2.1 Summary of the Original Research

Idiopathic pulmonary fibrosis (IPF) is a devastating disease of unknown etiology, for which there is no curative pharmacological therapy. Bleomycin, an antineoplastic agent that causes lung fibrosis in human patients has been used extensively in rodent models to mimic IPF. The conventional therapy has been steroids and immunosuppressive agents. But only a minority of patients responds to such a therapy. IPF is a progressive, ultimately fatal disorder for which substantive medical therapy is desperately needed. Fisetin is a flavonol which inhibits the activity of several proinflammatory cytokines. The polyphenol curcumin is used to treat inflammatory diseases, abdominal disorders, and a variety of other ailments. The aim of this study was to evaluate the beneficial effect of fistine, curcumin, and mesoporous carbon nanoparticle (MCN)-loaded fistin as an anti-inflammatory agent against bleomycin-induced changes in mice with IPF. In our study, flavonoids showed their antifibrotic action. The inflammatory cell count was greatly increased for bleo-treated individuals, and effectiveness of fistin was increased after the addition of MCN particles with it; curcumin also showed anti-inflammatory effects. In another experiment, bleomycin effectively inhibits the cellular recruitment to the spleen and treatment with fistin, and curcumin increases the cellular recruitment in spleen. Colony count was also increased in MCN + fistin-treated groups, and it was statistically significant. We also observed the increased level of cytokines with fistin treatment, with curcumin treatment, and with MCN + fistin treatment as compared to the bleo-treated sample. In conclusion, the present research suggests that fistin and curcumin and MCN-loaded fistin may be a promising therapeutic agent for bleomycin-induced changes in mice with IPF. This will open up new perspectives for a potential role of these drugs as a molecular target in idiopathic pulmonary fibrosis.

2.2 Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and lethal lung disorder of unknown etiology [1–3]. IPF primarily occurs between 60 and 70 years of age and is slightly more predominant in males [2, 4]. Data from around the world demonstrate that IPF favors no particular race or ethnic group. IPF is considered a complex disease where both genetic and environmental factors are believed to contribute to disease susceptibility [3]. The disease appears to be driven by abnormal and/or dysfunctional alveolar epithelial cells.
(AECs) that promote fibroblast recruitment, proliferation, and differentiation, resulting in scarring of the lung, architectural distortion, and irreversible loss of function [5]. Exercise-induced breathlessness and chronic dry cough are the prominent symptoms. The onset of symptoms is slow, but symptoms become progressively worse over time. The initial presentation of breathlessness is commonly attributed to aging, cardiac disease, or emphysema which results in typical delays of diagnosis. At the cellular level, IPF is characterized by alveolar epithelial injury, initiation of inflammatory cascades, exaggerated profibrotic cytokine expression, increased extracellular matrix (ECM) deposition, and the development of fibrotic lesions known as fibroblast “foci” [6]. Injured epithelium could release growth factors, cytokines, and matrix metalloproteinase, which caused the activation or proliferation of mesenchymal cell, deposition of extracellular matrix, and the accumulation of fibroblasts [7]. The disease progresses toward chronic restrictive respiratory failure and death [8, 9]. IPF is associated with a median survival of only 3–5 years following diagnosis [10]. In their study, Japanese physicians were the first to describe acute, unexpected deterioration in patients with IPF. This phenomenon has been called the “acute exacerbation” or, more euphemistically, the “terminal complication” of IPF [3].

IPF belongs to a family of lung disorders known as the interstitial lung diseases (ILDs) or, more accurately, the diffuse parenchymal lung diseases (DPLDs). Within this broad category of diffuse lung diseases, IPF belongs to the subgroup known as idiopathic interstitial pneumonia (IIP). During IPF, airway remodeling occurs which can be defined as changes in the composition, content, and organization of the cellular and molecular constituents of the airway wall [3, 14].

The pathogenesis of the disease remains poorly understood, although current paradigms focus on the importance of alveolar epithelial cell injury as a critical initiating event with subsequent dysregulated wound healing and fibrosis, resulting in distortion of the lung architecture. The activation of cell-signaling pathways through tyrosine kinases such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) has been implicated in the pathogenesis of the disease [11–13]. IPF not only destroys the normal lung parenchyma but also affects the pulmonary vasculature with aberrant microvascular and macrovascular remodeling [14]. Previous studies showed that IPF develops from chronic epithelial cell injury and aberrant activation of progressive fibrosis [15]. Therefore, the therapeutic strategy against IPF has shifted from corticosteroids and/or immunosuppressants to antifibrotic agents [16, 17].

Current medical therapy for IPF is poorly effective. However, IPF is a progressive, ultimately fatal disorder for which substantive medical therapy is desperately needed. The only care options endorsed by guidelines published in 2011 were pulmonary rehabilitation, long-term oxygen therapy, lung transplantation, and enrollment in a clinical trial [8].

Animal models play an important role in the investigation of diseases, and many models are established to examine pulmonary pathobiology. Chronic diseases such as IPF are more difficult to model, since the etiology and natural history of the disease are unclear, and no single trigger is known that is able to induce IPF in animals. Different models of pulmonary fibrosis have been developed over the years. Common methods include radiation damage, instillation of bleomycin, silica or asbestos, and transgenic mice or gene transfer employing fibrogenic cytokines. So far, the standard agent for induction of experimental pulmonary fibrosis in animals is bleomycin [18].

Bleomycin (bleo) is a glycopeptide-derived antibiotic, isolated from the soil fungus Streptomyces verticillus. It is a chemotherapeutic agent with side effects especially on skin. The most serious complication of bleomycin is pulmonary fibrosis and impaired lung function. It has been suggested that bleomycin induces sensitivity to oxygen toxicity and some studies support the role of the proinflammatory cytokines IL-18 and IL-1β in the mechanism of bleomycin-induced lung injury [19]. Animal models are often used to
investigate pulmonary fibrosis, and they play an important role in understanding the pathogenesis of this disease. Bleomycin model is the most widely used animal model of pulmonary fibrosis. It is widely used as an inducer in the animal models [18, 20]. Lung fibrosis induced by bleomycin delivered to animals via different routes has different pattern of foci distributions. Using the bleomycin-induced pulmonary fibrosis model, it has been previously reported that pulmonary inflammation and fibrosis are mediated by the secretion of the proinflammatory and profibrotic cytokine IL-1β through Nlrp3 inflammasome activation and IL-1R1/MyD88 signaling [21–23]. Fibrosis associated with bleomycin treatment has also been linked to toxic reactive oxygen and nitrogen species produced by infiltrating inflammatory cells [24]. Thus, agents that depress oxidative stress are also of potential clinical value and could have additional protective effects against bleomycin-induced pulmonary fibrosis.

Plants polyphenols are a class of molecules characterized by the presence of multiple phenol groups in their structural moiety. Over the past several years, polyphenols have been studied for their potential to modulate the production and activity of inflammatory molecules [25]. Fisetin (3, 7, 3′, 4′-tetrahydroxyflavone) is a flavonol, a structurally distinct chemical substance that belongs to the flavonoid group of polyphenols found in many plants fruits and vegetables, such as strawberries, apples, persimmons, onions, and cucumbers. Fisetin inhibits the activity of several proinflammatory cytokines, including tumor necrosis factor alpha, interleukin-6, and nuclear factor kappa B [26, 27]. In addition, fisetin is a potent natural anticancer agent.

The polyphenol curcumin is the active ingredient in the herbal remedy and dietary spice turmeric. Chemically, curcumin exhibits keto-enol tautomerism having a predominant keto form in acidic and neutral solutions and stable enol form in alkaline medium [28]. This vibrant yellow spice, derived from the rhizome of the plant Curcuma longa, has a long history of use in traditional medicines of China and India, where it is used to treat inflammatory diseases, abdominal disorders, and a variety of other ailments [29]. Curcumin has been shown to exhibit antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, and anticancer activities and thus has a potential against various malignant diseases, diabetes, allergies, arthritis, Alzheimer’s disease, and other chronic illnesses [30, 31]. Extensive scientific research over the past decade has shown the ability of this compound to modulate multiple cellular targets and hence possesses preventive and therapeutic value against a wide variety of diseases [28]. Curcumin has been shown to suppress TNF expression in in vitro, in vivo, and human studies. It can suppress TNF expression induced by numerous stimuli and by numerous cell types. Recent work has suggested that curcumin acts as a cancer chemopreventive and chemotherapeutic agent [32–35]. Curcumin inhibits activation of nuclear factor kB through blockade of IκB kinase and inhibits activation of cyclooxygenase 2 (COX2). It also alters activator protein 1 (AP1) complexes and inhibits Akt. In addition to the effects on transcription and cell signaling, curcumin possesses chemical features that may further modulate its chemopreventive activity [36–38].

In light of the promising properties and broad spectrum of activities of fisteine and curcumin, the aim of our study was to evaluate the beneficial effect of fisteine, curcumin, and mesoporous carbon nanoparticle (MCN)-loaded fistein as an anti-inflammatory agent against bleomycin-induced changes in mice with idiopathic pulmonary fibrosis.

2.3 Materials and Methods

Mice Balb/C mice from National Institute of Nutrition (NIN), Hyderabad, were used. The following numbers of animals used in each group were as follows: (a) control [3], (b) bleomycin-treated [4], (c) bleomycin + fistein-treated [4], (d) bleomycin + curcumin-treated [4], and (e) bleomycin + fistein + mesoporous carbon nanoparticle-treated [4]. Total 19 animals were maintained under pathogen-free condition and given food and water routinely. All experiments were
performed according to rules laid down by the Institutional and Departmental Animal Ethics Committee, and the animals housed under specific pathogen-free conditions at the animal house of the department of Zoology, University of Calcutta.

**Treatment of mice** Treatment was performed with adult Balb/C mice. 16 mice were anaesthetized using propofol received a single dose of bleomycin (naprobleo, miracalus) both intratracheally and intranasally, bleomycin 0.075 U/ml bleomycin dissolved in 1 ml of 0.09 % sterile saline water, from here 16 mice received a single dose of bleomycin at day 0.20 μl was administered intranasally and 40 μl from intratracheally. Mice were administered with 40 μl of fisetin, 40 μl of curcumin, and 40 μl of mesoporous carbon nanoparticle (MCN)-conjugated fisetin intratracheally at day 7, 14, and 21, and at the 28th day, they were killed, and organs such as lung, bone marrow, liver, bronchoalveolar lavage, and peripheral blood were collected. Weight and other parameter of mice were taken on a regular basis according to rodent health monitoring program (RHMP).

Mice developed marked interstitial and alveolar fibrosis, collagen content was detected by hydroxyproline estimation, blood smear preparation and cytospin sample staining with hematoxylin were used to quantify the differential cell count of blood, clonogenic potential was detected by colony-forming unit (CFU) assay, cytokine analysis of BALF and peripheral blood was used to study the cytokine profile, and weight of mice was taken in a regular basis to observe the development of fibrosis.

**Colony-forming unit (CFU)-c assay** For quantification of committed progenitors of all lineages, colony-forming units in culture (CFU-c) were performed using standard protocol. Briefly, after dissection, the tissues (spleen, lung) were immediately kept in Iscove’s modified Dulbecco’s media (IMDM) (purchased from Himedia, India) maintaining aseptic conditions. For bone marrow samples, the bone was taken into the biosafety cabinet (Vision Scientific, Korea) and flushed with PBS until the bone turns white. The cell suspension was then kept in IMDM. The tissues were minced, and the cell suspension was collected with the help of a nylon mesh. Spleen and lung samples were centrifuged at 5000 rpm for 5 min. Bone marrow was centrifuged at 5000 rpm for 10 min. Cell count was taken. Number of cells per well taken was $1 \times 10^4$. For bone marrow, $-4 \times 10^3$ cells were taken per well. CFU-c media were prepared using IMDM, supplemented with 30 % FBS (Himedia, India), 10 % BSA (Biosera), 1 % penicillin–streptomycin (Himedia, India), and 5 ng/ml murine SCF (Biovision). Lastly, 1.5 % methylcellulose (in powdered form purchased from Himedia, India) was added into the concoction. 1 ml CFU-c assay media and 500 μl cell suspension were plated in each 24-well cell culture plate. The 24-well plate (NEST Biotech Co., Ltd.) was kept in CO$_2$ incubator (Thermo Scientific) at 5 % CO$_2$ and 37 °C for 14 days. All colony types were counted using Floid Cell Imaging Station (Life Technologies, India.) and pooled to get total CFU-c.

**Total count/differential count** White blood cells (WBCs) are a heterogeneous group of nucleated cells. They play an important role in phagocytosis and immunity. Differential white blood cell count is an examination and enumeration of the distribution of leukocytes in a stained blood smear. The different kinds of white cells are counted and reported as percentages of the total WBCs examined. Increases in any of the normal leukocyte types, or the presence of immature leukocytes or erythrocytes in peripheral blood, are important diagnostically in a wide variety of inflammatory disorders. Differential count was taken using the following protocol. Blood was collected in 1X RBC lysis buffer. It was kept at room temperature for 5 min, then flushed with 1X PBS [137 mM NaCl (Merck), 2.7 mM KCl (Himedia), 10 mM Na$_2$HPO$_4$ (Qualigens), 2 mM KH$_2$PO$_4$ (Himedia)], and centrifuged (Cold Centrifuge Vision VS-15000CFN) at 4000 rpm for 5 min at 4 °C. The supernatant was discarded. The pellet is dissolved in 1X PBS and stored at 4 °C. $10^5$
cells, dissolved in PBS, were diluted in cold 1 % BSA-PBS. Quickly, 100 μl of each sample was added to appropriate wells of the cytopsin (Centurion Scientific), and the slides and filters were placed in the correct slots of the cytopsin. The slides were centrifuged at 2000 rpm for 3 min. The slides were removed and air-dried. They were then fixed with methanol (SRL) and air-dried before staining. The fixed slides were placed in 100 % ethanol in a Coplin jar for 5 min, followed by 10 min in 90 % ethanol. They were then stained with hematoxylin for 5 min, rinsed in 70 % ethanol, counterstained with eosin for 2 min, and again rinsed in 70 % ethanol. Then, they were placed in 100 % ethanol for 1 min and then observed under the microscope (Debro DX-200).

Collagen content estimation by hydroxyproline assay  Collagen is the major structural protein of the cellular matrix of the lung. The most common method for evaluating tissue fibrosis/collagen deposition is hydroxyproline quantification. 4-hydroxyproline is a major component of collagen, comprising around 13.5 % of its amino acid composition. The basis of hydroxyproline quantification is that total collagen can be assessed by acid hydralization of proteins followed by measurement of hydroxyproline content.

The hydroxyproline content of mouse lung was determined by standard methods. The pulmonary vasculature was perfused with PBS until free of blood. Lung tissue was excised, trimmed free of surrounding conducting airways, and homogenized in 1.8 ml of 0.5 mol/L glacial acetic acid. This mixture was dried in a speed vacuum and weighed. The dried sample was redissolved sample in 2 ml of 6 N HCl and hydrolyzed at high temperature overnight. 7 μl samples were transferred to eppendorf tubes, dried, and resuspended in citrate–acetate buffer (5 % citric acid, 1.2 % glacial acetic acid, 7.24 % sodium acetate, and 3.4 % NaOH dissolved in distilled water and adjusted to pH 6.0). Freshly prepared chloramine-T solution [0.282 g chloramine-T (Merck), 2 ml n-propanol, 2 ml distilled H2O to 20 ml with citrate–acetate buffer], 150 μl, was added and the sample allowed to stand at room temperature for 20 min. 150 μl freshly prepared Ehrlich’s solution [4.5 g 4-dimethylaminobenzaldehyde (Merck) dissolved in 18.6 ml n-propanol, 7.8 ml of 70 % perchloric acid] was added and the sample heated to 65 °C for 15 min. Optical density was measured at 550 nm. A standard curve of samples with known quantity of hydroxyproline (Sigma) was generated for each assay. Absorbance of unknown samples was compared to the standard curve, and hydroxyproline content per lung was calculated.

Cytokine analysis of BALF and peripheral blood  The BD CBA Mouse Th1/Th2 Cytokine Kit (Catalog NO 551287) was used to measure interleukin-2, (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interferon-γ (IFN-γ), and tumor necrosis factor (TNF) protein levels in both BALF and peripheral blood samples. This uses bead array technology to simultaneously detect multiple cytokines in the samples. 5 bead populations with distinct fluorescent intensities are coated with capture antibodies, specific for the above-mentioned proteins. The beads are mixed to form the bead array and resolved in a red channel of a flow cytometer. After addition of the sample to the sample assay tubes containing the capture beads, the mouse Th1/Th2 PE detection reagents were added to each tubes. The tubes were incubated for 1 h at room temperature, in the dark, and then washed with 1 ml of wash buffer, centrifuge at 200 g for 5 min. The supernatant was carefully discarded and 300 μl of wash buffer added to resuspend the bead pellet.

Statistical method  Results are given as means ± SD. The differences were assessed by 1-tail Student’s t-test, one-way at α = 5 % using GraphPad Prism 6. A P value of <0.05 was considered to indicate a significant difference. ⭐ denotes significance in samples compared to
control, ⭐ denotes significance in samples treated with only bleo, ⭐⭐ denotes significance in samples treated with fisetin, curcumin, and MCN-treated fisetin compared to bleo.

### 2.4 Result

Figure 2.1 shows comparison between control and treated sample with respect to their hydroxyproline concentration. We found that there is 19-fold increase in collagen concentration for bleo-treated group, sixfold increase for bleo + fisetin-treated group, threefold increase for bleo + fisetin + MCN-treated group, and ninefold increase for bleo + curcumin-treated group. Here, we observed that bleomycin is highly effective in causing fibrosis in mice as compared to control groups, and the plant flavonoids also showed their antifibrotic action.

The average weights of different groups of mice were significantly different at the 0th day. During the treatment mainly from the day 7 (Fig. 2.2), we observed that the weight of bleo-treated groups were significantly reduced. Weight reduced by 45, 42, and 19 % on 7th, 14th, and 21st day, respectively, as compared to control groups. When we challenged the bleo-treated group with flavonoids (curcumin and fisetin), the weight of the mice was significantly increased to 39, 37, and 5 % at day 7, 14, and 21, respectively, for the curcumin-treated group; 55, 33, and 9 % at the respective days for fisetin-treated groups; and 46, 33, and 14 % at the respective days for MCN-loaded fisetin groups. Significant weight loss indicates development of fibrosis after treating with bleomycin.

We found that total cell count in peripheral circulating blood increases by 1.7-fold in the bleo-treated group as compared to control. We also found that the count decreases by 1.43-fold with fisetin, 1.40-fold with MCN + fisetin, and 1.5-fold with curcumin when compared to only bleo. This indicates 1.3-fold increase with fisetin, 1.2-fold increase with MCN + fisetin, and onefold increase as compared to control-treated groups (Fig. 2.3). These findings indicate that after the development of fibrosis during bleomycin treatment, the total cell count was greatly increased which was decreased significantly after challenging with the plant flavonoids.

The polymorphonuclear (PMN) cell count was increased by 5.6 % and the mononuclear (MN) cell count by 2 % for bleo-treated as compared to placebo groups. Both PMN and MN cell counts decreased after treatment with fisetin, curcumin, and MCN-loaded fisetin. PMN cell count decreased 5 % by fisetin treatment and 4 % by both curcumin and MCN-loaded fisetin treatment, and the MN cell count decreased by 3 % for fisetin and 5 % by both curcumin and MCN-loaded fisetin as compared to only

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**Table 2.1** Concentration(µg/ml) of hydroxyproline in lung samples

<table>
<thead>
<tr>
<th></th>
<th>Concentration(µg/ml) of hydroxyproline in lung samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>73.33 ±17.64</td>
</tr>
<tr>
<td>Bleo</td>
<td>1377.50 ± 202.34</td>
</tr>
<tr>
<td>Bleo+F</td>
<td>465.00 ± 91.70</td>
</tr>
<tr>
<td>Bleo+M+F</td>
<td>245.00 ± 42.72</td>
</tr>
<tr>
<td>Bleo+C</td>
<td>682.50 ± 168.79</td>
</tr>
</tbody>
</table>

**Fig. 2.1** Graphical representation of hydroxyproline estimation of lung sample of different groups using GraphPad Prism software
**2.4 Result**

**Weight of mice**

*Weights of Animals During Experiment*

![Graph showing weight changes over time for different groups.](image)

<table>
<thead>
<tr>
<th>Duration of Experiment</th>
<th>Control</th>
<th>Bleo</th>
<th>Bleo+F</th>
<th>Bleo+MCN+F</th>
<th>Bleo+C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>24.83±2.59</td>
<td>25.00±2.89</td>
<td>25.13±0.13</td>
<td>22.63±2.19</td>
<td>27.00±1.22</td>
</tr>
<tr>
<td>7 days</td>
<td>24.83±2.59</td>
<td>21.63±2.72</td>
<td>25.13±0.13</td>
<td>24.88±0.92</td>
<td>24.38±1.20</td>
</tr>
<tr>
<td>14 days</td>
<td>27.50±3.82</td>
<td>21.13±3.03</td>
<td>25.75±0.43</td>
<td>25.75±0.63</td>
<td>26.88±0.63</td>
</tr>
<tr>
<td>21 days</td>
<td>27.50±3.82</td>
<td>23.75±1.61</td>
<td>25.38±1.07</td>
<td>26.75±0.25</td>
<td>25.00±1.02</td>
</tr>
<tr>
<td>28 days</td>
<td>27.33±2.33</td>
<td>25.38±1.69</td>
<td>23.13±0.63</td>
<td>24.38±1.20</td>
<td>25.63±0.63</td>
</tr>
</tbody>
</table>

Fig. 2.2 Represents the weight of mice for each group

**Results of blood cell count**

*Fig. 2.3 Total cell count of peripheral blood of all groups using hemocytometer*

<table>
<thead>
<tr>
<th>Total cell count of peripheral blood using hemocytometer</th>
<th>TC (x 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.87 ± 0.15</td>
</tr>
<tr>
<td>Bleo</td>
<td>1.48 ± 0.12</td>
</tr>
<tr>
<td>Bleo+F</td>
<td>1.03 ± 0.06</td>
</tr>
<tr>
<td>Bleo+MCN+F</td>
<td>1.05 ± 0.06</td>
</tr>
<tr>
<td>Bleo+C</td>
<td>0.95 ± 0.17</td>
</tr>
</tbody>
</table>
bleo-treated samples. Here, we found that the inflammatory cell count was greatly increased for bleo-treated individuals and the effectiveness of fisetin was increased after addition of MCN with it; curcumin also showed anti-inflammatory effects (Fig. 2.4).

The clonogenic potential in spleen decreases by 1.94-fold in the bleo treated as compared to control. The count decreases by 1.94-fold with fisetin, 1.07-fold with MCN + fisetin, and 1.4-fold with curcumin as compared to control. 1.80-fold increase with MCN + fisetin, and 1.38-fold increase with curcumin as compared to only bleo. Bleomycin effectively inhibits the cellular recruitment to the spleen and treatment with fisetin, and curcumin increases the cellular recruitment in spleen. Colony count was also increased in MCN + F-treated groups, and it shows a statistical significance as compared to both bleo and bleo + fisetin (Fig. 2.5a).

The clonogenic potential in lung decreases by twofold significantly ($p < 0.05$) than the untreated control groups after challenge with bleomycin, whereas the total number of lung progenitor cells

![Fig. 2.4](image-url)
increases by 1.35-fold with fisetin, 1.30-fold with MCN-loaded fisetin, and 1.66-fold with curcumin as compared to the bleo-treated groups. This denotes that bleomycin effectively decreases the clonogenic potential of the lung cells, and fisetin, MCN-loaded fisetin, and curcumin also show its

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Bleo</th>
<th>Bleo+F</th>
<th>Bleo+MCN+F</th>
<th>Bleo+C</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days</td>
<td>3.50±0.50</td>
<td>1.80±0.20</td>
<td>1.80±0.20</td>
<td>3.25±0.25</td>
<td>2.50±0.00</td>
</tr>
<tr>
<td>14 days</td>
<td>3.75±0.75</td>
<td>2.75±0.25</td>
<td>2.20 +0.00</td>
<td>3.90±0.90</td>
<td>2.65± 0.15</td>
</tr>
</tbody>
</table>

**Fig. 2.5** a Clonogenic potential of spleen samples. b Clonogenic potential of lung sample. c Clonogenic potential of bone marrow sample. d Clonogenic potential of peripheral blood sample
regenerative effect on it; among them, curcumin showed the best results (Fig. 2.5b).

The clonogenic potential of bone marrow (aspirated from the femur), the site of poiesis, decreases by twofold than the untreated control groups after bleomycin challenge, whereas in fisetin-treated groups, total cell number in bone marrow increases by 1.36-fold, in MCN + fisetin-treated groups, total cell number increases by fourfold, and in curcumin-treated group by 1.83-fold when compared to only bleo. This indicates a 126.43-fold decrease with fisetin and a 2.82-fold decrease with MCN + fisetin, as compared to only Ova. This shows that the
The synthesis of cells in the bone marrow, which decreases after challenge with bleomycin, is increased due to the treatment (Fig. 2.5c).

Clonogenic potential of peripheral blood was also decreased (2 fold) for bleo-treated groups, treatment with the plant flavonoids trying to show its regenerative affect against bleomycin, among the 3 groups here MCN-loaded fisetin showed the best affect, it increases the clonogenic potential by twofold as compared to bleo-treated groups, fisetin and curcumin also showed its regenerative affect by increasing the clonogenic potential to 1.9-fold and 1.7-fold, respectively, compared to bleo-treated groups (Fig. 2.5d).
Figure 2.6a demonstrated that, after challenging with bleomycin, there was a decrease in the levels of IL-4 (1.06-fold), IL-5 (1.16-fold), IFN-γ (1.01-fold), and TNF-α (1.08-fold) as compared to control except IL-2 (1.06-fold increase), and there was an increase in the level of cytokines with fisetin treatment (1.03-fold for IL-2, 1.04-fold for IL-4, 1.11-fold for IL-5, 1.11-fold for IFN-γ, and 1.03-fold for TNF-α), with curcumin treatment (1.03-fold for IL-2, 1.06-fold for IL-4, 1.14-fold for IL-5, 1.29-fold for IFN-γ, and onefold for TNF-α), and with MCN + F treatment (1.03-fold for IL-2, 1.01-fold for IL-4, 1.04-fold for IL-5, 1.15-fold for IL-2, 1.04-fold for IL-4, 1.11-fold for IL-5, 1.11-fold for IFN-γ, and 1.03-fold for TNF-α).
for IFN-γ, and 1.15-fold for TNF-α) as compared to the bleo-treated samples.

After challenging with bleomycin, there was an decrease in the levels of IL-2 (1.36-fold), IL-4 (1.39-fold), IL-5 (1.04-fold), IFN-γ (1.67-fold), and TNF-α (1.41-fold) as compared to control, and there is an increase in the level of cytokines with curcumin treatment (1.31-fold for IL-2, 1.30-fold for IL-4, 1.45-fold for IL-5, 1.35-fold for IFN-γ, and 1.17-fold for TNF-α), with fisetin treatment (1.29-fold for IL-2, 1.35-fold for IL-4, 1.29-fold for IL-5, 1.46-fold for IFN-γ, and 1.42-fold for TNF-α), and with MCN + fisetin treatment (1.38-fold for IL-2, 1.41-fold for IL-4, 1.45-fold for IL-5, 1.81-fold for IFN-γ, and 1.44-fold for TNF-α) as compared to the bleo-treated sample (Fig. 2.6b).

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IFN-γ</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>15.8 ± 1.90</td>
<td>15.6 ± 0.30</td>
<td>14.0 ± 0.70</td>
<td>8.5 ± 0.30</td>
<td>8.95 ± 1.15</td>
</tr>
<tr>
<td>BLEO</td>
<td>16.9 ± 0.20</td>
<td>14.6 ± 0.65</td>
<td>12.1 ± 0.30</td>
<td>8.4 ± 0.25</td>
<td>8.25 ± 1.05</td>
</tr>
<tr>
<td>BLEO+C</td>
<td>16.4 ± 0.40</td>
<td>15.6 ± 0.20</td>
<td>13.9 ± 0.35</td>
<td>10.9 ± 0.50</td>
<td>8.3 ± 1.00</td>
</tr>
<tr>
<td>BLEO+F</td>
<td>16.4 ± 0.00</td>
<td>15.2 ± 0.40</td>
<td>13.5 ± 1.05</td>
<td>10.7 ± 0.00</td>
<td>8.55 ± 0.35</td>
</tr>
<tr>
<td>BLEO+MCN+F</td>
<td>16.3 ± 0.55</td>
<td>14.8 ± 1.05</td>
<td>12.7 ± 2.30</td>
<td>9.7 ± 0.55</td>
<td>9.55 ± 0.55</td>
</tr>
</tbody>
</table>

Fig. 2.6  a Cytokine profile of BALF. b Cytokine profile of peripheral blood sample
2.5 Discussion

For the first time, our study presents evidence that fisetin, curcumin, and mesoporous carbon nanoparticle (MCN)-loaded fisetin could be a promising therapeutic option against idiopathic pulmonary fibrosis (IPF).

Bleomycin-induced pulmonary fibrosis is a well-established disease model for IPF and widely used in the investigation of the efficacy and mechanism of therapeutic candidates [39]. IPF is a progressive interstitial pneumonia of unknown etiology, while its pathogenesis is not fully understood [40]. The risk of developing IPF is likely due to both host and environmental factors, and their elucidation may lead to improved prevention and treatment strategies [41]. Elevated levels of TNF-α, a cytokine with inflammatory and fibrogenic properties, have been detected in the lungs in patients with IPF. IPF remains a major cause of morbidity and mortality around the world, and there is still a large unmet medical need. There is no definitive approach to the treatment of IPF because evidence for effective medical therapy is still lacking. A better understanding of the basic biology of IPF is crucial to enable the development of novel therapeutic agents for this disease. Animal models supported the concept not only of abnormal vascular remodeling as a pathogenic mechanism in pulmonary fibrosis (PF), with reports of newly formed vascular networks within the fibrotic lung, but also of increased

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Concentration pico gm/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peripheral blood</strong></td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td>17.2 ± 0.40 16.3 ± 0.25 13.0 ± 0.25 10.7 ± 0.50 8.3 ± 0.00</td>
</tr>
<tr>
<td>BLEO</td>
<td>12.6 ± 4.40 11.7 ± 4.75 92.5 ± 3.25 6.4 ± 3.85 5.85 ± 4.05</td>
</tr>
<tr>
<td>BLEO+C</td>
<td>16.5 ± 0.60 15.2 ± 0.55 13.5 ± 0.30 8.7 ± 1.40 6.9 ± 1.30</td>
</tr>
<tr>
<td>BLEO+F</td>
<td>16.2 ± 0.10 15.9 ± 1.10 12.0 ± 0.40 93.50 ± 1.25 8.35 ± 0.35</td>
</tr>
<tr>
<td>BLEO+MCN+F</td>
<td>16.6 ± 0.60 16.5 ± 0.60 13.5 ± 1.00 11.6 ± 0.15 8.45 ± 0.15</td>
</tr>
</tbody>
</table>

Fig. 2.6 (continued)

![Graph showing cytokine profile of peripheral blood](image)
capillary irregularity and dilatation [42]. Progressive fibrosis with loss of normal lung tissue leads to restricted ventilation, impaired gas exchange, respiratory symptoms and exercise limitation, poor quality of life, and ultimately death [41]. Bleomycin is a chemotherapeutic drug used clinically for a variety of human malignancies, including lymphoma. It has been reported that administration of a high dose of bleomycin often leads to lethal lung injury and pulmonary fibrosis in human patients, as well as in rodent [19]. Therefore, it has been widely used in making pulmonary fibrosis animal models [43]. The bleomycin model has the advantage that it is quite easy to perform, widely accessible, and reproducible, and therefore fulfills important criteria expected from a good animal model [44]. Bleomycin inhibits the incorporation of thymidine, causing DNA fragmentation resulting in apoptosis with the release of chemical mediators for the recruitment of immune cells. On the other hand, the epidermal atrophy could be related to the effect of bleomycin, which has been shown to cause cessation of the epidermal cell cycle, with induction of epidermal cell apoptosis [45, 46]. The bleomycin model has contributed tremendously to elucidate the roles of cytokines, growth factors, and signaling pathways involved in pulmonary fibrosis. For instance, it has helped to determine transforming growth factor (TGF) β as one of the key factors in the development of pulmonary fibrosis [47].

Unfortunately, no pharmacologic therapy exists at this time that has been proven to improve survival. Due to the heterogeneity of IPF’s clinical course, lack of complete understanding of the pathogenesis, and infrequency of the disease itself, there is a lack of large-scale randomized controlled trials. During the last decade, several clinical trials for IPF have been conducted worldwide to determine an effective treatment regimen for IPF, but the results have been disappointing. Clinical trial failures may arise for many reasons, including disease heterogeneity, lack of readily measurable clinical end points other than overall survival, and, perhaps most of all, a lack of understanding of the underlying molecular mechanisms of the progression of IPF [48]. Some clinical trials are presently assessing the utility of novel agents in the treatment of IPF. Most medications are not recommended due to their side effects and lack of proven benefit [49].

Fisetin has recently received attention for its beneficial effects against several diseases. In the past years, fisetin was a subject of research because of its antiproliferative [50–52], apoptotic [52, 53], and antioxidant [54] activities. Several studies indicate that fisetin is a promising novel antioxidant. Fisetin has been reported to inhibit human low-density lipoprotein (LDL) oxidation in vitro [55]. It induced quinone oxidoreductase activity in murine hepatoma 1c1c7 cells in a time- and dose-dependent manner, and the induction of activity was associated with increase in mRNA expression. It was found that fisetin prevented LDL from oxidation, in part, through reducing CD36 gene expression in macrophages, a possible effect in ameliorating atherosclerosis [56]. Curcumin is the active ingredient in the traditional herbal remedy and dietary spice turmeric. Research has revealed that curcumin has a surprisingly wide range of beneficial properties, including anti-inflammatory, antioxidant, chemopreventive, and chemotherapeutic activity. These activities have been demonstrated both in cultured cells and in animal models and have paved the way for ongoing human clinical trials [35]. Previous report showed that curcumin inhibits the production of proinflammatory monocyte/macrophage-derived cytokines in PMA- or LPS-stimulated peripheral blood monocytes and alveolar macrophages [57]. Clinical trials of curcumin in humans have been promising. Phase I studies demonstrated virtually no toxicity in humans consuming up to 8 g curcumin per day for 3 months or a single dose of up to 12 g [58, 59]. Based on the encouraging preclinical and phase I clinical data, several additional human trials have been initiated and are currently enrolling patients. This includes trials testing the activity of curcumin in patients with colon cancer, pancreatic cancer, multiple myeloma, and myelodisplasia [32]. Nanoparticles have promising applications in medicine [60]. Recently, targeted and triggered drug delivery systems accompanied by nanoparticle technology
have emerged as prominent solutions to the bioavailability of therapeutic agents. The factors affecting the immune response are complex, including particle composition, size, surface chemistry, plasma protein binding, and exposure route. Investigation of the relationship between properties of nanoparticles and systemic immune response is crucial for their application in medicine and other areas.

In our study, we found that bleomycin is highly effective in causing fibrosis in mice as compared to control groups and the plant flavonoids also showed their antifibrotic action (Fig. 2.1). The average weights of different groups of mice were significantly different at the 0th day, and the weight of bleo-treated groups was significantly reduced mainly from the day 7 (Fig. 2.2). We demonstrated that total cell count in peripheral circulating blood increases by 1.7-fold in the bleo-treated group as compared to control. The count decreases by 1.43-fold with fisetin, 1.40-fold with MCN + fisetin, and 1.5-fold with curcumin as compared to only bleo. This indicates 1.3-fold increase with fisetin, 1.2-fold increase with MCN + fisetin, and onefold increase as compared to control-treated groups (Fig. 2.3). These indicates that after the development of fibrosis during bleomycin treatment, the total cell count was greatly increased which was decreased significantly after challenging with the plant flavonoids. In this study, the inflammatory cell count was greatly increased for bleo-treated individuals and the effectiveness of fisetin was increased after addition of MCN particles with it; curcumin also showed anti-inflammatory effects (Fig. 2.4). In another experiment, bleomycin effectively inhibits the cellular recruitment to the spleen and treatment with fisetin, and curcumin increases the cellular recruitment in spleen. Colony count was also increased in MCN + fisetin-treated groups, and it was statistically significant (Fig. 2.5a). Figure 2.5b demonstrated that bleomycin effectively decreases the clonogenic potential of the lung cells, and fisetin, MCN-loaded fisetin, and curcumin also showed its regenerative effect on it; among them, curcumin showed the best results. We also observed the increased level of cytokines with fisetin treatment, with curcumin treatment, and with MCN + fisetin treatment as compared to the bleo-treated sample (Fig. 2.6a). In other experimental assays, fisetin, curcumin, and MCN + fisetin showed promising results in bleomycin-induced idiopathic pulmonary fibrosis in mice.

It is important to emphasize that probably no single agent is going to work in this disease and that a combination, including agents fisetin and curcumin, will be necessary. Improvement of our knowledge about the biopathological principles of the disease will increase the opportunities of finding new agents.

In conclusion, idiopathic pulmonary fibrosis is a challenging, terminal disease characterized by progressive dyspnea and cough and the present research suggests that fisetin and curcumin may be a promising therapeutic agent for bleomycin-induced changes in mice with IPF. Additionally, the administration of mesoporous carbon nanoparticle-loaded fisetin enhanced the beneficial effects. This may open up new perspectives for a potential role of these drugs as a molecular target in idiopathic pulmonary fibrosis.

References

References


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