tokyo, Japan) light microscope and photographed with a DS-Ri2 model digital camera (Yildirim et al. 2016).

Quantitative immunohistochemistry

Eight lung sections taken from the paraffin blocks of each rat were selected. TGF-β1, NF-κB p65, and α-SMA were evaluated in terms of immunostaining intensities. Five photographs were taken from each stained slide, and the immunoreactivity of five fields from each photograph was measured. Four hundred fields were evaluated for each group. ImageJ Program was used for density measurements. Statistical analyses of the obtained data were performed.

Biochemical analysis

Determination of myeloperoxidase (MPO) enzyme activity

A manual spectrophotometric method was used. The principle in this method is as follows: it is based on the principle that while hydrogen peroxide is oxidized by MPO in the tissue homogenate, o-dianisidine in the reaction mixture is reduced and the reduced dianisidine absorbs light at a wavelength of 410 nm (Koike et al. 1994).

Determination of tissue malondialdehyde (MDA) levels

Tissue MDA levels were measured with the use of a manual spectrophotometric method. The principle of this method is as follows. It is based on the spectrophotometric measurement of the red colour due to the complex formed by MDA in the sample with thiobarbituric acid by binding the proteins in the supernatant with sodium dodecyl sulfate under conditions where the ambient pH is 3.5 (Yildirim et al. 2006).

Determination of glutathione (GSH) levels

Measurement of glutathione (GSH) levels was performed with spectrophotometric method. The principle of this method is based on the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) added to the reaction medium by sulfhydryl groups, resulting in the formation of 1 mol of 2-nitro-5-mercaptobenzoic acid for 1 mol of sulfhydryl. The nitro-mercaptobenzoic acid formed is bright yellow in colour and can be used to measure sulfhydryl groups by spectrophotometric method (Kalantar et al. 2021).

Determination of hydroxyproline (HYP) levels

Rat hydroxyproline ELISA kit was used from analysis. Analysis of tissue hydroxyproline levels was performed following the procedures specified in the ELISA kit.

Table 1 Primers from selected base sequences for target and reference gene

Oligonucleotide ID	Nucleotide sequence
TIMP-1	5′-GCAACTCGGACCTGGTTAT-3′
	5′-GTCGAATCCTTTGAGCATCTT-3′
MMP-2	5′-GAGTTGGCAGTGCAATACCT-3′
	5′-CCAAAGAACTTCTGCATCTTCT-3′
MMP-9	5′-CTTCGAGGGCCACTCCTACT-3′
	5′-CAGTGACGTCGGCTCGAGT-3
GAPDH	5′-CCTCAAGATTGTCAGCAAT-3′
	5′-CCATCCACAGTCTTCTGAGT-3′

Bronchoalveolar lavage fluid (BAL) analysis

After the rats were euthanized at the end of the experiment, the trachea was cannulated using a blunt needle syringe and BAL was performed with 4–5 ml PBS. The cell-free supernatant was collected after centrifugation at 5000 rpm for 10 min at 4 °C. The BAL fluid obtained was used for cell counting. Total and differential cell counts were performed using a May-Grünwald-Giemsa hemocytometer. In each smear, 100 cells were counted × 100 magnification. Accordingly, alveolar macrophage, neutrophil and lymphocyte ratios were evaluated (Chitra et al. 2013; Cortijo et al. 2001). The images were taken under a Nikon Ni-U (Nikon, Tokyo, Japan) model light microscope with a DS-Ri2 model digital camera at 100 magnification.

RT-PCR analysis

Tissue homogenisation and RNA isolation

Fibrotic genes in lung tissue were also analysed by RT-PCR. MMP-2, MMP-9, and TIMP-1 genes (Table 1) were analysed. The tissues were stored at -80 C until the day of the study.

Tissues were homogenised with the lysis buffer in the kit using a refrigerated homogeniser (Bertin/Precellys Evolution). The samples were kept for 5–10 min to ensure complete lysis. Then, 0.2 ml chloroform was added, vortexed for 30 s, and centrifuged at $12,000 \times g$ for 10 min at 4 °C. Approximately 540 µl of supernatant was transferred to a 1.5-ml RNase-free centrifuge tube. Eight hundred ten microliters of absolute alcohol (100% ethanol) was added to the supernatant and pipetted several times. The mRNA isolation of the samples was performed using the High Pure RNA Isolation Kit (12033674001 High Pure RNA Tissue Kit/Germany). The steps were performed in the following order according to the kit manual.

cDNA synthesis from mRNA

The isolated mRNAs were used for cDNA synthesis according to the commercial kit OneScript plus cDNA synthesis kit (ABM/Canada). All components included in the kit were prepared in the amounts specified in the kit content for each sample. $5 \times$ RT buffer 4 µl, DNTP 1 µl, random hexamer primers 1 µl, isolated RNA 2 ng, One-Script Plus Rtase 1 µl, and nuclease-free water were added to 20 µl. At 55 °C for 15 min and 85 °C for 5 min, cDNA was obtained in a thermal cycler (Bio-Rad, USA) at 4 °C and measurements were made with nanodrop.

Real-time PCR

SYBR Green Master Mix 10 µl, forward primer 500 nM 1 µl, reverse primer 500 nM 1 µl, cDNA 5 µl, and ddH20 3 µl were prepared according to the commercial kit Fast-Start Essential SYBR Green.

Master Mix (Roche, Germany) was used to ensure amplification of cDNAs for the reference gene and to mark the target and reference regions.

Statistical analysis

Power analysis for the number of animals to be used was determined with G*Power 3.1.9.7 software. According to the statistical method to be used (ANOVA), power analyses were taken as an example (Parlar et al. 2021). Accordingly, the power analysis calculated using the G*Power (version 3.1.9.7) program in our study showed that at least 40 animals were required to obtain a power of 0.95 and an effect size of 0.75 at a significance level of 0.05. Therefore, it was determined that 40 animals were appropriate for our study.

JASP 0.14.1 program was used to analyse the data. It was evaluated whether the data showed normal distribution by looking at the skewness and kurtosis values. After these evaluations, data were expressed as mean $\pm$ SD and analysed with one-way ANOVA test and Tukey's post hoc test for parametric tests. For non-parametric tests, the Kruskal-Wallis test with median minimum-maximum values was applied, and the Mann-Whitney U test was used for pairwise comparisons (rank values were used in RT-PCR analyses). $p < 0.05$ was considered significant in the analysis.

Results

Histopathology

Haematoxylin-eosin, Masson trichrome staining, scoring, and body weight data

Sh group was similar to normal lung architecture. There were areas of thickening in the interalveolar septum and small areas of increased connective tissue (Fig. 2). In the BLM group, fibrotic areas were dense; mononuclear cell infiltration, alveolar disruption, and cell spillage into the alveolar lumen were observed. In the BLM + PFD group, similar findings were observed as in the BLM group. Infiltration of inflammatory cells, vascular congestion, increase in connective tissue, cell shedding in the alveolar lumen, and fluid accumulation were observed. Infiltration of inflammatory cells, cell shedding in the bronchiole lumen, and cell shedding in the alveolar lumen were observed in the fisetin group. Although the histopathological findings were similar to those in the BLM-only group, partial improvements were observed in the groups receiving BLM in combination with FST and PFD (Fig. 2). We would also like to point out that some parts of the tissue were better, while fibrotic areas were dense in some parts. Histopathological improvements were better in the groups that received BLM and pirfenidone and fisetin together. When statistical analyses of the Ashcroft scoring (Table 2), which assesses tissue fibrosis, were examined, there was a significant difference between all experimental groups and the sham group. When we compared the BLM group with the other groups, there was a significant difference between BLM + PFD and BLM + PFD + FST groups. The highest scoring value belonged to the BLM group (Table 2). MT staining showed the accumulation of collagen fibres due to BLM. There was a significant decrease in collagen fibre accumulation in the BLM + PFD + FST group (Fig. 3). HYP content was also high in the BLM group and decreased in the other treatment groups (Table 2). Weights were measured on the first day (day 0) and the last day (day 14) of the experiment. In general, there was a decrease in weight in all groups on day 14 compared to day 0 (Table 3). When these data were evaluated collectively, it was seen that the combined use of fisetin and pirfenidone at the doses we used for the treatment of pulmonary fibrosis was more effective than their individual use.

Immunohistochemistry

It was observed that α-SMA was expressed in blood vessels in the Sh group in lung tissue, whereas it was highly expressed in fibroblastic foci outside the vessels in the other groups (Fig. 4). In our study, α-SMA expression was highest in the BLM group and lowest in the Sh group. There was no significant difference between BLM + PFD and BLM + FST groups, while there was a significant difference between the other groups. In the BLM + PFD + FST group, it was observed that the BLM + PFD + FST group was closer to the Sh group. When TGF-β1 expression is examined, it is seen as positive brown-stained structures around the light bronchi and in some interstitial macrophages and inflammatory cells (Fig. 4). For TGF-β1 expression, it was observed that TGF-β1 expression was significantly increased in the BLM group compared to the Sh group, while there was no significant difference between the other groups and the Sh group. In addition, when the BLM group was compared with the other groups, there was a significant difference between the BLM group and the BLM + PFD + FST group. When PFD and FST were given together, TGF-β1 expression was closer to the Sh group. Under normal physiological conditions, NF-κB p65 is weakly expressed in the lungs. When we look at NF-κB p65 expression, positive brown-stained structures around the bronchi and in some interstitial macrophages and inflammatory cells show NF-κB p65 expression (Fig. 4). NF-κB p65 expression was found to be at the highest level in the BLM group. There was a difference between the sham group and BLM, BLM + PFD, and BLM + FST groups. There was also a significant difference between BLM and BLM + PFD + FST group (Table 4).

Biochemical results

GSH

The level of GSH content in lung tissue decreased with BLM treatment and the decrease was significant compared with the Sh group ($p < 0.001$). There was a significant difference between all groups. As shown in Table 5, GSH content was highest in the Sh group (34.93 ± 0.11 nmol/mg protein) and lowest in the BLM group (18.22 ± 0.07 nmol/mg protein). There were increases in GSH content in BLM + PFD, BLM + FST, and BLM + PFD + FST groups compared to BLM-treated group. In the BLM + PFD + FST group, there was an increase closer to the Sh group.

MPO

MPO activity, which indicates polymorphonuclear leukocyte (PMN) activity, increased with exposure to BLM (7.16 ± 0.26 U/g) compared to the Sh group (2.13 ± 0.22 U/g) ($p < 0.001$). As shown in Table 5, there was a statistically significant difference between BLM and Sh groups
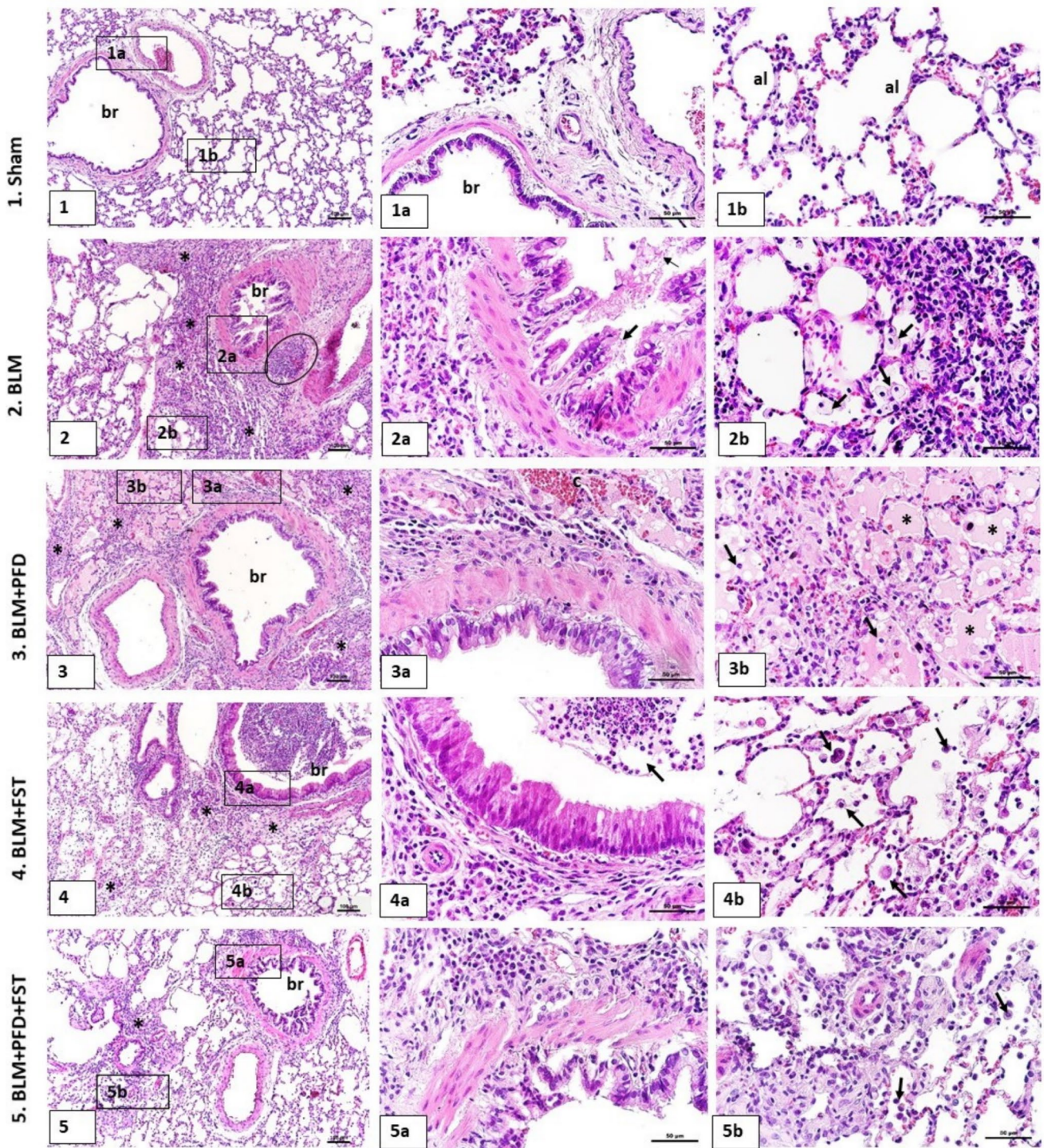
**Fig. 2 1** Infiltration of inflammatory cells (*). **1a** Bronchiole epithelium and muscle layer under the epithelium are seen. b, bronchiole. **1b** Alveoli and interalveolar septum are seen. a, alveoli. **2** Round shape: dense mononuclear cell infiltration; infiltration of inflammatory cells (*). **2a** Sloughing of bronchial epithelium (thin arrow), disruption of bronchiolar epithelium (thick arrow). **2b** Alveolar closure and alveolar cell shedding, with shed cells visible in the lumen (arrows). **3** Infiltration of inflammatory cells (*). **3a** Congestion of vessels (c), increase in connective tissue. **3b** Cell shedding into the alveolar lumen (arrow) and fluid accumulation (*). **4** Infiltration of inflammatory cells (*). **4a** Cell shedding in the lumen of the bronchioles. **4b** Sloughed cells in the alveolar lumen. **5** Infiltration of inflammatory cells (*). **5b** Impaired alveoli and cell shedding next to normal alveoli (arrows). **1**, **2**, **3**, **4**, **5**: bar, 100 µm; **1a**, **1b**, **2a**, **2b**, **3a**, **3b**, **4a**, **4b**, **5a**, **5b**: bar, 50 µm

Table 2 Ashcroft scoring values and HYP content

Groups	Sham	BLM	BLM+PFD	BLM+FST	BLM+PFD+FST	p
Score	$1.30 \pm 0.73^{+}$	$5.02 \pm 1.24^{*}$	$4.41 \pm 1.11^{*,+}$	$4.60 \pm 1.06^{*}$	$4.38 \pm 1.17^{*,+}$	0.001
HYP (ng /ml)	7.43 ± 0.21^{a}	7.81 ± 0.05^{b}	6.85 ± 0.94^{abcd}	6.57 ± 1.23^{c}	5.85 ± 0.55^{d}	0.002

Data are expressed as mean ± standard deviation. Abbreviations: *BLM* bleomycin, *PFD pirfenidone*, *FST* fisetin, *HYP* hydroxyproline. *When compared with sham, $p < 0.001$ was considered significant. $^{+}p < 0.001$ was considered significant when compared with BLM. The same letters indicate that there is no significant difference between the groups and different letters indicate that there is a significant difference between the groups
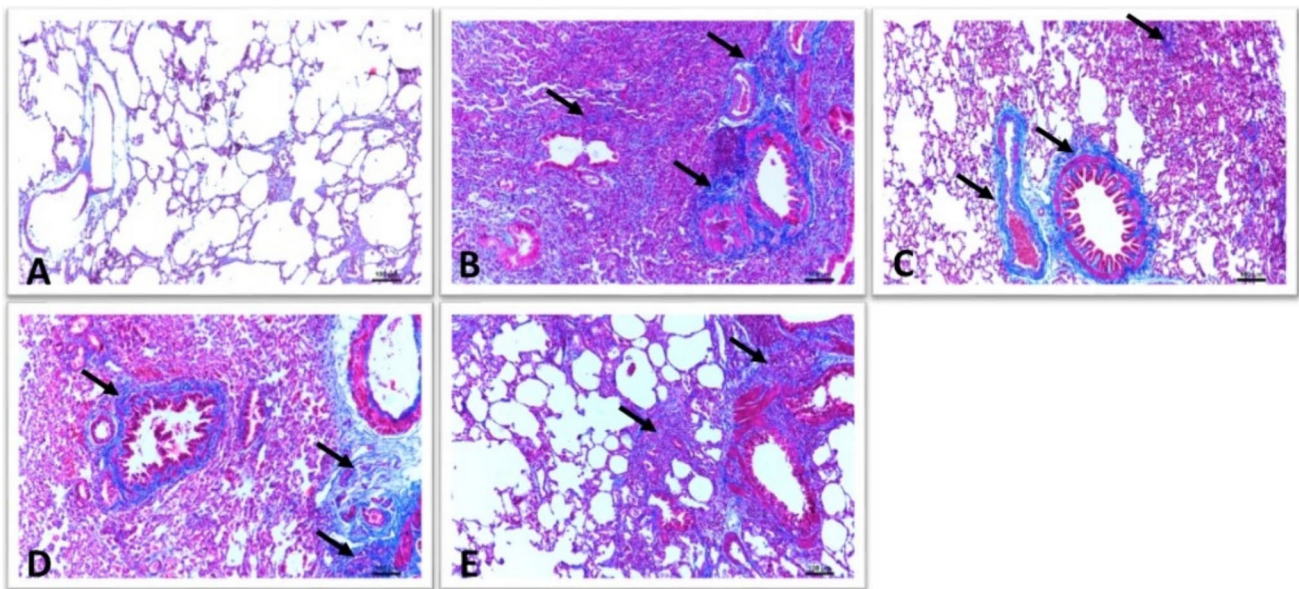
Fig. 3 Masson trichrome–stained lung tissue (×20). Bar = 100 μm. Blue areas (arrows) show collagen content in the tissue. Increased collagen is seen around the vessels and fibrotic areas. **A** Group I: sham group; **B** group II: BLM group; **C** group III: BLM+PFD; **D** group IV: BLM+FST; **E** group V: BLM+PFD+FST

Table 3 Weight change of the experimental groups on the first day (day 0) and the last day (day 14) of the experiment

	Sham0	Sham14	BLM0	BLM14	B+P0	B+P14	B+F0	B+F14	B+P+F0	B+P+F14
n	8	8	8	8	8	8	8	8	8	8
$\bar{x}$ (g)	256.14	223.71	265.00	230.00	265.00	220.86	259.33	224.33	261.83	223.33
Standard deviation (g)	36.70	21.86	41.46	38.17	28.05	23.76	32.42	31.85	21.48	12.06

and all groups. There was no statistically significant difference between the BLM+PFD and BLM+FST groups. In the BLM+PFD+FST group, there was a decrease closer to the Sh group.

MDA

MDA levels, which reflect lipid peroxidation end products, increased with exposure to BLM (10.36 ± 0.51 nmol/g tissue) compared with the Sh group (5.71 ± 0.02 nmol/g tissue) (Table 5). There was no statistically significant difference

between the BLM+PFD and BLM+FST groups. Elevated tissue MDA levels were significantly reduced in the BLM+PFD, BLM+FST, and BLM+PFD+FST groups compared with the BLM-only group. The BLM+PFD+FST group (6.14 ± 0.02 nmol/g tissue) showed a decrease closer to that of the Sh group (5.71 ± 0.02 nmol/g tissue).

In other words, GSH, MPO, and MDA levels were positively affected when pirfenidone and fisetin were given separately. However, when these two compounds were administered simultaneously, the rate of effect on these parameters increased significantly.
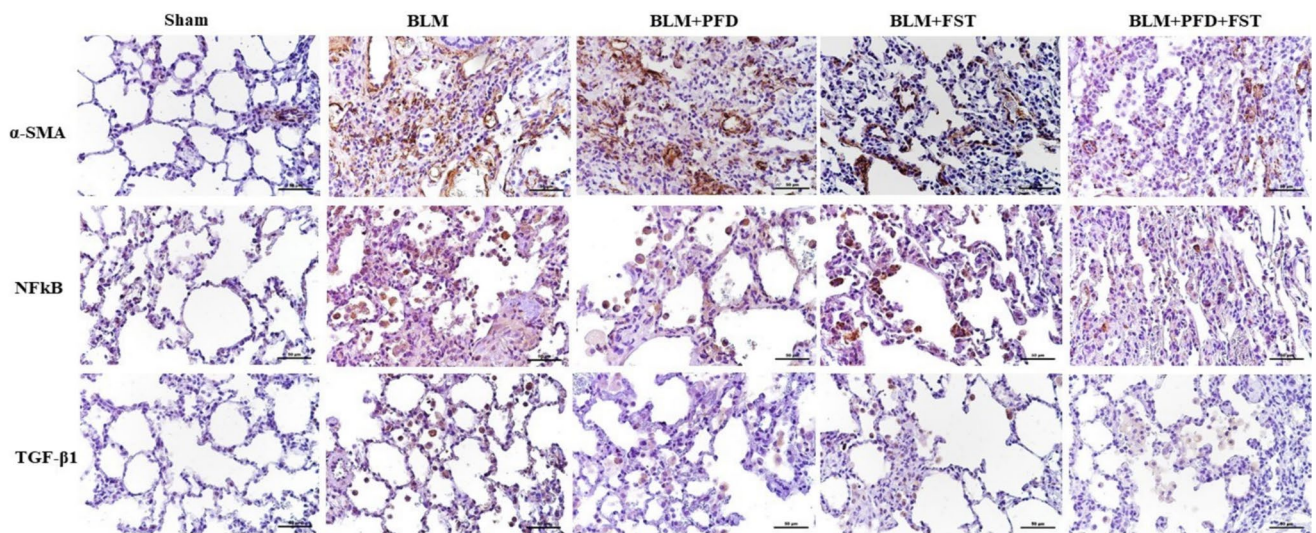
Fig. 4 α-SMA, NF-κB p65, and TGF-β1 expressions in lung tissue in all groups. Immunohistochemistry. ×40, scale bar 50 μm

Table 4 α-SMA, NF-κB p65, and TGF-β1 immunoreactivity intensity measurement values in lung tissue

Groups	Sham	BLM	BLM+PFD	BLM+FST	BLM+PFD+FST	p
α-SMA	85.34 ± 8.15^a	108.77 ± 11.97^b	101.07 ± 8.60^c	98.52 ± 7.51^c	91.22 ± 14.39^d	0.001
NF-κB p65	$107.71 \pm 14.40^+$	$118.86 \pm 11.44^*$	$115.53 \pm 10.46^*$	$114.72 \pm 11.13^*$	$111.80 \pm 12.30^+$	0.001
TGF-β1	$113.96 \pm 9.65^+$	$122.40 \pm 14.03^*$	119.44 ± 9.6	116.21 ± 14.25	$114.94 \pm 11.71^+$	0.003

Data are expressed as mean ± standard deviation. Abbreviations: *BLM* bleomycin, *PFD* pirfenidone, *FST* fisetin. No significant difference between groups with the same superscript (for α-SMA)

*$p < 0.05$ was considered significant compared to Sham

+$p < 0.001$ was considered significant compared to BLM (for TGF-β1 and NF-κB p65)

Table 5 Changes in GSH, MDA, and MPO activities between groups

Groups	Sham	BLM	BLM+PFD	BLM+FST	BLM+PFD+FST	p
GSH (nmol/mg protein)	34.93 ± 0.11^a	18.22 ± 0.07^b	24.13 ± 0.07^c	24.89 ± 0.06^d	28.63 ± 0.15^e	0.001
MDA (nmol/g tissue)	5.71 ± 0.02^a	10.36 ± 0.51^b	8.17 ± 0.04^c	8.02 ± 0.04^c	6.14 ± 0.02^d	0.001
MPO (U/g tissue)	2.13 ± 0.22^a	7.16 ± 0.26^b	5.59 ± 0.14^c	5.79 ± 0.06^c	3.68 ± 0.04^d	0.001

Data are expressed as mean ± standard deviation. Abbreviations: *BLM* bleomycin, *PFD* pirfenidone, *FST* fisetin. There is no significant difference between the groups shown with the same superscript

BAL analysis results

When the BAL cell counts were analysed, it was observed that the total cell count, lymphocyte count, neutrophil count, and alveolar macrophage count were highest in the BLM group and lowest in the Sh group (Table 6). According to the results of BAL analysis of total cell counts, there was a statistical difference between the groups ($p < 0.01$). Bonferroni test and Kruskal-Wallis test were used for statistical analysis. The cell count was significantly higher between the Sh group and the BLM, BLM+FST groups ($p < 0.05$)

(Table 7). When the BLM group was compared with all groups, there was a significant difference between them ($p < 0.01$) (Table 7). Figure 5 shows the microscope images of the cells in BAL.

Real-time qPCR results

According to Kruskal–Wallis *H* test analyses, there was a statistically significant difference between all groups for TIMP-1 ($\chi^2(4) = 9.232$; $p < 0.05$), MMP-2 ($\chi^2(4) = 13.912$; $p < 0.01$), and MMP-9 ($\chi^2(4) = 14.838$; $p < 0.01$) (Table 8).
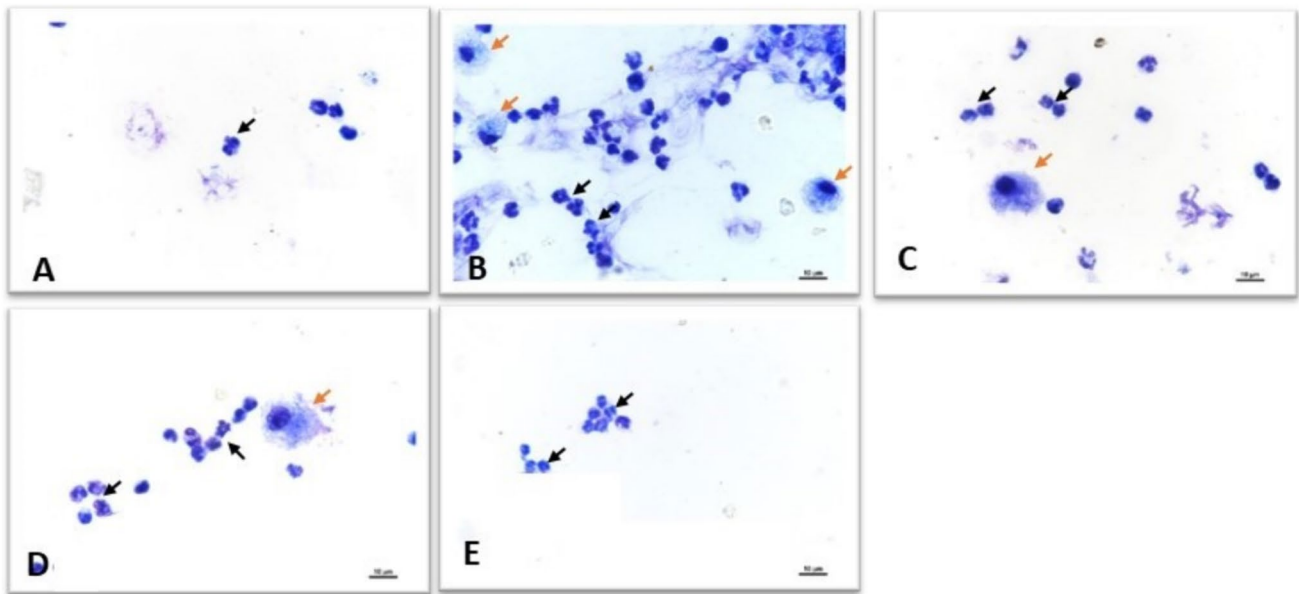
Fig. 5 Sh (**A**), BLM (**B**), BLM + PFD (**C**), BLM + FST (**D**), and BLM + PFD + FST (**E**) groups: appearance of neutrophil and macrophage cells in BAL fluid (black arrows indicate neutrophils; orange arrows indicate macrophages); May-Grünwald-Giemsa, ×100. Bar, 10 μm

Table 6 Results of differential cell counts in BAL

Groups	Total cell counts	Lymphocyte %	Neutrophils %	Alveolar macrophage %
Sh (n=6)	406 (244–421)	1 (1–2)	1 (1–2)	98 (97–98)
BLM (n=6)	1064 (626–1330)	5 (4–5)	10 (10–11)	85 (84–85)
BLM + PFD (n=6)	450 (305–560)	3 (3–4)	4 (3–5)	93 (91–95)
BLM + FST (n=6)	625 (478–702)	4 (4–5)	5 (4–6)	91 (88–94)
BLM + PFD + FST (n=6)	427 (302–482)	2 (2–3)	3 (2–3)	95 (92–100)

Data are presented as median and (range) of groups

Table 7 Statistical analysis results of total cell counts in BAL

Group	Sh (n=6)	BLM (n=6)	BLM + PFD (n=6)	BLM + FST (n=6)	BLM + PFD + FST (n=6)	p-value
Total cell count 'mean (standard deviation)'	372,875 (65,855)[+]	1018,750 (222,517)*	438,166 (99,258)+	599 (89,995)*+	409,166 (69,522)[+]	0.000

*$p < 0.05$ was considered significant when compared with Sham. [+]$p < 0.001$ was considered significant when compared with BLM

When the rank values for TIMP-1 were analysed, BLM (28.42), BLM + F (11.82), and BLM + P + F (14.75) were the highest and Sh (10.25) was the lowest (Fig. 6).

Different from TIMP-1, when MMP-2 was evaluated, BLM (26.08) was the highest and Sh (7.15) was the lowest (Fig. 6). Regarding MMP-9, BLM (28.90) was the highest and Sh (7.38) was the lowest (Fig. 6).

Considering all the results, it was observed that Sh was the lowest in terms of TIMP-1, MMP-2 and MMP-9 variables (although the values varied). The BLM group was found to be the highest. Although the values of the BLM + P, BLM + F, and BLM + P + F groups were close to each other, their values were lower than those of the BLM group. It was observed that fisetin inhibited TIMP-1, MMP-2, and MMP-9 genes slightly more than pirfenidone (Fig. 6).

Table 8 Analysis results of TIMP-1, MMP-2, and MMP-9 variables

Group	n	TIMP-1	MMP-2	MMP-9
Sh	8	6.91c	7.154c	7.380c
BLM	8	28.425a	26.087a	28.904a
BLM+P	8	19.69ab	17.97ab	23.31a
BLM+F	8	11.825b	14.75b	15.64ab
BLM+P+F	8	14.75ab	12.764b	22.90a
		$\chi^2(4) = 9.232$, $p = 0.032*$	$\chi^2(4) = 13.912$, $p = 0.004**$	$\chi^2(4) = 14.838$, $p = 0.002**$

$*p < 0.05$; $**p < 0.01$ significance level. The same letters indicate the closeness of the groups to each other; different letters indicate the distance

Discussion

The aim of this study was to investigate the combined effect of pirfenidone, a drug used in the treatment of pulmonary fibrosis, and fisetin, a flavonoid, in BLM-induced pulmonary fibrosis in rats.

Histopathological deterioration is an important indicator for the diagnosis and prognosis of PF. In the histopathological evaluation, H-E staining of the sections in the Sh group showed mild thickening of the alveolar septum and MT staining showed very little collagen in the pulmonary interstitial area. In the model groups, H-E staining showed significant pathological changes such as thickening in diffuse interalveolar areas, infiltration of inflammatory cells, and alveolar haemorrhage. MT staining showed increased collagen deposition. BLM-treated rats showed severe histopathological damage, increased inflammatory cell infiltration, and lung fibrosis. Similar to our study, studies on BLM-induced pulmonary fibrosis have observed thickening of the alveolar space, deterioration in lung structure, and a fibrotic lung phenotype compared to the control group (Song et al. 2018; Mousa 2016). Studies have also shown that PFD treatment can attenuate bleomycin-induced fibrotic effects in rat lungs. It has also been reported that HYP was also increased in the model groups and that pirfenidone reduced the increase in HYP (Mousa 2016; Bassiouny et al. 2023). Studies on the histopathological effects of FST in lung tissue are limited. In a study with BLM-induced pulmonary fibrosis, some effects of FST were shown.

FST treatment has been shown to significantly reduce inflammatory cell infiltration, interstitial thickness, and collagen deposition. FST was also shown to have a protective effect on lung microstructure, with a reduction in inflammation and fibrosis scores (Zhang et al. 2020). In another study, fisetin was shown to have antifibrotic effects. The total number of blood cells in the peripheral circulation was shown to increase in the BLM-treated group compared with the control group. Fisetin was found to reduce this number (Banerjee et al. 2015).

Histopathologically, some areas in the BLM + PFD and BLM + FST groups were fibrotic and some areas were
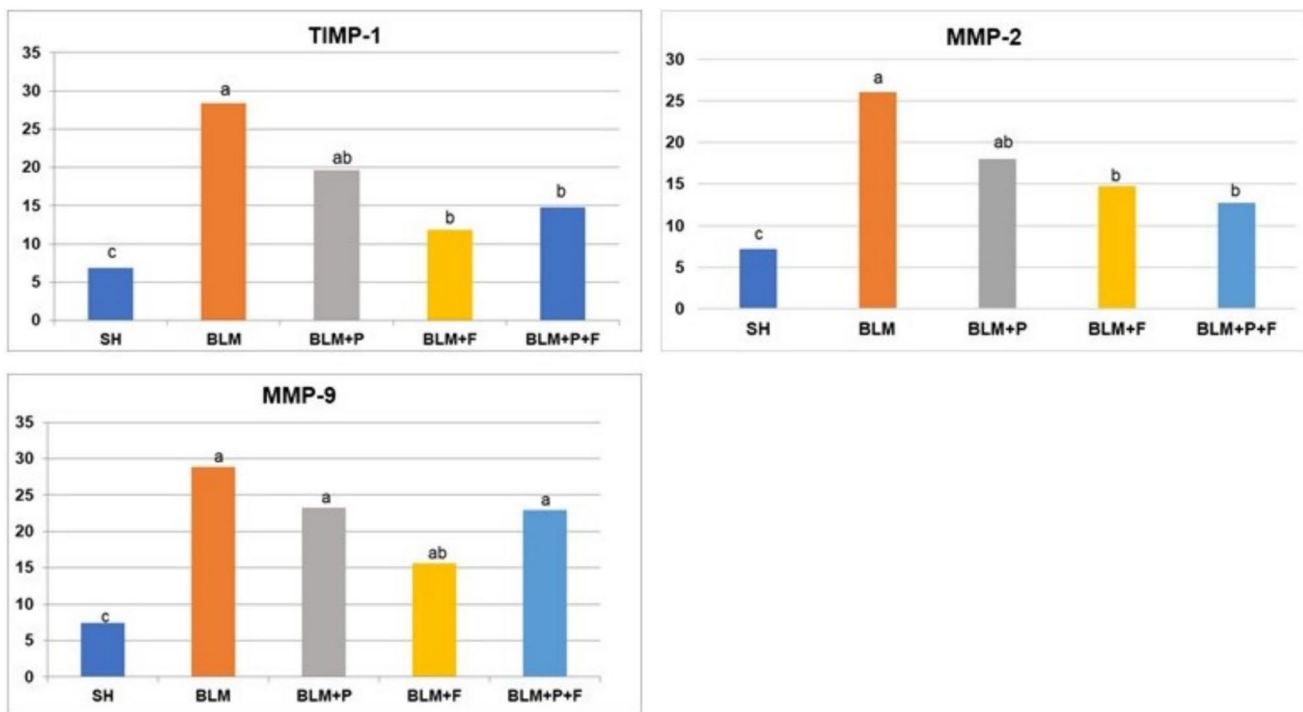
Fig. 6 Comparison of TIMP-1, MMP-2, and MMP-9 gene analysis results between groups

similar to the Sh group. Scoring results showed that the other groups were better than the BLM group. Especially in the groups where we administered PFD and FST together, less histopathological damage and a decrease in the accumulation of inflammatory cells in the interstitial area and alveolar space were observed. HYP content was higher in the BLM group and lower in the other groups (Banerjee et al. 2015). Based on the above findings, it can be concluded that fisetin and pirfenidone have protective effects on BLM-induced pulmonary fibrosis in rats. Myofibroblast differentiation is another marker induced by TGF-β1 activation of fibroblasts and determined by overexpression of α-SMA (Evans et al. 2003). In a study conducted in mice with intratracheal BLM injection, it was shown that pulmonary fibrosis was induced and the observed increase in collagen and α-SMA levels was suppressed by PFD treatment (Xiao et al. 2020). In a study using BLM and FST, it was found that FST suppressed the secretion of inflammatory cytokines, followed by decreased α-SMA expression and reduced collagen deposition in fibroblasts. However, the results supported the view that FST treatment can ameliorate the abnormal inflammatory microenvironment to maintain normal fibroblast function and reduce ECM deposition (Zhang et al. 2020).

In our study, α-SMA expression was highest in the BLM group and lowest in the Sh group. The groups in which we administered PFD and FST individually were similar. According to BLM, α-SMA expression was suppressed. However, in the group in which we administered BLM + PFD + FST together, α-SMA expression showed a decrease similar to the Sh group. These data suggest that FST and PFD can effectively attenuate the development of BLM-induced IPF. It also suggests that PFD and FST more effectively suppress the transdifferentiation of fibroblasts into myofibroblasts when given together.

The mechanism of occurrence of PF is not clear; it is presumed to be related to the activation of TGF-β (Kim et al. 2011). TGF-β1 has been shown to induce PF and overexpression of proteins that are effective in fibrosis such as α-SMA and collagen (Mou and Mou 2020). Pirfenidone inhibits both profibrotic and proinflammatory cytokines (Oku et al. 2008). Our results showed that treatment with fisetin and PFD reduced α-SMA and TGF-β1 expression. This suggests that when FST and PFD are used together, they may reduce fibrogenic proteins by inhibiting TGF-β1 expression and thus alleviate IPF.

Nuclear factor-κappa B (NF-κB) is a signalling molecule that is found in the cytoplasm of cells, but can pass into the nucleus when activated. Fisetin (FST) inhibits NF-κB activity and other proinflammatory cytokines (Gupta et al. 2014; Sahu et al. 2014; Kar et al. 2015). PFD treatment in mice with BLM-induced lung injury suppressed NF-κB expression (Kang et al. 2020). Activation of NF-κB with a gradual increase in NF-κB expression together with inflammation and fibrotic lesions of pulmonary fibrosis suggests that it is effective in the mechanism of pulmonary fibrosis (Wang et al. 2021). According to our study, co-administration of fisetin and pirfenidone to experimental animals inhibits NF-κB 65 activation. Both NF-κB p65 activity and TGF-β1 expression were inhibited in a coordinated manner. Previous research has also suggested that antioxidants may have an antifibrotic effect through the blockade of NF-κB 65 activation (El-Khouly et al. 2012).

ROS and oxidative stress are important for fibrotic responses in the lung, especially those induced by BLM. Flavonoids act as potent antioxidants, providing exceptional protection against oxidative and free radical damage (Chen et al. 2004). It was found that the combination of fisetin and pirfenidone with BLM generally resulted in a significant decrease in BLM-induced lipid peroxidation through a decrease in MDA levels and a significant increase in antioxidant status through an improvement in GSH. In rats administered BLM alone, MPO content, a marker of neutrophil accumulation, was significantly increased compared to controls. However, co-administration of fisetin and pirfenidone significantly prevented the increase in tissue MPO activity. These two compounds inhibited neutrophil migration and prevented the associated damage by preventing free radical production.

Upon analysis of GSH, MDA, and MPO values, it was observed that the values in the BLM + PFD + FST group closely resembled those of the Sh group. Therefore, the combined use of fisetin and pirfenidone in pulmonary fibrosis is advantageous in terms of enhancing antioxidant parameters and reducing leukocyte activity, thereby demonstrating the antifibrotic effects of antioxidants.

In PF, there is a change in the number of inflammatory cells in BAL fluid (Ohshimo et al. 2009). It was reported that the total cell count in BAL fluid reached the highest value on the 14th day, and neutrophils were at the highest levels between the 7th and 14th days (Hagiwara et al. 2000). BLM treatment with pirfenidone has been shown to decrease the number of macrophages in BAL on day 14 (Inomata et al. 2014). Our study also shows that PFD decreases the number of macrophages in BAL. Additionally, in a model of allergic airway inflammation, fisetin significantly reduced the total number of cells in BAL (Goh et al. 2012). The study demonstrated that FST reduced the total cell count in comparison to the BLM group. Additionally, both FST and PFD inhibited neutrophil and lymphocyte counts compared to BLM. When administered together, PFD and FST resulted in a more significant decrease in cell numbers than when administered individually. These two compounds may protect by inhibiting leukocyte accumulation in the lungs, production and release of chemical mediators, and formation of free oxygen radicals.

MMPs have been shown to regulate mechanisms involved in the process of PF formation and stimulate pulmonary fibrotic responses. They are also believed to induce ECM degradation and promote fibrocyte migration (Yao et al. 2019). However, MMP-9 is a multifunctional enzyme involved in various physiological pathways. MMP-9 expression has been reported to be regulated by NF-κB p65, because the DNA binding sites of NF-κB p65 are located on the same DNA strand as the MMP-9 gene (Nagao et al. 2000).

MMP-2 and MMP-9 gene and protein expression were found to be upregulated in IPF tissues (Selman et al. 2006). Batimastat, a synthetic MMP inhibitor, was able to significantly inhibit bleomycin-induced pulmonary fibrosis in mice by downregulating two gelatinases (MMP-2 and MMP-9) (Corbel et al. 2001). MMP inhibitors derived from natural products have been tested in studies. Grape seed extract and naringin have been shown to improve fibrotic foci in lung tissue by reducing MMP-9 and TGF-β1 (Liu et al. 2017; Chen et al. 2004). It is thought that the antifibrotic effect of PFD may be due to the reduced expression of profibrogenic procollagen, TGF-β1 mRNA, MMP-9, and MMP-2 through downregulation (Corbel et al. 2001; Di Sario et al. 2004). Imbalances in MMPs and TIMPs can lead to the development of specific disease states (Bassiouni et al. 2021). TIMPs are important regulators of MMP activity, and TIMP-1, a member of the TIMP family, acts on all active MMPs and is widely available (Mahalanobish et al. 2020). Our data show that fisetin and pirfenidone significantly inhibited TIMP-1 expression. For TIMP1 gene, according to the results of statistical analysis, although the B + F group is lower, there is no difference between them and the B + P + F group. It is similar to B + P. Both TIMP-1 and MMP-9 mRNA expressions were increased in the lungs as a result of BLM injury. PFD and FST treatments inhibited TIMP-1, MMP-2, and MMP-9 mRNA expression.

Although MMP-2 and MMP-9 are different, it is seen that there is a decrease in all groups in general. However, the difference in MMP-9 in the B + F + P group may be interpreted as an increase in ECM degradation due to a different reason. It may also be due to a different unknown effect of the mixture of treatments (BLM, PFD, and FST). However, in general, we can say that all treatments were effective on MMPs and TIMP1 when compared with the BLM group. However, the inhibitory effect of FST on MMPs is slightly better than PFD.

Fisetin reduces macrophage recruitment and infiltration by decreasing the expression of MMP-2 and MMP-9, which are critical for macrophage migration. This is the basic mechanism by which fisetin exerts its anti-inflammatory effects. By downregulating these MMPs, fisetin can reduce mononuclear cell infiltration, lower proinflammatory cytokine levels, and alleviate oxidative stress (Hada et al. 2021). This effect, combined with its effects on signalling pathways and cellular processes, makes fisetin a promising compound for fibrosis prevention and treatment strategies.

In our study, downregulation of NF-κB p65, TGF-β1, and α-SMA; downregulation of TIMP-1, MMP-2, and MMP-9 genes; downregulation of HYP, MPO, and MDA activity; and upregulation of GSH by PFD and FST show that they have antifibrotic, antioxidant, and anti-inflammatory effects. Downregulation of histopathological analysis and scoring results also show that it provides an improvement in the tissue. In our study, it was quite remarkable that pirfenidone and fisetin showed similar effects on these parameters and the effect of both was increased when they were used together. We do not have very clear information about the interactions of PFD and FST. However, the fact that both compounds have antioxidative and, anti-inflammatory effects is one of the reasons why their combined use in the treatment of PF shows beneficial effects. Our study is very important in terms of showing the combined effect of PFD, an antifibrotic drug used in the treatment of PF, and FST, a flavonoid. The use of FST together with antifibrotic products may increase its antioxidant effect. This has been shown to be potentially beneficial. Fisetin may be a potential hope for the treatment of PF, but it is important to say that more clinical research and evidence are needed.

Conclusion

In conclusion, our results show that the combined use of PFD and FST reduces fibroblast differentiation and ECM accumulation by inhibiting fibroblast proliferation; downregulates levels of gelatinases and their inhibitors, which are thought to be highly effective in epithelial-mesenchymal transition; and leads to an improvement in BLM-induced pulmonary fibrosis by significantly improving antioxidant defence parameters. PFD and FST showed almost similar effects in all parameters we evaluated in the groups in which we administered pirfenidone and fisetin individually. Increasing the doses of PFD and FST in combination may be more effective for the treatment of PF. We think that it may be useful to perform higher dose trials than the doses used in this study.

It would be beneficial to give fisetin supplements to PF patients treated with PFD and cancer patients continuing treatment with BLM along with their medication, and it can be recommended to patients.

Abbreviations BAL: Bronchoalveolar lavage fluid; BLM: Bleomycin; DMSO: Dimetilsülfoksit; FST: Fisetin; GSH: Glutathione; H-E: Haematoxylin-eosin; HYP: Hydroxyproline; MDA: Malondialdehyde; MMPs: Matrix metalloproteinases; MPO: Myeloperoxidase; MT: Masson trichrome; NF-κB: Nuclear factor kappa-B; PF: Pulmonary fibrosis; PFD: Pirfenidone; ROS: Reactive oxygen species;

TGF-β1: Transforming growth factor-β1; α-SMA: α-Smooth muscle actin; RT-PCR: Real-time polymerase chain reaction; MMP-2: Matrix metalloproteinase-2; MMP-9: Matrix metalloproteinase-9; TIMP-1: Tissue inhibitor of metalloproteinases; ECM: Extracellular matrix

Declarations

Competing interests The authors declare no competing interests.

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