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Research Article

Nintedanib reduces corticosteroid resistance pulmonary fibrosis induced by bleomycin in mice by increasing the expression of β3 and β6 integrins

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ABSTRACT

BACKGROUND: Pulmonary fibrosis is a progressive lung disorder that occurs by lung injury and scarring of the lung tissue. This injury makes the lung thickened and stiff causing difficulty to breathe. Corticosteroid resistance pulmonary fibrosis is a major health problem. Bleomycin is an anti-cancer agent, it induces pulmonary fibrosis resistance to corticosteroid therapy, this study aimed to determine the effectiveness of nintedanib (NIN) a tyrosine kinase inhibitor on corticosteroid resistance pulmonary fibrosis induced by bleomycin in mice.

METHODS: Sixty albino male adult mice were used in this study. The mice were divided into five groups (n=12) at random. control group, the BLM group received a single dose of bleomycin (BLM); 2U/kg by endotracheal instillation, the BLM+MP group received bleomycin and methylprednisolone (MP) 10mg/kg/day by gastric gavage, BLM+NIN group received BLM and NIN, 60mg/kg/day by gastric gavage and the combined treatment group received BLM, NIN, and MP. All groups were sacrificed after the last dose of treatment on day 7 (acute stage) and day 28 (chronic stage). The lung tissues were obtained for biochemical analysis, gene expression, and histopathological examination at the end of the experiment.

RESULTS: After 7 days of treatments, BLM+NIN and BLM + NIN + MP groups showed a significant ($P<0.05$) decrease in the contents of interleukin-2, interleukin-4, interferon-gamma, tumor necrosis factor-alpha, Malondialdehyde with an increase in the glutathione content in lung tissue compared to MP group. Also, they showed a significant decrease in the lung water content compared to the BLM group treated with MP. After 28 days, both NIN groups showed a significant reduction in hydroxyproline, and transforming growth factor beta (β) contents in the lung tissues compared to the MP group, and they showed a positive effect on the expression of β3 and β6 integrins compared to the negative effect of the MP group. Upon histopathology examination, the BLM+NIN group and BLM + NIN + MP groups showed significant improvement compared to the MP group by hematoxylin and eosin (H and E) and Masson's trichrome stains. Immunohistochemical staining revealed negative BCL-2 expression in the cytoplasm of bronchiolar epithelium in BLM+NIN and BLM + NIN + MP groups after 7 and 28 days of treatment. Morphometric studies of lung tissue showed significant improvement in pathological changes induced by BLM in the BLM+NIN group and BLM + NIN + MP groups.

CONCLUSION: Altogether, our data indicate that NIN overcame corticosteroid resistance pulmonary fibrosis induced by bleomycin in mice mainly by decreasing TGF-β and improving the expression of β3 and β6 integrins.

1. INTRODUCTION

Pulmonary fibrosis is a disease of the lower respiratory system (Green, F. H. 2002). It happens when the tissue of the lung becomes damaged, scar, and fibrous tissue accompanied by impairment in the respiratory capacity, severe shortness of breath, and low quality of life, with an increase in the rate of death. It is characterized by diffuse, progressive remodeling of the parenchyma of the lung with extracellular matrix deposition and scarring of tissue (Meyer, 2017).

Increased glucocorticoid resistance is reported in patients with severe asthma, COPD, COVID-19, and cystic fibrosis. The oxidative stress leads to a significant decrease in activity and expression of HDAC2 which causes resistance to the action of glucocorticoid. However, the dissociated glucocorticoids have been under investigation to decrease side effects, but it is so

difficult to dissociate anti-inflammatory effects from adverse effects. Patients with glucocorticoid resistance must use alternative anti-inflammatory treatments as well as drugs that may reverse the molecular mechanism of glucocorticoid resistance(Barnes, P. J. 2010).

Bleomycin is a drug of cancer treatment that produces poisonousness of pulmonary tissue prompting severe progressive pulmonary fibrosis (Della Latta, et al., 2015).

Mainly, TGF-β, PDGF receptor-A (PDGFR-A), and tumor necrosis factor- ά (TNF-ά) are stimulating the transformation, proliferation, and accumulation of fibroblasts, which leads to the participation of extracellular matrix. The advanced precipitation of collagen matrix causes alteration and destruction of alveolar structures and, finally, loss of function of the lung. The bleomycin-induced lung fibrosis model indicates that some fibroblasts in fibrosis may be formed from bone marrow progenitors, as well as from epithelial cells through epithelial-mesenchymal transition(Ojo, et al., 2020). Corticosteroid treatment of pulmonary fibrosis induced by bleomycin cannot reduce the IL-13-mediated myofibroblast differentiation and IL-4 is not inhibited by corticosteroids (Wilson and Wynn, 2009; Hosoya, T. 1997). Nettelbladt and co-workers found that there is no impact of methylprednisolone therapy on bleomycin-induced lung fibrosis in a rodent model. Also, prednisolone treatment was found to have few effects on bleomycin-induced lung fibrosis in rats(Nettelbladt, et al. 1990). Langenbach et al found that several mice in the methylprednisolone-treated group showed particularly severe fibrosis (Langenbach, et al., 2007).

The current work employed a bleomycin-induced mouse model, which is the most commonly used and internationally known animal model for studying pulmonary fibrosis mechanisms (Jenkins, et al., 2017).

Nintedanib is an intracellular inhibitor of tyrosine kinases that target PDGF receptors A and FGF receptors 1–3, VEGF receptors 1–3, TGF-β, c-Abelson, and Src family kinases. Nintedanib exhibits significant therapeutic effects on modulating myofibroblast differentiation and extracellular matrix (ECM) secretion in vitro and attenuating bleomycin- and silica-induced pulmonary fibrosis in animal models (Richeldi et al., 2014). It could act as a potential treatment for idiopathic pulmonary fibrosis (IPF). Also, nintedanib has a role in oncology treatment, it is a strong inhibitor of 3 proangiogenic pathway receptors (VEGFR, FGFR, and PDGFR) and was considered to act as an antiangiogenic anticancer therapy(Roth, et al., 2015; Landi, et al., 2020).

In this study assessment of the potential therapy of nintedanib either alone or combined with methylprednisolone on bleomycin-induced corticosteroid resistance pulmonary fibrosis in mice was done.

The present study aimed to determine the effectiveness of nintedanib (NIN) on corticosteroid resistance pulmonary fibrosis induced by bleomycin in mice.

2. MATERIALS AND METHODS

2.1 Animals

Sixty albino adult male mice 8 to 10 weeks (20-30g) (Kilkenny, et al. 2009). Mice were obtained from the animal house from the Faculty of Pharmacy, King Abdulaziz University, Jeddah, K.S.A. Animals were kept under a 12-hours light/dark cycle at room temperature (22±2°C) and 55±5% humidity, with provided food and water free. The animal experiments were approved by Institutional Animal Ethical Committee from the Faculty of Pharmacy, King Abdulaziz University, Jeddah, K.S.A. (PH-1442-56).

2.2 Drugs and chemicals

Bleomycin sulfate powder was purchased from Shanghai Huirui Chemical Technology Co., Ltd, nintedanib powder with a purity of 99% was purchased from Shanghai Huirui Chemical Technology Co., Ltd, and methylprednisolone powder was purchased from Pfizer, New York suspended in a 0.5% solution of carboxymethylcellulose sodium as a vehicle. Formaldehyde 100% was purchased from Zoad International Co for the medical supplier, KSA, and di-methyl-ether was purchased from Molekula Ltd. Lingfield Way, Darlington, DL1 4XX, United Kingdom.

2.3 After seven days of adaptation of mice, 60 mice were divided into five groups as follows:

Control mice group (n=12):
The mice received 0.3ml of PBS solution by endotracheal instillation then mice received daily by gastric gavage with the vehicle- the vehicle was saline and 0.5% carboxymethylcellulose from day 0(Izbicki, et al., 2002). 6 mice were killed on day 7 (acute stage) and the other 6 mice were killed on day 28 (chronic stage).

Bleomycin group (BLM; n=12):
The mice received a single dosage (2 U/kg) in 50 µl of PBS by endotracheal instillation then they received daily by gastric gavage with the vehicle- the vehicle was saline and 0.5% carboxymethylcellulose from day 0(Izbicki et al., 2002). 6 mice were killed on day 7 (acute stage) and the other 6 mice were killed on day 28 (chronic stage).

Bleomycin + methylprednisolone group (BLM+MP; n=12):
BLM was given by endotracheal instillation. Then mice were treated with 10 mg/kg methylprednisolone (prepared in fresh vehicle 0.5% (carboxymethylcellulose,0.5%) and dissolved in saline) every day before treatment and it was administered by gastric gavage 10mg/kg of methylprednisolone from day 0 (Zhao, et al., 2019) . 6 mice were killed on day 7 (acute stage) and the other 6 mice were killed on day 28 (chronic stage).

Bleomycin + nintedanib group (BLM+NIN; n=12):
It was performed as previously described doses.

Bleomycin + (methylprednisolone and nintedanib) (BLM+(MP+NIN); n=12):
BLM was endotracheal instillation then this group was treated with a combination of nintedanib and methylprednisolone at the same previous doses.

2.4 Induction of pulmonary fibrosis by bleomycin

The mice were anesthetized using diethyl ether. 0.3ml of bleomycin or sterile PBS was loaded into a sterile 200 µL pipet tip. It was performed as previously described (Liu, et al., 2017)

Outcome measures
After sacrificing the animals, the lung was rapidly removed then, the left lung for histological examination and the right lung for analysis by ELIZA, PCR, and wet/dry ratio.

Biochemical parameters measurement, at 7 days post-treatment
Measurement of interleukin-2 (IL-2), interleukin-4 (IL-4), interferon-gamma (INF-***γ***), tumor necrosis factor-alpha (TNFα), malondialdehyde (MDA), glutathione (GSH) contents were assessed by using the Mouse ELISA (Enzyme-Linked Immunosorbent Assay) kits. For the enzyme-linked immunosorbent assays mouse tissue homogenates were prepared as previously described(Tsoutsou et al., 2006), (Huang, et al., 2010) and (Mayr et al., 2016).
The Enzyme-Linked Immunosorbent (ELIZ) kits: The Mouse IL-2 ELIZA kit was purchased from My-bio-source Inc. The Mouse IL-4 ELIZA kit purchased from My-bio-source Inc. Mouse IFN-γ (Interferon Gamma) ELISA Kit purchased from My-bio-source Inc. Abcam's tumor necrosis factor-alpha Mouse ELISA (TNF-α): TNF-α Immunoassay ELISA purchased from Abcam's Co. Mouse Malondialdehyde (MDA) ELISA Kit (competitive ELIZA) purchased from My-bio-source Inc. Glutathione ELISA Kit in mice purchased from Elab-science Co.USA.

Biochemical parameters measurement, after 28 days post-treatments
Measurement of transforming growth factor beta (TGF-β) and hydroxyproline contents was assessed by using the Mouse ELISA (Enzyme-Linked Immunosorbent Assay) kits is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of Mouse in Tissue Homogenates was performed as previously described(Sisson et al., 2010) and(Saraiva, et al., 2018). The Enzyme-Linked Immunosorbent (ELIZ) kits: Lung Transforming growth factor-β1 (TGF-β1) in mice purchased from Picokine Co. Bio-vision's hydroxyproline ELISA Kit in mice purchased from bio-vision's Inc.

Tissue homogenate
The lung tissue was rinsed in ice-cold PBS (0.01M, pH=7.4) to eliminate excess blood thoroughly. The 100mg tissue was rinsed with 1x PBS, homogenized in 1 ml of 1x PBS, and stored immediately at -20°C. Next two freeze-thaw cycles were performed to break the cell membranes; the homogenates were centrifuged for 5 min at 5000×g at 2 - 8°C to get the supernatant for immediately assayed or stored at -20°C or -80°C to avoid loss of bioactivity and contamination. Centrifuge the samples again before they are assayed, and precautions were taken to avoid multiple freeze-thaw cycles.

2.5 Wet-to-dry lung weight ratio at day 7 and day 28 post-treatments:

The lung tissue was dried in an oven for 5 days at 60°C and re-weighed as dry weight. The W/D weight ratio was calculated by dividing the wet by the dry weight (Matsuyama et al., 2008).

Gene expression of β3 and β6 integrins profiling by real-time quantitative polymerase chain reaction (qPCR) at day 28 post-treatment:
Lung tissue was removed from each mouse using a dissecting scissor and forceps. The dissecting was performed by the same investigator for all animals to limit discrepancies in the sample collection. Tissue from three mice was pooled for each biological replicate (12 animals per group were used to obtain 3 biological replicates). The lung tissues were transferred to liquid nitrogen directly. Lung tissue was homogenized in a homogenizer on ice and total RNA was isolated using Thermo Scientific GeneJET RNA Purification Kit #K0731 (USA). RNA samples having a 260/280 ratio of 1.8 to 2.0 were accepted for analysis. 1 µg of total RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA). RT-qPCR was performed (AriaMx real-time PCR system) as previously described (Bi et al., 2019; Bi et al., 2016).

Lung histopathology:
The left lung specimens from each animal were fixed in a 10 % formalin solution. Fixation was followed by dehydration, clearing, and embedding in paraffin. Serial sections of 5 µm thickness were cut and then stained using the following stains and

measurements were performed by a pathologist blinded to the study groups. H and E (Hematoxylin and eosin) and trichrome blue slides were reviewed and subjectively analyzed for general signs of inflammation and fibrosis. The histological analysis and lung damage severity scores for acute after one week and chronic after 4 weeks were the same (Aubin Vega, et al., 2019) and (Polosukhin, et al., 2012). Visualization and photographing of slides were done using an Olympus light BX61 microscope.

2.6 Immunohistochemical analysis for BCL-2 at day 7 and day 28 post-treatment:

The left lung specimens from each animal after one week and after 4 weeks of bleomycin and treatment administration were fixed in 10 % formalin solution. Fixation was followed by dehydration, clearing, and embedding in preparation for 5um paraffin section slides. The immunohistochemical technique for detection of Bcl-2 expression was performed using the labeled streptavidin-biotin technique (Zymed) Cat No. 18-0193 with the monoclonal antibody (Bcl-2). The localization of the Bcl-2 protein was demonstrated as a yellowish-brown color area(Ahmed and Anwar, 2004).

2.7 Morphometric measurement after 7 days, the mean thickness of the interalveolar septa in (µm) and the mean alveolar space surface area (µm 2) after 7 days As the same mentioned in morphometric analysis (Zakaria, et al., 2020) Blind histological analysis was performed on the lung paraffin sections from mice after 7 days for estimation of parenchymal distortion and airway (peri-bronchial) inflammation using specific semi-quantitative scores: Analysis of Lung parenchymal distortion: It was assessed by analysis of ten sequential non-overlapping tissue fields using x200 magnification. Each tissue field was scored using a 0-to-4-point system. Mean scores for all fields were calculated for each mouse (Polosukhin et al., 2012). Analysis of airway inflammation: It was estimated by individual assessment of each airway in the tissue section using a 0-to-3-point system. Mean scores for all analyzed airways were calculated for each animal (Polosukhin et al., 2012).

2.8 Measurement of area percentage of PCL-2 immunostaining after 7 days.

The intensity of immunohistochemical staining was graded semi-quantitatively as follows: grade 0 = no staining present or less than 10% of the cells are positive; grade 1 = 10% of the cells are positive; grade 2 = more than 10% and less than 50% of the cells are positive and grade 3 = more than 50% of the cells are positive (Safaeian et al., 2008).

2.9 Morphometric analysis after 28 days: measurement of the area percent of collagen fibers in Masson trichrome stain as same mentioned (Zakaria et al., 2020). They were measured by using the NIH Image J (v1.50) program and measurement of subepithelial connective tissue volume density (VVsub) in Masson trichrome stain after 28 days. Airway wall remodeling was evaluated by measurement of VV sub as the difference in the area, delimited by the basement membrane and the outer edge of the airway adventitia, divided by the length of the subepithelial basement membrane (Polosukhin et al., 2012). They were measured from x 40 photomicrographs using Digimizer 4.3.2. image analysis software (MedCalc Software BVBA, Belgium).

Statistical analysis Statistical comparisons among experimental groups were performed by one way of variance (ANOVA) followed by Tukey's multiple. Student's t-test for paired comparisons was performed. The log2-transformed data was used for the RT-qPCR statistical analysis (Rieu and Powers 2009). The values were presented as mean ± standard error of the mean (SEM). The proportion of the lung tissues present was analyzed by Fisher's exact test. $P \leq 0.05$ was considered statistically significant. All analyses used SPSS 25.0 statistical software (IBM Corp., Armonk/ N.Y., USA). Significant differences between experimental groups were determined at P. value ≤ 0.05. The graphs were made by using the prism version.9 Correlations between measured parameters were made using Pearson correlations. P. values ≤ 0.05 were considered significant.

3. RESULTS

3.1 The effect of nintedanib (NIN) and methylprednisolone (MP) either alone or in combination on inflammatory markers and cytokines contents in lung tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin after 7-days post-treatment:

Figure 1 showed a significant reduction in the contents of IL-2, IL-4, INF-gamma, and TNF-α in the bleomycin group treated with NIN either alone or combined with MP compared to the bleomycin group treated with MP ($P < 0.05$). No significant

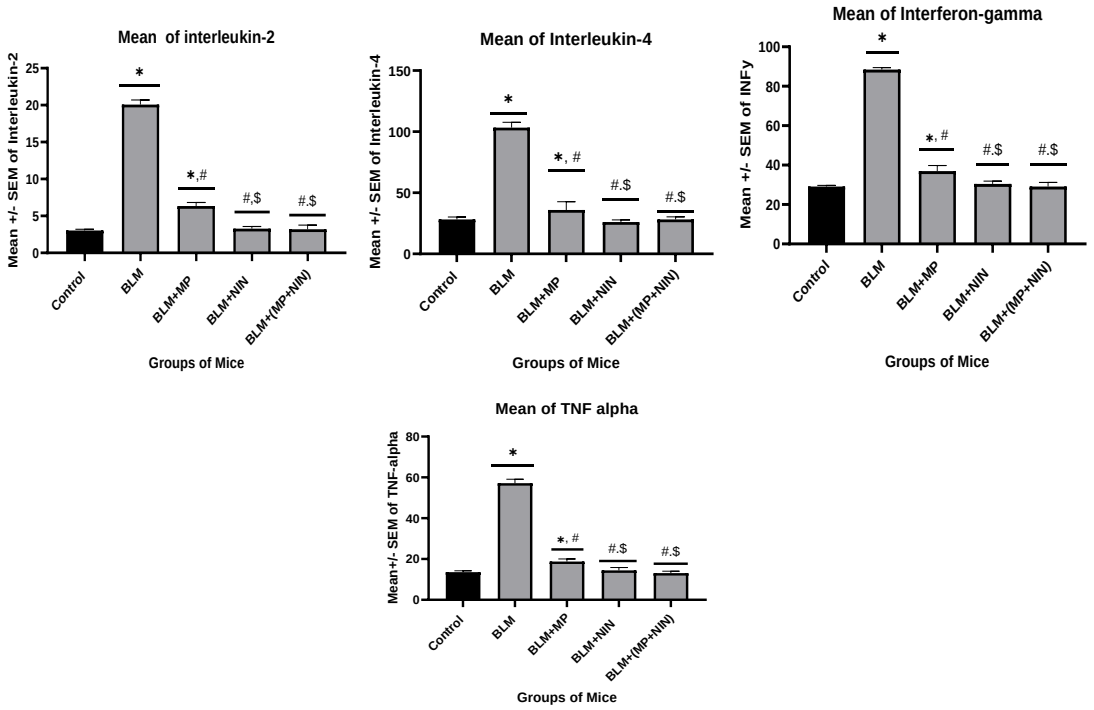

Figure1: showed the effect of nintedanib and methylprednisolone either alone or in combination on inflammatory markers and cytokines contents in lung tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin 7-days post treatments.

differences were detected between the bleomycin-treated group with NIN either alone or combined with MP.

3.2 The effect of nintedanib (NIN) and methylprednisolone (MP) either alone or in combination on oxidative stress contents in lung tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin after 7-days post-treatments:

Figure 2 showed a significant reduction in the content of MDA in the bleomycin group treated with NIN alone or combined with MP compared to the bleomycin group treated with MP alone and it showed a significant increase in the content of GSH in the bleomycin group treated with NIN alone or in combined with MP compared to bleomycin group treated with MP alone ($P < 0.05$). No significant differences were detected between the bleomycin-treated group with NIN either alone or combined with MP.

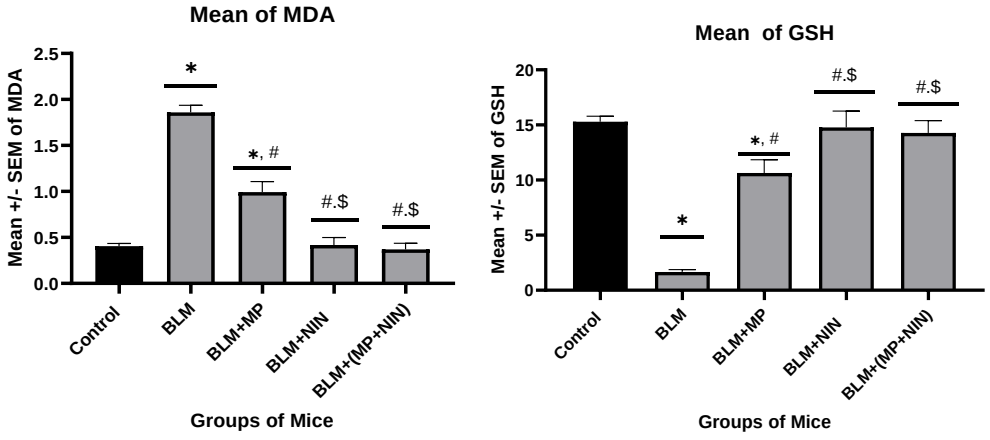

Figure 2: showed the effect of nintedanib either alone or in combination with methylprednisolone on level of MDA and GSH in lung tissue on corticosteroid resistance pulmonary fibrosis resistance to corticosteroid.

3.3 The effect of nintedanib (NIN) and methylprednisolone (MP) either alone or in combination on transforming growth factor beta and hydroxyproline contents in lung tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin after 28-days post-treatments:

Figure 3 showed a significant reduction in the contents of TFG-β and hydroxyproline in the bleomycin group treated with NIN alone or combined with MP compared to the bleomycin group treated with MP alone ($P < 0.05$). No significant differences were detected between the bleomycin-treated group with NIN either alone or combined with MP.

3.4 The effect of nintedanib and methylprednisolone either alone or in combination on the lung water content in lung tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin after seven (7) and 28 days post treatments:

Figure 4 showed a significant decrease in the content of water in the bleomycin group treated with MP, the bleomycin group treated with NIN, and the bleomycin group treated with NIN+ MP compared to the bleomycin group at p. value <0.05. No significant difference between the bleomycin group treated with MP and the bleomycin group treated with NIN and the bleomycin group treated with NIN+ MP after 7 days post-treatment. Figure 4 showed a significant reduction in the contents of the water in the bleomycin group treated with NIN alone or combined with MP compared to the bleomycin group treated with MP alone. No significant difference between the bleomycin group treated with MP and the bleomycin group. No significant difference between the bleomycin group treated with MP and the bleomycin group treated with NIN and the bleomycin group treated with NIN+ MP after 28 days post-treatment.

3.5 The effect of nintedanib (NIN) and methylprednisolone (MP) either alone or in combination on gene expression of β3 and β6 integrins compared to the control group and bleomycin group in lung tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin 28-days post-treatments:

Figure 5 showed the negative effect of the bleomycin group on wound healing was associated with a significant decrease in the expression of β3 and β6 integrins compared to the control group at p. value <0.00. Also, it showed the bleomycin group treated with MP, the bleomycin group treated with nintedanib, and the bleomycin treated with (MP+NIN) downregulation of the expression of β3 and β6 integrins compared to the control group, in addition, there was downregulation in β3 integrin gene in the bleomycin group treated with MP compared to bleomycin group but upregulation in β3 and β6 integrins gene in the bleomycin group treated with NIN and the bleomycin treated with (MP+NIN) compared to bleomycin group.

3.6 Histological Results after 7 days post treatments:

Hematoxylin and eosin (H and E) stained sections. (BLM group): revealed marked distorted bronchiole with obliteration of the lumen. Moreover, most of the bronchial passages were seen surrounded by marked aggregation of mononuclear cells, and marked inflammatory cellular infiltration in the adventitia of bronchioles was observed. Also, cellular aggregates were detected in marked thickened inter-alveolar septa. Notice marked obliteration of most alveoli with some alveoli appearing collapsed and widening of other alveoli seen. Congested dilated blood vessels with thickened walls were noticed in all sections (Figs6. 1B and 2B).

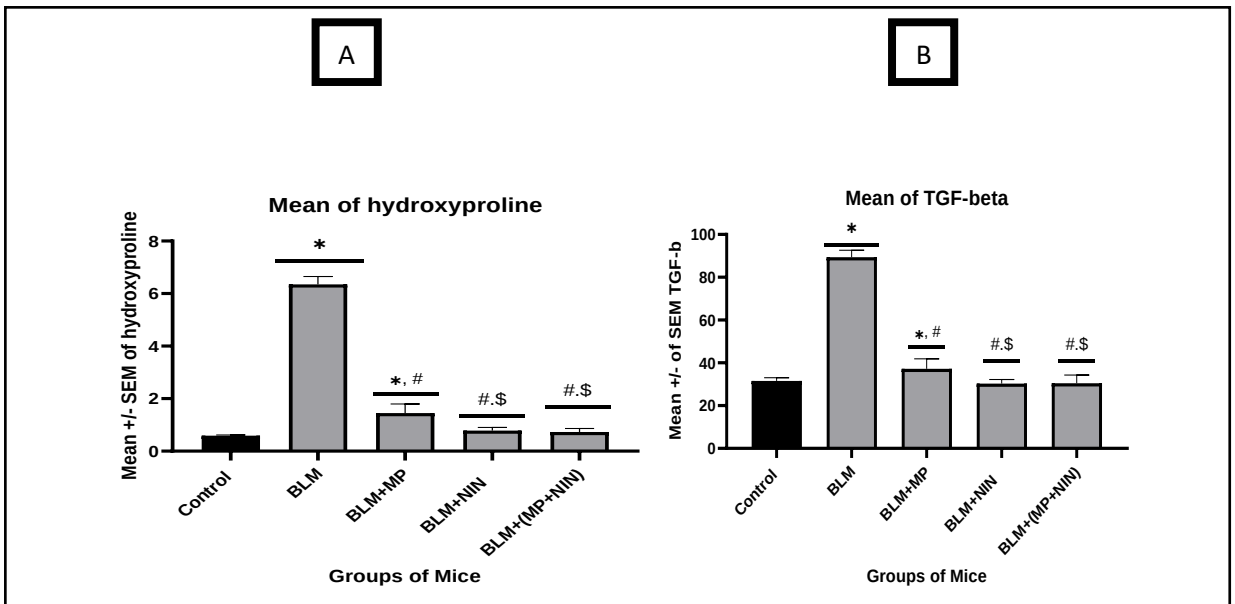

Figure 3: showed the effect of nintedanib either alone or in combination with methylprednisolone on level of TGF-β and hydroxyproline in lung tissue on corticosteroid resistance pulmonary fibrosis resistance to corticosteroid.

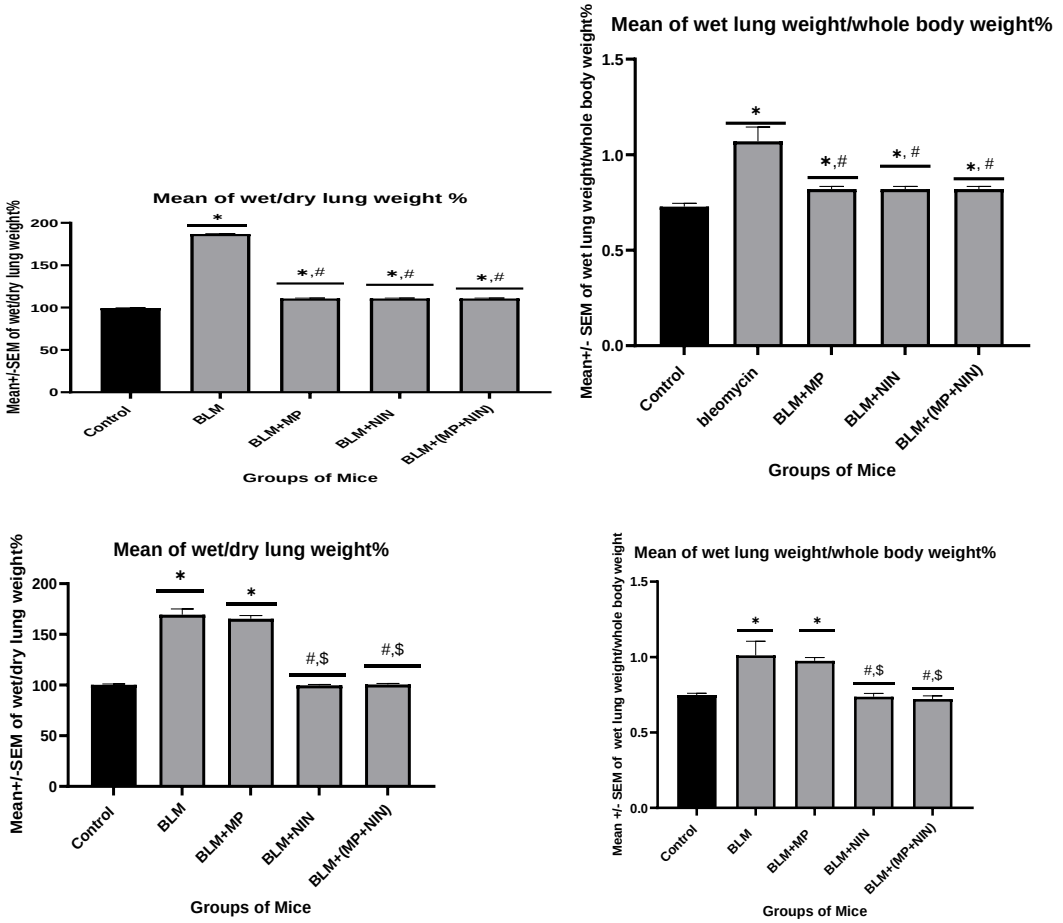

Figure 4 showed the effect of nintedanib and methylprednisolone either alone or in combination on the lung water content in lung tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin after seven (7) and 28 days post treatments.

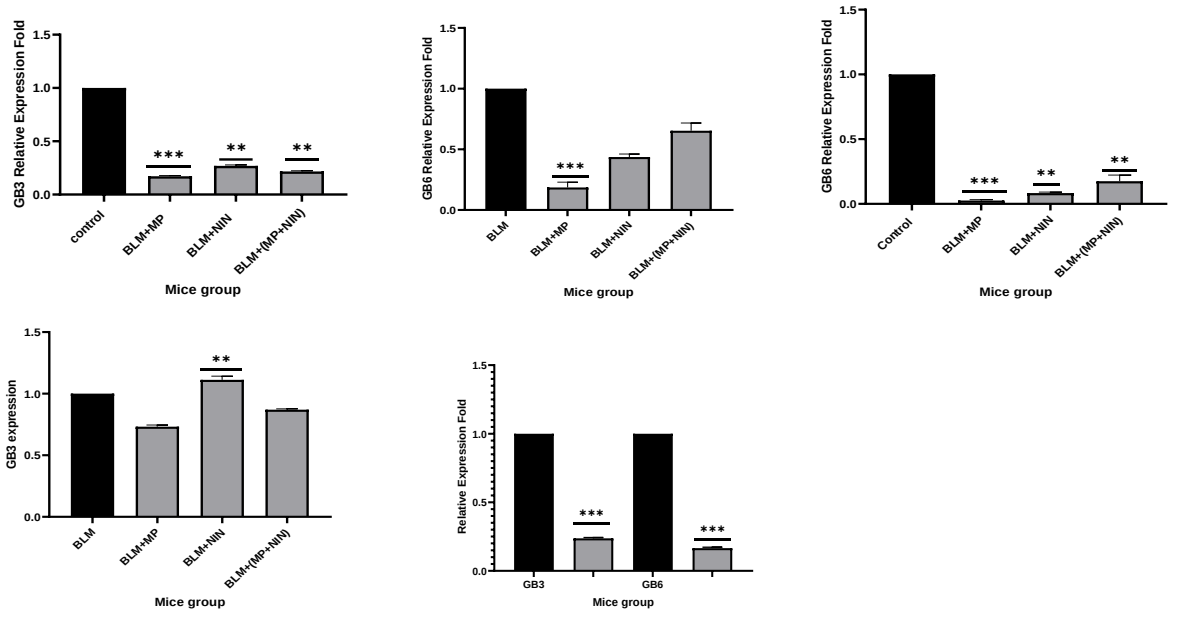

Figure 5: showed the effect of nintedanib (NIN) and methylprednisolone (MP) either alone or in combination on gene expression of β3 and β 6 integrin compared to control group and bleomycin group in lung tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin 28 days post treatments.

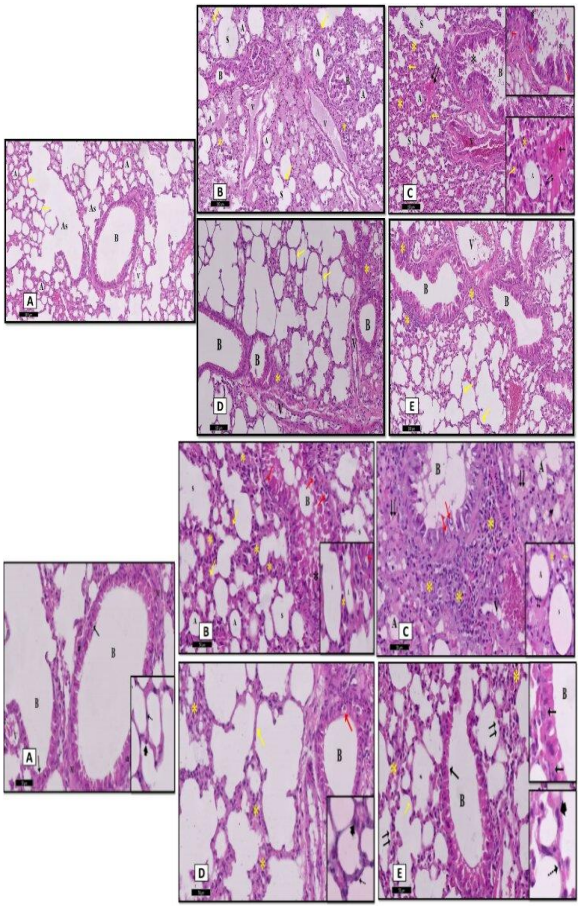

Figure 6:
(1) A photomicrograph of the histology (H and E x 10; scale bar 100 µm)
(2) photomicrograph of the histology (H and E x 20; scale bar 50 µm)

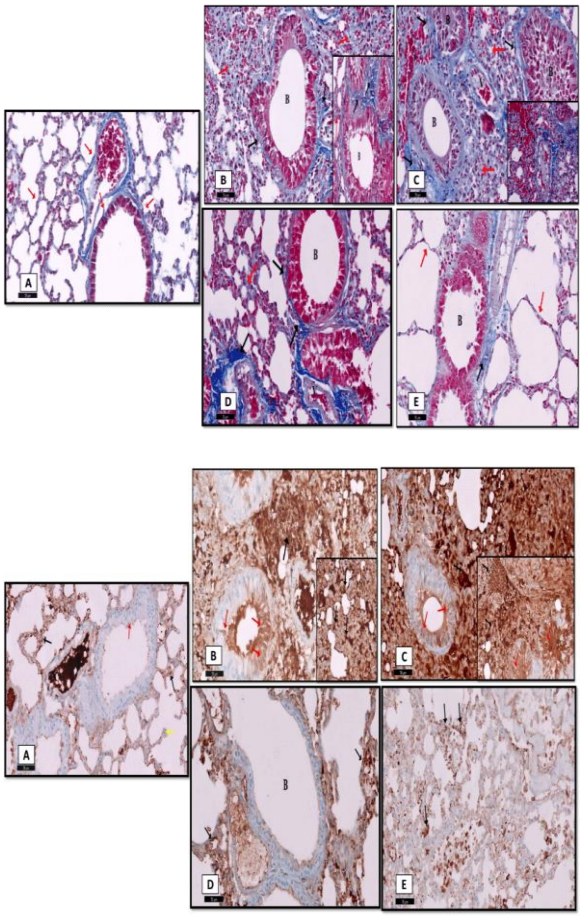

Figure 7: (1) Masson trichrome stained x 20; scale bar 50 µm section of a mouse lung after 28 days showing.

(2) Immunohistochemical staining for BCL-2 x10; scale bar 50 µm of a section in the lung of a mouse lung after 28 days showing

(BLM+MP): as BLM group with massive cellular aggregates detected in marked thickened inter-alveolar septa. Eosinophilic exudates and extravasations of red blood cells in the inter-alveolar septa were frequently seen (Figs 6. 1C and 2C). **(BLM+NIN)** showed moderate improvement in lung architecture, but not fully complete histological recovery as compared to the control group. Notice most of the alveoli are apparent nearly as a control group (Figs6. 1D and 2D). **(BLM+MP+NIN)** exerted an ameliorating effect on the lung structure. Few focal areas of inflammatory cellular infiltration were noticed surrounding the bronchi. Moreover, the lining epithelium of the bronchi was nearly comparable to that of the control group. Notice most of the alveoli are apparent nearly as a control group with thin inter-alveolar septa (Figs6. 1E and 6.2E).

Masson's trichrome stain:

Examination of mice lung sections of **(the control group)** revealed few collagen fibers in the perivascular areas. Scanty collagen fibers in the interalveolar septa and around bronchiolar passages were evident as well (Fig9. 1A). While **(the BLM group) and (BLM+MP)** exhibited an apparent increase of collagen fibers deposition in the lung interstitium, in areas surrounding the bronchioles (denoting lung fibrosis), and surroundings the alveoli as compared to that in the control group (Figs9. 1B and 1C). Meanwhile, sections of **(BLM+NIN)** showed an apparent decrease of collagen fibers in the interalveolar septa as well as around the bronchi (Fig9. 1D). While in **(BLM+(MP+NIN))** some collagen fibers were seen in the interalveolar septa as well as around the wall of bronchi (Fig9. 1E). Nearly both BLM+NIN and BLM+MP+NIN groups appeared as control group after 28 days.

Immunohistochemical analysis for BCL-2

In the immunohistochemical analysis for BCL-2, sections from the **(control group)** showed very few cells with positive immune reactions in the cytoplasm of the alveolar epithelium lining of the alveoli and bronchiolar epithelium. Notice a few strong positive reactions in the interstitial tissue in the lumen of some alveoli (Fig.9.1A). However, in **(the BLM group) and (BLM+MP)** most

of the cells with strong positive immune reactions were seen in the inter-alveolar septa and the interstitium of the lung. Marked positive immune reactions were seen in the epithelium lining the bronchiole lumen (Figs9. 1B and 1C). Meanwhile, in **(BLM+NIN)**, few cells with strong positive BCL-2 immune reaction were seen in the inter-alveolar septa (Fig9. 1D). Also, sections of **(BLM+MP+NIN)** showed few cells with a strong positive immune reaction for BCL-2, in interalveolar septa (Fig9. 1E).

3.7 The effect of nintedanib and methylprednisolone either alone or in combination on the mean thickness of the interalveolar septa in (µm) in lug tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin after day 7 post-treatments:

There was a non-significant (P=0.95) difference between treated mice with nintedanib alone and the group of mice treated with nintedanib and methylprednisolone as shown in Table 1.

The effect of nintedanib and methylprednisolone either alone or in combination on the mean alveolar space surface area (µm^2) in lug tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin after day 7 post-treatments:

There was a significant increase (P<0.01) in the mean alveolar space surface area in the group of mice treated with nintedanib and methylprednisolone compared to the group of bleomycin treated with nintedanib alone as shown in Table 1.

The effect of nintedanib and methylprednisolone either alone or in combination on the scoring of lung parenchymal degeneration and the scoring of airway inflammation in lug tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin after day 7 post-treatments:

No significant difference between treated mice with nintedanib alone and the group of mice treated with nintedanib and methylprednisolone.

The effect of nintedanib and methylprednisolone either alone or in combination on the scoring of airway inflammation in lug tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin after day 7 post-treatments:

No significant (P=0.17) difference between treated mice with nintedanib alone and the group of mice treated with nintedanib and methylprednisolone.

Table 1: Showed the effect of nintedanib and methylprednisolone either alone or in combination on the mean thickness of the interalveolar septa in (µm), the mean alveolar space surface area (µm^2), the scoring of lung parenchymal degeneration and the scoring of airway inflammation in lug tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin after day 7:

Mice groups N=6	The mean thickness of the interalveolar septa in (µm) Mean ± SEM	The mean alveolar space surface area (µm^2) Mean ± SEM	Scoring of Lung parenchymal degeneration Mean± SEM	Scoring of Airway inflam-mation Mean ± SEM
Control group	3.36±0.24	782.83±17.29	000	000
BLM group	30.87±1.38 *	343.66±9.43 *	2.50±0.22	2.16± 0.31
BLM+MP group	37.77±1.29 *, #	226.22±17.98 *, #	3.66±0.22	3.00± 0.00 #
BLM+NIN group	3.62±0.18 #, $	533.61±19.25 #, $	1.50±0.33#, $	1.33± 0.21#, $
BLM+ (MP+NIN) group	3.68±0.14 #, $	601.94± 26.39 #, $, @	1.00±0.36 #, $	1.00± 0.26 #, $

Data Values were mean ± standard error of mean (SEM); N = number of animals. BLM; bleomycin, MP; methylprednisolone, NIN; nintedanib. (%) = mean percentage change compared to control group. ANOVA one-way, accompanied by a multiple comparison test by Tukey. * Control group compared to other groups. # BLM group compared to treatments group. $ BLM+MP group compared to BLM+NIN and BLM+(MP+NINI). @ BLM+NIN compared to BLM+ (MP + NIN) group.

3.8 The effect of nintedanib and methylprednisolone either alone or in combination on the mean area percentage of positive BCL2 cells and the grading system of the intensity of immunohistochemical staining in lung tissues in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin after day 7 post-treatments:

The expression of BCL-2 was significantly upregulated in bronchiolar cells, alveolar epithelial cells, interstitial myofibroblasts, and inflammatory cells after bleomycin instillation as compared to the bleomycin-treated mice with nintedanib alone and bleomycin-treated mice with methylprednisolone and nintedanib. Interestingly, the expression of BCL-2 was significantly (P<0.0001) upregulated in inflammatory cells in bleomycin-treated mice with nintedanib alone as compared to bleomycin-treated mice with methylprednisolone and nintedanib. No significant (P>0.05) difference in the expression of BCL-2 in the bronchiolar epithelium, alveolar epithelial cells, and interstitial myofibroblasts in bleomycin-treated mice with nintedanib alone as compared to

bleomycin-treated mice with methylprednisolone and nintedanib as shown in ***Table 2.***

The mean area percentage of positive BCL2 cells (Mean ± SEM)					
Groups (N=6)		Bronchiolar cells	Alveolar epithelial cells	Interstitial myofibroblasts	Inflamma-tory cells
Control group	F	2.86± 0.23	3.28±0.06	1.51± 0.18	1.79±0.24
	G	0	0	0	0
BLM group	F	68.14 ±2.63 *	27.13±0.31 *	81.31 ±2.06 *	77.69±2.08 *
	G	3	2	3	3
BLM+MP group	F	73.00 ±1.60 *, #	27.09±0.22 *	80.67 ±1.24 *	77.45±0.82*
	G	3	2	3	3
BLM+NIN group	F	2.70± 0.25 #, $	10.02±0.01#, $	12.73 ±0.22 #, $	18.80±1.47#, $
	G	0	1	2	2
BLM+(MP +NIN) group	F	1.71± 0.21 #, $	10.33±0.18 #, $	11.86± 0.17#, $	12.64±0.54 #, $, @
	G	0	1	2	2

F: Frequency expressed as percentage of immunoreactive cells; G: Staining grade expressed as: Grade 0: No staining or less than 10% positive cells; Grade 1: 10% positive cells, Grade 2: 10% - 50% positive cells, Grade 3: More than 50% positive cells. Data Values were mean ± standard error of mean (SEM); N = number of animals. BLM; bleomycin, MP; methylprednisolone, NIN; nintedanib. (%) = mean percentage change compared to control group.

3.9 The effect of nintedanib and methylprednisolone either alone or in combination on the area percentage of collagen fibers in lung tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin after day 28 post-treatments:

The bleomycin group and bleomycin group treated with methylprednisolone showed a significant increase in the mean area percentage of collagen fibers as compared to the treated group with nintedanib alone and to the group of mice treated with nintedanib and methylprednisolone. Interestingly, there was a significant increase between treated mice with nintedanib alone as compared to the group of mice treated with nintedanib and methylprednisolone as shown in Table 3.

Table 3: showed the effect of nintedanib and methylprednisolone either alone or in combination on the area percentage of collagen fibers and the mean of the subepithelial connective tissue volume density (VVsub) (mm) in lung tissue in a mice model of corticosteroid resistance pulmonary fibrosis induced by bleomycin:

Groups (N=6)	The area percentage of collagen fibers Mean ± SEM	The mean of the subepithelial connective tissue volume density (VVsub) (mm) Mean± SEM
Control group	4.47±0.12	0.01± 0.001
BLM group	31.82± 0.88*	0.09± 0.002*
BLM+MP group	35.56± 0.34 *, #	0.0920± 0.002*
BLM+NIN group	7.99± 0.27 #, $	0.04±0.002 #, $
BLM+(MP+NIN) group	5.18± 0.04 #, $, @	0.02±0.002#, $, @

Data Values were mean ± standard error of mean (SEM); N = number of animals. BLM; bleomycin, MP; methylprednisolone, NIN; nintedanib. (%) = mean percentage change compared to control group. ANOVA one-way, accompanied by a multiple comparison test by Tukey. * Control group compared to other groups. # BLM group compared to treatments group. $ BLM+MP group compared to BLM+NIN and BLM+(MP+NINI). **@** BLM+NIN compared to BLM+ (MP + NIN) group.

4. DISCUSSION

In the present study, we found that significant reduction in the contents of cytokines in lung tissue in nintedanib either alone or in combination with methylprednisolone compared to the bleomycin group treated with MP alone on day 7 post-treatment.

However, Kim et al., 2018, found that the contents of cytokines in the lung tissue were studied with the reverse-transcriptase polymerase chain reaction. Contents of promotor cytokines, such as IL-2, IL-4, INF-y, and TNFα were significantly higher in lung tissue in the bleomycin group. The expression of these cytokines in the glucocorticoid group was low, especially the peak value, but the expression of IL-4 was high in the bleomycin group and was not reduced in the glucocorticoid group(Kim, et al., 2018). But, they found a significantly decreased in inflammatory cytokines after nintedanib treatment because it acts as an anti-inflammatory effect as in the study(Kim, et al. 2018; Ubieta, et al., 2021, found that nintedanib inhibited the release of cytokines such as IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12p70 and IL-13, which may cause a clinical benefit in pulmonary fibrosis in interstitial lung diseases. Also, IL-2 has able to bind to lung fibroblasts, and the proliferation of fibroblasts is induced by IL-2. IL-4 acts

directly on the lung fibroblast to induce a fibrogenic response. However, IFN-γ, which is also reduced by nintedanib, exhibits potent antifibrotic activity by inhibiting the synthesis of collagen in fibroblasts (Ubieta, et al., 2021).

In the current study, there was a significant reduction in the content of MDA in lung tissue in the BLM+MP group compared to the bleomycin group in addition there was a more significant reduction in the content of MDA in lung tissue in groups of BLM+NIN and BLM+MP+NIN compared to bleomycin group and BLM+MP group.

Also, there was a significant increase in the content of GSH in lung tissue in the BLM+MP group compared to the bleomycin group in addition there was a significant increase in the content of GSH in lung tissue in BLM+NIN and BLM+MP+NIN groups compared to bleomycin group. There was a significant increase in the content of GSH in the BLM+NIN and BLM+MP+NIN groups compared to the bleomycin group and BLM+MP group. The present study agrees with them they found that the production of MDA in lung tissues was increased and GSH production in lung tissues was decreased after bleomycin treatment (Liu, et al., 2017) In the present study, there was a significant decrease in the content of TGF-β in the BLM+MP group compared to the bleomycin group, in addition, there was a more significant reduction in the content of TGF-β in lung tissue in BLM+NIN and BLM+MP+NIN groups compared to bleomycin group and BLM and MP group alone after 28 days post-treatment. There was a significant decrease in the content of hydroxyproline in the BLM+MP group compared to the bleomycin group, in addition, there was a more significant reduction in the content of hydroxyproline in lung tissue in BLM+NIN and BLM+MP+NIN groups by 88% compared to bleomycin group and BLM+MP group after 28 days post-treatment. Khalil et al.,1993, found that the corticosteroid therapy administered in the advanced stages of the disease would likely not suppress the TGF-b production by alveolar macrophages. The relative resistance to corticosteroid therapy in pulmonary inflammatory and fibrotic responses seen in many human lung diseases may be caused by the corticosteroid insensitivity of TGF-b production by alveolar macrophages(Khalil, et al., 1993) .

The water content in lung tissue after 28 days post-treatment, was a significant reduction in water content in the bleomycin group with nintedanib either alone or in combination with methylprednisolone compared to the bleomycin group, and bleomycin treated with MP alone in addition there was no significant difference between bleomycin group and bleomycin group treated with MP. Our findings agree with what they found that dexamethasone treatment did not prevent bleomycin-induced edema (Aubin Vega et al., 2019).

Similarly, to dexamethasone, methylprednisolone did not elicit any beneficial effect on lung edema in bleomycin mice. Also, our findings agree with a previous study found that the effect of nintedanib in a rat model of lung fibrosis induced by bleomycin and they showed a significant decrease in water content compared to the bleomycin group(Pittelli et al., 2017).

Our study's histological findings supported earlier research showing BLM causes an acute inflammatory response in the early stages (7–10 days), then the inflammation subsides, and fibrotic alterations develop and remain in the late stages (3-4 weeks)(Aono et al., 2005; Chaudhary, et al. 2006; Moeller, et al., 2008; Mouratis and Aidinis, 2011). Similarly, Galuppo et al.2011, reported that 7 days after bleomycin administration, the pulmonary lesions observed in mice consisted of multifocal areas of severe inflammation and intense fibrosis. In these areas, an intense thickening of alveolar septa with evident infiltration of inflammatory cells, and some eosinophils was observed. Masson's trichrome staining confirmed the presence of intense fibrosis in the inflammatory focal areas when compared with sham-operated animals(Galuppo et al., 2011).

The initial objective of our research was to look at the effects of daily methylprednisolone therapy in a mouse model of pulmonary fibrosis. Our findings showed that methylprednisolone was unable to enhance the histological outcomes of the bleomycin model of lung fibrosis after 7 and 28 days in the current research. This was in line with prior research by (Aubin Vega et al., 2019; Bahtouee et al., 2018; Dik, W.A. 2003). MP therapy increased the expansion of alveolar airspaces and decreases lung compliance, according to(Langenbach, et al., 2007). Also, Aubin Aubin Vega, et al. (2019), investigated the effect of corticosteroids on bleomycin outcomes during the acute exudative phase after 3 and 7 days. They found that exposure to bleomycin and dexamethasone exhibited severe injury scores and alveolar damage on days 3 and day 7. They stated that dexamethasone failed to reduce inflammatory cell infiltration and alveolar epithelial injury induced by bleomycin. Dexamethasone affected the expression of β3- and β6-integrins, key proteins of alveolar repair(Aubin Vega, et al., 2019). Previous research has demonstrated that long-term corticosteroid treatment, either before or at the same time as bleomycin treatment, reduced the development of lung fibrosis in rats(Cross, et al., 1985; Phan, et al., 1981; Shaker and Sourour, 2011). Moreover, in the present study, methylprednisolone does not reverse the bleomycin-repair impairment, but it further worsened the bronchiolar epithelial condition. The epithelial lining of the bronchiolar passages showed marked disorganization and degeneration in which their epithelial lining exhibited vacuolation with eukaryotic nuclei. Their lumen appeared obliterated by detached epithelial cells. Dexamethasone has been found to have a negative influence on repair processes in bronchiolar epithelial cells, which is consistent with our findings (Kadmiel, 2016; J. Liu et al., 2013). According to these findings, bleomycin-induced pulmonary fibrosis is resistant to suppression by concurrent MP therapy. In the present study, there was a significant reduction in the β3 integrin gene in lung tissue in the bleomycin group compared to the control group, with the negative effect of methylprednisolone alone compared to a control group, in addition to the effect of nintedanib alone and combination with methylprednisolone was upregulation of β3 integrin gene in lung tissue compared to bleomycin group. B6 integrin gene, was a significant decrease in β6 integrin gene in lung tissue in the bleomycin group compared to the control group, with negative effect of

methylprednisolone alone compared to control group, in addition the effect of nintedanib alone was increased in β6 integrin gene in lung tissue compared to bleomycin group and the effect of NIN+ MP was better increased in β6 integrin gene in lung tissue compared to bleomycin group. We discovered that nintedanib reduced BLM-induced alveolar inflammation and pulmonary fibrosis. In comparison to the BLM group, the mice in the nintedanib-treated and nintedanib and MP groups had reduced inflammatory cell infiltration and collagen deposition. This came in accordance with previous study(Chen et al., 2020; Rangarajan, et al., 2016; Redente et al., 2018; Wollin, et al., 2013)

Chen et al.2020, looked into the function of nintedanib in bleomycin-induced pulmonary fibrosis, comparing lung sections from pulmonary fibrosis mice who received nintedanib by gastric gavage with those from mice with pulmonary fibrosis who did not. They discovered lung inflammation and fibrosis seven days after bleomycin-induced pulmonary fibrosis, which was substantially decreased by nintedanib therapy. They also discovered that animals given nintedanib had considerably lower lung damage scores, as well as reduced pulmonary fibrosis as measured by Masson's trichrome staining and the Ashcroft score seven days following bleomycin administration. They discovered that nintedanib not only had antifibrotic properties but also anti-inflammatory properties(Chen et al., 2020).

Moreover, Wollin *et al.* 2013, investigated nintedanib at 30 mg/kg or 60 mg/kg as a preventive treatment (from day 0 to day 14) and as a treatment (from day 7 to 21) in a mouse model of lung inflammation and fibrosis by a single intratracheal administration of bleomycin. They found that therapeutic therapy at 60 mg/kg had similar inhibitory effects as a preventative treatment, while the effect magnitude was lower at 30 mg/kg. They found that nintedanib successfully decreased pulmonary inflammation and fibrosis in mice whether given as a preventative or therapeutic therapy. Nintedanib's anti-inflammatory and anti-fibrotic properties may influence the progression of fibrotic lung disorders such as IPF(Wollin et al., 2013).

Apoptosis is a form of cell death that plays a critical function in the maintenance of cellular homeostasis. The Bcl-2 family of proteins is one of the most essential regulators of the apoptosis process (Kluck, R. 2010). Bcl-2 protein is an intracellular membrane-associated protein that inhibits cell death when overexpressed (Dewson, 2011; Nemec and Khaled, 2008; Youle and Strasser, 2008). Bcl-2 family members appear to play a critical role in the pathogenesis of inflammation, apoptosis, and fibrosis produced by different causes of interstitial lung disorders, according to an increasing body of data (Safaeian, et al., 2014)

In the current study, in the BLM group and BLM group treated with MP, there was upregulation of positive immune reaction in the inter-alveolar septa and the interstitium of the lung. While few positive immune reactions were seen in the epithelium lining the bronchiole lumen.

High expression of the anti-apoptotic gene Bcl-2 and low expression of the pro-apoptotic gene Bax prevented apoptosis in lung fibroblasts following bleomycin instillation, which is a major characteristic of chronic fibrotic illnesses, according to our findings, which are similar with previous research(Aguilar et al., 2009; Hu and Zhu, 2020; Safaeian et al., 2009; Zhou et al., 2013).

Furthermore, the current findings were consistent with those of Safaeian et al., (2009), who discovered BCL-2 immunoreactivity in a variety of cells, including bronchiolar epithelial cells and lymphocytes, macrophages, neutrophils, alveolar epithelial cells, and myofibroblasts, and found that their interactions are linked to the development of interstitial fibrosis after bleomycin instillation(Safaeian et al., 2008). Predescu et al., on the other hand, detected no alterations in Bcl-2 and Bcl-xL during Fas-mediated apoptosis in primary lung fibroblasts(Predescu et al., 2017).

The extracellular matrix-producing myofibroblasts that collect in fibrotic lung lesions gain resistance to apoptosis in pulmonary fibrosis (Potter-Perigo et al., 2010; Thannickal & Horowitz, 2006). Nintedanib has been found to inhibit fibroblast proliferation, migration, and transformation, although its effects on apoptosis have yet to be investigated(Wollin et al., 2015).

In the present study, the treatment with nintedanib and the combined treatment of methylprednisolone and nintedanib were more effective in a model of pulmonary fibrosis. There was an apparent decrease of the positive immune reaction for BCL-2 in inter alveolar septa with negative BCL-2 of bronchiolar epithelium. The present results were similar to the findings of studies by (Milara et al., 2018).

Milara et al. (2018) have discovered that JAK2 and STAT3 are activated in IPF in a rat model of bleomycin-induced lung fibrosis and that their dual inhibition could be an appealing strategy for inhibiting fibroblast migration, preventing increases in fibroblast senescence and Bcl-2 expression, and improving impaired autophagy. Rangarajan et al. discovered that nintedanib enhances the apoptotic clearance of fibrocytes and lung-resident myofibroblasts, slowing the development of TGF-induced pulmonary fibrosis. In fibroblasts isolated from IPF lungs, nintedanib was reported to activate autophagy(Rangarajan et al., 2016).

Furthermore, nintedanib can prevent fibrocyte migration, lowering the number of fibrocytes in the lungs during bleomycin-induced pulmonary fibrosis(Sato et al., 2017). This study has potential limitations. The safety of using nintedanib in combination with corticosteroid was not studied. IL-13 a biochemical marker for corticosteroid resistance was not measured. The bronchoalveolar fluid was not measured. In summary of our finding, the corticosteroid resistance pulmonary fibrosis induced by bleomycin was solved by using another treatment in combination with corticosteroids. Thus, it was successful to decrease the resistance of corticosteroids, by improve in wound healing in lung tissue, cytokines results were better than methylprednisolone alone, and it was able to decrease the water content in lung tissue with increased expression of integrins β3 and β6 and it was ably decreased the collagen formation in lung tissue compared to bleomycin

group treated with MP as showed in histological finding.

5. CONCLUSIONS AND RECOMMENDATION

Altogether, our data indicate that nintedanib overcame the corticosteroid resistance pulmonary fibrosis induced by bleomycin in mice mainly by decreasing TGF-β and improving the expression of β3 and β6 integrins. Our data suggest that nintedanib in combination with corticosteroids is helpful to reduce the resistance of pulmonary fibrosis to corticosteroids. We recommend future studies to study the safety of use the of nintedanib in combination with corticosteroid in pulmonary fibrosis.

COMPETING INTERESTS:

The authors declare that they have no competing interests.

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REFERENCES

Aguilar, S., Scotton, C. J., McNulty, K., Nye, E., Stamp, G., Laurent, G., Bonnet, D., & Janes, S. M. (2009). Bone Marrow Stem Cells Expressing Keratinocyte Growth Factor via an Inducible Lentivirus Protects against Bleomycin-Induced Pulmonary Fibrosis. PLoS ONE, 4(11), e8013. https://doi.org/10.1371/journal.pone.0008013.

Ahmed, B. S., & Anwar, N. M. (2004). Immuno-histochemical study of the expression of Bcl-2 in the Leydig's interstitial cells and primary spermatocyte cells of adult male rabbit under the effect of oral Gossypol intake. The Egyptian Journal of Hospital Medicine, 14(1), 34–44. https://doi.org/10.21608/ejhm.2004.18219.

Aono, Y., Nishioka, Y., Inayama, M., Ugai, M., Kishi, J., Uehara, H., Izumi, K., & Sone, S. (2005). Imatinib as a Novel Antifibrotic Agent in Bleomycin-induced Pulmonary Fibrosis in Mice. American Journal of Respiratory and Critical Care Medicine, 171(11), 1279–1285. https://doi.org/10.1164/rccm.200404-531oc.

Aubin Vega, M., Chupin, C., Pascariu, M., Privé, A., Dagenais, A., Berthiaume, Y., & Brochiero, E. (2019). Dexamethasone fails to improve bleomycin-induced acute lung injury in mice. Physiological Reports, 7(21). https://doi.org/10.14814/phy2.14253.

Bahtouee, M., Fatemikia, H., Movahed, A., Esmaili, A., Hassan, Y., Zarei, M., & Seyedian, R. (2018). A Comparative Analysis of Saffron and Methylprednisolone on Bleomycin-Induced Pulmonary Fibrosis in Rats. Iranian Journal of Toxicology, 12(3), 9–13. https://doi.org/10.32598/ijt.12.3.76.3.

Barnes, P. J. (2010). Mechanisms and resistance in glucocorticoid control of inflammation. The Journal of Steroid Biochemistry and Molecular Biology, 120(2-3), 76–85. https://doi.org/10.1016/j.jsbmb.2010.02.018.

Bi, J., Dai, J., Koivisto, L., Larjava, M., Bi, L., Häkkinen, L., & Larjava, H. (2019). Inflammasome and cytokine expression profiling in experimental periodontitis in the integrin β6 null mouse. Cytokine, 114, 135–142. https://doi.org/10.1016/j.cyto.2018.11.011.

Bi, J., Koivisto, L., Owen, G., Huang, P., Wang, Z., Shen, Y., Bi, L., Rokka, A., Haapasalo, M., Heino, J., Häkkinen, L., & Larjava, H. S. (2016). Epithelial Microvesicles Promote an Inflammatory Phenotype in Fibroblasts. Journal of Dental Research, 95(6), 680–688. https://doi.org/10.1177/0022034516633172.

Chaudhary, N. I., Schnapp, A., and & Park, J. E. (2006). Pharmacologic Differentiation of Inflammation and Fibrosis in the Rat Bleomycin Model. American Journal of Respiratory and Critical Care Medicine, 173(7), 769–776. https://doi.org/10.1164/rccm.200505-717oc.

Chen, W.-C., Chen, N.-J., Chen, H.-P., Yu, W.-K., Su, V. Y.-F., Chen, H., Wu, H.-H., & Yang, K.-Y. (2020). Nintedanib Reduces Neutrophil Chemotaxis via Activating GRK2 in Bleomycin-Induced Pulmonary Fibrosis. International Journal of Molecular Sciences, 21(13), 4735. https://doi.org/10.3390/ijms21134735.

Cross, C.E., Warren, D., Gerriets, J.E., Wilson, D.W., Halliwell, B., & Last, J.A. (1985) Deferoxamine injection does not affect bleomycin-induced lung fibrosis in rats. J Lab Clin Med. 106(4):433-8. PMID: 2413151.

Della Latta, V., Cecchettini, A., Del Ry, S., & Morales, M. A. (2015). Bleomycin in the setting of lung fibrosis induction: From biological mechanisms to counteractions. Pharmacological Research, 97, 122–130. https://doi.org/10.1016/j.phrs.2015.04.012.

Dewson, G. (2011). Interplay of Bcl-2 proteins decides the life-or-death fate. The Open Cell Signaling Journal, 3. 3-8. DOI: 10.2174/1876390101103010003

Kilkenny, C., Parsons, N., Kadyszewski, E., Festing, M. F., Cuthill, I. C., Fry, D., ... & Altman, D. G. (2009). Survey of the quality of experimental design, statistical analysis and reporting of research using animals. *PloS one*, *4*(11), e7824. DOI: 10.1371/journal.pone.0007824

Kluck, R. (2010). Bcl-2 family-regulated apoptosis in health and disease. Cell Health and Cytoskeleton, 9. https://doi.org/10.2147/chc.s6228.

Dik, W. A. (2003). Short course dexamethasone treatment following injury inhibits bleomycin induced fibrosis in rats. Thorax, 58(9), 765–771. https://doi.org/10.1136/thorax.58.9.765.

Flaherty, K. R., Wells, A. U., Cottin, V., Devaraj, A., Walsh, S. L. F., Inoue, Y., Richeldi, L., Kolb, M., Tetzlaff, K., Stowasser, S., Coeck, C., Clerisme-Beaty, E., Rosenstock, B., Quaresma, M., Haeufel, T., Goeldner, R.-G., Schlenker-Herceg, R., & Brown, K. K. (2019). Nintedanib in Progressive Fibrosing Interstitial Lung Diseases. New England Journal of Medicine, 381(18), 1718–1727.

https://doi.org/10.1056/nejmoa1908681.

Galuppo, M., Esposito, E., Mazzon, E., Di Paola, R., Paterniti, I., Impellizzeri, D., & Cuzzocrea, S. (2011). MEK inhibition suppresses the development of lung fibrosis in the bleomycin model. Naunyn-Schmiedeberg's Archives of Pharmacology, 384(1), 21–37. https://doi.org/10.1007/s00210-011-0637-7

Greos, L. S., Vichyanond, P., Bloedow, D. C., Irvin, C. G., Larsen, G. L., Szefler, S. J., & Hill, M. R. (1991). Methylprednisolone Achieves Greater Concentrations in the Lung Than Prednisolone: A Pharmacokinetic Analysis. American Review of Respiratory Disease, 144(3_pt_1), 586–592. https://doi.org/10.1164/ajrccm/144.3_pt_1.586.

Green FH. (2002) Overview of pulmonary fibrosis. Chest. Dec;122(6 Suppl):334S-339S.
doi: 10.1378/chest.122.6_suppl.334s. PMID: 12475811.

Hosoya T. (1997) Steroid resistance and lung-tissue cytokines in experimental bleomycin-induced lung fibrosis. Nihon Kyobu Shikkan Gakkai Zasshi. Jul;35(7):766-75. Japanese. PMID: 9341282.

Hu, X., & Huang, X. (2019). Alleviation of Inflammatory Response of Pulmonary Fibrosis in Acute Respiratory Distress Syndrome by Puerarin via Transforming Growth Factor (TGF-β1). Medical Science Monitor, 25, 6523–6531. https://doi.org/10.12659/msm.915570.

Hu, X., & Zhu, D. (2020). Rehmannia Radix Extract Relieves Bleomycin-Induced Pulmonary Fibrosis in Mice via Transforming Growth Factor β1 (TGF-β1). Medical Science Monitor, 26. https://doi.org/10.12659/msm.927240.

Huang, S.-H., Cao, X.-J., Liu, W., Shi, X.-Y., & Wei, W. (2010). Inhibitory effect of melatonin on lung oxidative stress induced by respiratory syncytial virus infection in mice. Journal of Pineal Research, 48(2), 109–116. https://doi.org/10.1111/j.1600-079x.2009.00733.x.

Izbicki, G., Segel, M. J., Christensen, T. G., Conner, M. W., & Breuer, R. (2002). Time course of bleomycin-induced lung fibrosis. International Journal of Experimental Pathology, 83(3), 111–119. https://doi.org/10.1046/j.1365-2613.2002.00220.x.

Jenkins, R. G., Moore, B. B., Chambers, R. C., Eickelberg, O., Königshoff, M., Kolb, M., Laurent, G. J., Nanthakumar, C. B., Olman, M. A., Pardo, A., Selman, M., Sheppard, D., Sime, P. J., Tager, A. M., Tatler, A. L., Thannickal, V. J., & White, E. S. (2017). An Official American Thoracic Society Workshop Report: Use of Animal Models for the Preclinical Assessment of Potential Therapies for Pulmonary Fibrosis. American Journal of Respiratory Cell and Molecular Biology, 56(5), 667–679. https://doi.org/10.1165/rcmb.2017-0096st.

Kadmiel, M., Janoshazi, A., Xu, X., & Cidlowski, J. A. (2016). Glucocorticoid action in human corneal epithelial cells establishes roles for corticosteroids in wound healing and barrier function of the eye. Experimental Eye Research, 152, 10–33. https://doi.org/10.1016/j.exer.2016.08.020.

Khalil, N., Whitman, C., Zuo, L., Danielpour, D., & Greenberg, A. (1993). Regulation of alveolar macrophage transforming growth factor-beta secretion by corticosteroids in bleomycin-induced pulmonary inflammation in the rat. Journal of Clinical Investigation, 92(4), 1812–1818. https://doi.org/10.1172/jci116771.

Kim, H.-Y., Kim, M.-S., Kim, S.-H., Joen, D., & Lee, K. (2018). Protective Effects of Nintedanib against Polyhexamethylene Guanidine Phosphate-Induced Lung Fibrosis in Mice. Molecules, 23(8), 1974. https://doi.org/10.3390/molecules23081974.

Kim, M.-S., Kim, S.-H., Jeon, D., Kim, H.-Y., & Lee, K. (2018). Changes in expression of cytokines in polyhexamethylene guanidine-induced lung fibrosis in mice: Comparison of bleomycin-induced lung fibrosis. Toxicology, 393, 185–192. https://doi.org/10.1016/j.tox.2017.11.017.

King, C. S., Freiheit, E., Brown, A. W., Shlobin, O. A., Aryal, S., Ahmad, K., Khangoora, V., Flaherty, K. R., Venuto, D., & Nathan, S. D. (2021). Association Between Anticoagulation and Survival in Interstitial Lung Disease. Chest, 159(4), 1507–1516. https://doi.org/10.1016/j.chest.2020.10.019.

Landi, C., Bergantini, L., Cameli, P., d'Alessandro, M., Carleo, A., Shaba, E., Rottoli, P., Bini, L., & Bargagli, E. (2020). Idiopathic Pulmonary Fibrosis Serum proteomic analysis before and after nintedanib therapy. Scientific Reports, 10(1). https://doi.org/10.1038/s41598-020-66296-z.

Langenbach, S. Y., Wheaton, B. J., Fernandes, D. J., Jones, C., Sutherland, T. E., Wraith, B. C., Harris, T., Schuliga, M. J., McLean, C., & Stewart, A. G. (2007). Resistance of fibrogenic responses to glucocorticoid and 2-methoxyestradiol in bleomycin-induced lung fibrosis in miceThis article is one of a selection of papers published in the Special Issue on Recent Advances in Asthma Research. Canadian Journal of Physiology and Pharmacology, 85(7), 727–738. https://doi.org/10.1139/y07-065.

Li, L.-F., Kao, K.-C., Liu, Y.-Y., Lin, C.-W., Chen, N.-H., Lee, C.-S., Wang, C.-W., & Yang, C.-T. (2017). Nintedanib reduces ventilation-augmented bleomycin-induced epithelial-mesenchymal transition and lung fibrosis through suppression of the Src pathway. Journal of Cellular and Molecular Medicine, 21(11), 2937–2949. https://doi.org/10.1111/jcmm.13206.

Liu, J., Zhang, M., Niu, C., Luo, Z., Dai, J., Wang, L., Liu, E., & Fu, Z. (2013). Dexamethasone Inhibits Repair of Human Airway Epithelial Cells Mediated by Glucocorticoid-Induced Leucine Zipper (GILZ). PLoS ONE, 8(4), e60705. https://doi.org/10.1371/journal.pone.0060705.

Liu, T., De Los Santos, F. G., & Phan, S. H. (2017). The Bleomycin Model of Pulmonary Fibrosis. Fibrosis, 27–42. https://doi.org/10.1007/978-1-4939-7113-8_2.

Liu, Y., Lu, F., Kang, L., Wang, Z., & Wang, Y. (2017). Pirfenidone attenuates bleomycin-induced pulmonary fibrosis in mice by regulating Nrf2/Bach1 equilibrium. BMC Pulmonary Medicine, 17(1). https://doi.org/10.1186/s12890-017-0405-7.

Ojo, A. S., Balogun, S. A., Williams, O. T., & Ojo, O. S. (2020). Pulmonary Fibrosis in COVID-19 Survivors: Predictive Factors and Risk Reduction Strategies. Pulmonary Medicine, 2020, 1–10. https://doi.org/10.1155/2020/6175964

Matsuyama, H., Amaya, F., Hashimoto, S., Ueno, H., Beppu, S., Mizuta, M., Nobuaki Shime, Akitoshi Ishizaka, & Hashimoto, S. (2008). Acute lung inflammation and ventilator-induced lung injury caused by ATP via the P2Y receptors: an experimental study. 9(1). https://doi.org/10.1186/1465-9921-9-79

Mayr, M. J., Clemens Duerrschmid, Medrano, G., Taffet, G. E., Wang, Y., Entman, M. L., & Haudek, S. B. (2016). TNF/Ang-II synergy is obligate for fibroinflammatory pathology, but not for changes in cardiorenal function. 4(8), e12765–e12765. https://doi.org/10.14814/phy2.12765

Milara, J., Hernandez, G., Ballester, B., Morell, A., Roger, I., Montero, P., . . . & Morcillo, E. (2018). The JAK2 pathway is activated in idiopathic pulmonary fibrosis. *Respiratory research*, *19*(1), 1-12. DOI: 10.1186/s12931-018-0728-9

Moeller, A., Ask, K., Warburton, D., Gauldie, J., & Kolb, M. (2008). The bleomycin animal model: A useful tool to investigate treatment options for idiopathic pulmonary fibrosis? The International Journal of Biochemistry & Cell Biology, 40(3), 362–382. https://doi.org/10.1016/j.biocel.2007.08.011

Mouratis, M. A., & Aidinis, V. (2011). Modeling pulmonary fibrosis with bleomycin. *Current opinion in pulmonary medicine*, *17*(5), 355-361. DOI: 10.1097/MCP.0b013e328349ac2b

Nemec, K. N., & Khaled, A. R. (2008). Therapeutic Modulation of Apoptosis: Targeting the BCL-2 Family at the Interface of the Mitochondrial Membrane. Yonsei Medical Journal, 49(5), 689. https://doi.org/10.3349/ymj.2008.49.5.689

Nettelbladt O, Tengblad A, Hällgren R. (1990). High-dose corticosteroids during bleomycin-induced alveolitis in the rat do not suppress the accumulation of hyaluronan (hyaluronic acid) in lung tissue. Eur Respir J. 1990 Apr;3(4):421-8.https://doi.org/10.1183/09031936.93.03040421

Phan, S. H., Thrall, R. S., & Williams, C. (1981). Bleomycin-induced pulmonary fibrosis. Effects of steroid on lung collagen metabolism. 124(4), 428–434. https://doi.org/10.1164/arrd.1981.124.4.428

Pittelli, M. G., Pitozzi, V., Caruso, P., Bonatti, M., Aquino, G., Biagetti, M., . . . Lagrasta, C. (2017). Effect of Nintedanib in a rat model of lung fibrosis induced by single or double bleomycin administration. *The European Respiratory Journal*, *50*, PA2956 . DOI: 10.1183/1393003.congress-2017.PA2956

Predescu, S. A., Zhang, J., Bardita, C., Patel, M., Godbole, V., & Predescu, D. N. (2017). Mouse Lung Fibroblast Resistance to Fas-Mediated Apoptosis Is Dependent on the Baculoviral Inhibitor of Apoptosis Protein 4 and the Cellular FLICE-Inhibitory Protein. Frontiers in Physiology, 8. https://doi.org/10.3389/fphys.2017.00128

Polosukhin, V. V., Degryse, A. L., Newcomb, D. C., Jones, B. R., Ware, L. B., Lee, J. W., Loyd, J. E., Blackwell, T. S., & Lawson, W. E. (2012). Intratracheal bleomycin causes airway remodeling and airflow obstruction in mice. Experimental Lung Research, 38(3), 135–146. https://doi.org/10.3109/01902148.2012.658595

Potter-Perigo, S., Johnson, P. T., Evanko, S. P., Chan, C., Braun, K. R., Wilkinson, T. J., Altman, L. C., & Wight, T. N. (2010). Polyinosine-Polycytidylic Acid Stimulates Versican Accumulation in the Extracellular Matrix Promoting Monocyte Adhesion. 43(1), 109–120. https://doi.org/10.1165/rcmb.2009-0081oc

Rangarajan S, Kurundkar A, Kurundkar D, Bernard K, Sanders YY, Ding Q, Antony VB, Zhang J, Zmijewski J, Thannickal VJ. (2016) Novel Mechanisms for the Antifibrotic Action of Nintedanib. Am J Respir Cell Mol BiolJan;54(1):51-9. doi: 10.1165/rcmb.2014-0445OC.

Redente, E. F., Aguilar, M. A., Black, B. P., Edelman, B. L., Bahadur, A. N., Humphries, S. M., Lynch, D. A., Wollin, L., & Riches, D. W. H. (2018). Nintedanib reduces pulmonary fibrosis in a model of rheumatoid arthritis-associated interstitial lung disease. American Journal of Physiology-Lung Cellular and Molecular Physiology, 314(6), L998–L1009. https://doi.org/10.1152/ajplung.00304.2017

Richeldi, L., du Bois, R. M., Raghu, G., Azuma, A., Brown, K. K., Costabel, U., Cottin, V., Flaherty, K. R., Hansell, D. M., Inoue, Y., Kim, D. S., Kolb, M., Nicholson, A. G., Noble, P. W., Selman, M., Taniguchi, H., Brun, M., Le Maulf, F., Girard, M., & Stowasser, S. (2014). Efficacy and Safety of Nintedanib in Idiopathic Pulmonary Fibrosis. New England Journal of Medicine, 370(22), 2071–2082. https://doi.org/10.1056/nejmoa1402584

Rieu, I., & Powers, S. J. Real-time quantitative RT-PCR: design, calculations, and statistics. Plant Cell. (2009) Apr;21(4):1031-3. doi: 10.1105/tpc.109.066001.

Roth, G.J., Binder, R., Colbatzky, F., Dallinger, C., Schlenker-Herceg, R., Hilberg F., Wollin, S.L., & Kaiser, R (2015). Nintedanib: from discovery to the clinic. J Med Chem. Feb 12;58(3):1053-63. doi: 10.1021/jm501562a.

Safaeian, L., Abed, A., & Vaseghi, G. The role of Bcl-2 family proteins in pulmonary fibrosis. Eur J Pharmacol. 2014 Oct 15;741:281-9. doi: 10.1016/j.ejphar.2014.07.029.

Safaeian, L., Jafarian, A., Rabani, M., Mirmohammad, S. H., Torabinia, N., & Alavi, S.A. (2009). The effect of AT1 receptor blockade on bax and bcl-2 expression in bleomycin induced pulmonary fibrosis. .DARU Vol. 17, No. 1 2009 53-59.

Safaeian, L., Jafarian, A., Rabbani, M., Sadeghi, H.M., Torabinia, N., & Alavi, S.A. (2008). The role of strain variation in BAX and BCL-2 expression in murine bleomycin-induced pulmonary fibrosis. Pak J Biol Sci. 2008 Dec 1;11(23):2606-12. doi: 10.3923/pjbs.2008.2606.2612.

Sato, S., Shinohara, S., Hayashi, S., Shun Morizumi, Abe, S., Okazaki, H., Chen, Y., Goto, H., Aono, Y., Ogawa, H., Koyama, K., Nishimura, H., Kawano, H., Toyoda, Y., Uehara, H., & Nishioka, Y. (2017). Anti-fibrotic efficacy of nintedanib in pulmonary fibrosis via the inhibition of fibrocyte activity. Respiratory Research, 18(1). https://doi.org/10.1186/s12931-017-0654-2

Saraiva, G. N., Rosário, N. F. d., Medeiros, T., Leite, P. E. C., Lacerda, G. d. S., Andrade, T. G. d., . . .& Xavier, A. R. (2018). Restoring inflammatory mediator balance after sofosbuvir-induced viral clearance in patients with chronic hepatitis C. *Mediators of inflammation*, *2018*.

Shaker, O.G., & Sourour, D.A. (2011) Effect of leukotriene receptor antagonists on lung fibrosis in rats. J Appl Toxicol. Oct;31(7):678-84. doi: 10.1002/jat.1625.

Tsoutsou, P.G., Gourgoulianis, K.I., Petinaki, E., Germenis, A., Tsoutsou, A.G., Mpaka, M., Efremidou, S., & Molyvdas, P.A. Cytokine levels in the sera of patients with idiopathic pulmonary fibrosis. Respir Med. 2006 May;100(5):938-45. doi: 10.1016/j.rmed.2005.06.016.

Thannickal. V.J., Horowitz, J.C.,(2006) Evolving concepts of apoptosis in idiopathic pulmonary fibrosis. Proc Am Thorac Soc. 2006 Jun;3(4):350-6. doi: 10.1513/pats.200601-001TK.
Ubieta, K., Thomas, M. J., & Wollin, L. (2021). The Effect of Nintedanib on T-Cell Activation, Subsets and Functions. Drug Design, Development and Therapy, Volume 15, 997–1011. https://doi.org/10.2147/dddt.s288369

Wilson, M.S., and Wynn, T.A. (2009) Pulmonary fibrosis: pathogenesis, etiology and regulation. Mucosal Immunol. 2009 Mar;2(2):103-21. doi: 10.1038/mi.2008.85.

Wollin, L., Maillet, I., Quesniaux, V. & Ryffel, B. (2013). Nintedanib reduces bleomycin-induced lung inflammation and fibrosis in mice. Eur Respiratory Soc. 42: P682.

Wollin, L., Wex, E., Pautsch, A., Schnapp, G., Hostettler, K. E., Stowasser, S., & Kolb, M. (2015). Mode of action of nintedanib in the treatment of idiopathic pulmonary fibrosis. European Respiratory Journal, 45(5), 1434–1445. https://doi.org/10.1183/09031936.00174914.

Wuyts, W. A., Agostini, C., Antoniou, K. M., Bouros, D., Chambers, R. C., Cottin, V., Egan, J. J., Lambrecht, B. N., Lories, R., Parfrey, H., Prasse, A., Robalo-Cordeiro, C., Verbeken, E., Verschakelen, J. A., Wells, A. U., & Verleden, G. M. (2012). The pathogenesis of pulmonary fibrosis: a moving target. European Respiratory Journal, 41(5), 1207–1218. https://doi.org/10.1183/09031936.00073012.

Wynn, T. A. (2011). Integrating mechanisms of pulmonary fibrosis. The Journal of Experimental Medicine, 208(7), 1339–1350. https://doi.org/10.1084/jem.20110551.

Youle, R. J., and Strasser, A. (2008). The BCL-2 protein family: opposing activities that mediate cell death. Nature Reviews Molecular Cell Biology, 9(1), 47–59. https://doi.org/10.1038/nrm2308.

Zakaria, D. M., Zahran, N. M., Arafa, S. A. A., Mehanna, R. A., & Abdel-Moneim, R. A. (2020). Histological and Physiological Studies of the Effect of Bone Marrow-Derived Mesenchymal Stem Cells on Bleomycin Induced Lung Fibrosis in Adult Albino Rats. Tissue Engineering and Regenerative Medicine, 18(1), 127–141. https://doi.org/10.1007/s13770-020-00294-0.

Zhao, L., Mu, B., Zhou, R., Cheng, Y., & Huang, C. (2019). Iguratimod ameliorates bleomycin-induced alveolar inflammation and pulmonary fibrosis in mice by suppressing expression of matrix metalloproteinase-9. International Journal of Rheumatic Diseases, 22(4), 686–694. https://doi.org/10.1111/1756-185x.13463.

Zhou, Y., Huang, X., Hecker, L., Kurundkar, D., Kurundkar, A., Liu, H., Jin, T.-H., Desai, L., Bernard, K., & Thannickal, V. J. (2013). Inhibition of mechanosensitive signaling in myofibroblasts ameliorates experimental pulmonary fibrosis. Journal of Clinical Investigation, 123(3), 1096–1108. https://doi.org/10.1172/jci66700.