Epigallocatechin-3-gallate exhibits anti-fibrotic effect by attenuating bleomycin-induced glycoconjugates, lysosomal hydrolases and ultrastructural changes in rat model pulmonary fibrosis

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Abstract
Pulmonary fibrosis is characterized by excessive deposition of extracellular matrix components in the alveolar space, which hampers normal respiration process. Pathophysiological enzymes, glycoprotein moieties and matrix degrading lysosomal hydrolases are key markers and play a crucial role in the progression of fibrosis. Bleomycin is an anti-neoplastic drug, used for the treatment of various types of cancers and induces pulmonary fibrosis due its deleterious side effect. Tea catechin epigallocatechin-3-gallate (EGCG) is known for its wide array of beneficial effects. The present study was intended to evaluate the beneficial efficacy of EGCG against bleomycin-induced glycoconjugates, lysosomal hydrolases and ultrastructural changes in the lungs of Wistar rats. Intratracheal instillation of bleomycin (6.5 U/kg body weight) to rats increased the activities of pathophysiological enzymes such as aspartate transaminase, alanine transaminase, lactate dehydrogenase and alkaline phosphatase, which were attenuated upon EGCG treatment. The increased level of hydroxyproline and histopathological parameters in bleomycin-induced rats were decreased upon EGCG treatment. Bleomycin-induced increase in the level of glycoconjugates was restored closer to normal levels on EGCG treatment. Furthermore, the increased activities of matrix degrading lysosomal enzymes in bleomycin-induced rats were reduced upon EGCG supplementation. Treatment with EGCG also attenuated bleomycin-induced ultrastructural changes as observed from transmission electron microscopy studies. The results of the present study put-forward EGCG as a potential anti-fibrotic agent due to its attenuating effect on potential fibrotic markers.

1. Introduction
Pulmonary fibrosis is characterized by chronic scar formation and deposition of extracellular matrix (ECM), in the alveolar wall, resulting in impaired lung function and respiratory failure [1,2]. During the end stages of pulmonary fibrosis, ECM or collagenous components are usually accompanied by non-collagenous components. Majority of the non-collagenous component comprise of glycoproteins, which are predominantly protein in nature with one or more heterosaccharide chains containing hexose, hexosamine, sialic acid and fucose. Glycoproteins play a crucial role in the pathogenesis of pulmonary fibrosis. Earlier report suggests that glycoproteins are integral part of ECM and are believed to contribute to the structure of the matrix [3]. Glycoproteins incorporate into fibrils during collagen synthesis and cements fibrils into fibers, an interaction that accounts for certain biologic properties of fibers [4]. Besides glycoproteins, lysosomal enzymes recruited from the cysteine and aspartic proteinase families play an important role in matrix degradation processes [5–8]. The cysteine proteinases, cathepsins B, L and S are known to contribute to the degradation of several types of collagen and elastin in the extracellular space [7,8]. Lysosomal proteinases intracellularly complete the extracellular cleavage of collagens, elastin and proteoglycan core proteins by serine and metalloproteinases [8].

Bleomycin is an anticancer agent prescribed for various cancers, including that of the lung. However, this drug has a dose-dependent pulmonary toxicity that includes lung fibrosis, which limits its clinical use [9]. Many research groups have reported that during the early stages of bleomycin-induced lung damage, several biochemical and functional changes occurs such as inflammatory cell infiltration, increased collagen content, reduced lung volume and compliance [10–12], that resembles human pulmonary fibrosis. Flavonoids are ubiquitous group of polyphenolic substances, which are present in a variety of plants. Green tea contains catechin-based flavonoids with epigallocatechin-3-gallate (EGCG) being present in greatest amounts. Various studies have shown flavonoids, like EGCG, to be powerful antioxidants [13] that exhibits multi-
tionary properties such as anti-inflammatory [14,15], anti-bacterial [16] and anti-thrombotic [17]. Earlier reports have proved that natural compounds with potential antioxidant property can attenuate fibrosis by limiting ECM production [18,19].

Recently, we have reported the protective role of EGCG against experimental pulmonary fibrosis [13]. We have also documented the anti-inflammatory ability of EGCG through Nrf2-Keap1 signalling in bleomycin-injured animals [20]. However, glycoconjugates and lysosomal enzymes play an important role during the progression of pulmonary fibrosis, which is poorly documented. To our knowledge, none of the report directly addresses the role of EGCG in attenuating these parameters, during bleomycin-induced pulmonary fibrosis. Hence, this study is focused to evaluate the modulatory efficacy of EGCG on glycoconjugates, lysosomal enzymes and ultrastructural changes in the lungs of bleomycin-induced rats.

2. Materials and methods

2.1. Animals

Male albino Wistar rats, weighing around 180–220 g, were used in this study. The rats were housed in individual cages and acclimatized for 7 days before the start of the experiment. The rats were maintained on standard chow diet (M/s. Hindustan Lever Ltd., Bangalore, India) and were allowed free access to water. The experiments were designed and conducted according to ethical norms approved by the Ministry of Social Justice and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (Approval no. 360/01/A/CPCSEA).

2.2. Induction of pulmonary fibrosis

An animal model of bleomycin-induced pulmonary fibrosis as previously described [21] was followed in the present study, with little modifications. For induction of pulmonary fibrosis, male Wistar rats (n = 6) received a single dose of bleomycin sulfate (6.5 U/kg body weight) dissolved in 0.3 ml 0.9% NaCl solution by intratracheal instillation on day 1 of the experimental period. Control rats were given a single intratracheal dose of saline alone.

2.3. Experimental design

A pilot study was conducted with five different doses of EGCG (10, 15, 20, 25 and 30 mg/kg body weight), post-treated 6 h after bleomycin induction and administered intraperitoneally for 28 days to determine the dose-dependent effect in bleomycin-induced rats. It was observed that EGCG treatment at a dose of 20 mg/kg body weight, significantly (p < 0.05) altered bronchoalveolar lavage fluid (BALF) hydroxyproline level and serum activities of alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) to near normal (BALF) hydroxyproline level and serum activities of alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) to near normal values in bleomycin-induced rats after 28 days of experimental study (data not shown). Hence, the dose of 20 mg/kg was chosen for our study.

The rats were divided into four groups (six rats in each group) as follows,

**Group I:** Control rats.
**Group II:** Bleomycin-induced rats (6.5 U/kg body weight).
**Group III:** Bleomycin-induced rats were treated intraperitoneally with EGCG, administered 6 h after bleomycin induction, at a dosage of 20 mg/kg body weight, once daily throughout the experimental period.
**Group IV:** Control rats treated with EGCG (dosage and duration as mentioned for Group III).

After 28 days of the experimental period, the rats in all the groups were sacrificed. Blood was collected in clean sterilized tubes from which serum was derived. Bronchoalveolar lavage fluid was isolated from both the lungs. A small portion of both the lungs was cut and used for histological studies after which the lungs were homogenized in 0.1 M Tris–HCl buffer (pH 7.4) and were used for biochemical measurements.

2.4. Scoring of fibrotic markers

The level of hydroxyproline, a marker of parenchymal collagen deposition, in the lung tissue homogenate was estimated by the method of Neuman and Logan [22]. Fixed tissue sections were stained with hematoxylin and eosin (H&E) and were used for histopathological analysis that was evaluated using a light microscope (Nikon XDS-1B). The histopathological gradings were evaluated based on inflammation, alveolar damage, and vascular congestion. The severity of lung injury was evaluated by using a blind semi-quantitative scoring system according to the previously defined criteria: no injury = 0, mild injury = 1, moderate injury = 2, and severe injury = 3 [23].

2.5. Assay of marker enzymes

The activities of pathophysiological enzymes such as aspartate transaminase (AST) and alanine transaminase (ALT) were assayed in the serum [24], while the activities of alkaline phosphatase (ALP) [25] and lactate dehydrogenase (LDH) [26] were assayed in both serum and BALF.

The LDH isoenzymes were separated by agarose gel electrophoresis as described earlier [27]. Serum samples from all the group of rats were subjected to 1% agarose gel electrophoresis. After the separation, the gel was incubated in dark for 30 min (37 °C) in a staining solution containing 1.0 ml of 1.0 M lithium lactate, 1.0 ml of 0.1 M sodium chloride, 1.0 ml of 5.0 mM magnesium chloride, 2.5 ml of 0.1% (w/v) nitro blue tetrazolium, 0.25 ml of 0.1% phenazine methosulphate, 2.5 ml of 0.5 M phosphate buffer, pH 7.5 and 10 mg of NAD in a total volume of 10 ml. The isoenzymes separated were visualized as purple bands.

2.6. Analysis of glycoprotein components

To the weighed amount of defatted tissues, 2 ml of 4N HCl was added and the mixture was refluxed at 100 °C for 4 h in a test tube with suitable marble lids. The hydrolysate was neutralised with sodium hydroxide. Aliquots of the neutralised samples were taken for the analysis of hexose and hexosamine. For estimation of sialic acid, the tissues were added with 2.0 ml of 0.1N sulphuric acid and hydrolysed for 1 h at 80 °C.

The level of hexose was estimated by the method of Niebes [28]. Hexosamine was estimated by the method of Wagner [29]. Sialic acid and fucose was estimated by the method of Warren [30] and Dische and Shettles [31], respectively.

Paraffin embedded tissue sections of 4 µM thickness were rehydrated using xylene and graded ethanol solutions. The slides were then stained using 2% periodic acid and Schiff’s reagent in dark for 20 min following the method of Meloan et al. [32]. The slides were then visualized under a light microscope (Nikon XDS-1B).

2.7. Assay of lysosomal hydrolases

Lysosomal fraction was isolated by the method of Wattiaux et al. [33]. The activity of β-d-glucuronidase was assayed by the method of Kawai and Anno [34]. β-d-Galactosidase activity was
assayed by the method of Conchie et al. [35]. The activities of N-acetyl-β-D-glucosaminidase [36] and cathepsin-D [37] were also assayed.

2.8. Ultrastructural studies using transmission electron microscopy

Randomly selected portions from upper and lower lobes of right lungs of control and experimental groups of rats were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 18 h. The lungs were dissected into small pieces and postfixed for 1.5 h in 1% osmium tetroxide dissolved in 0.1 M phosphate buffer (pH 7.4), then dehydrated through a series of graded ethanol solutions and embedded in araldite (epoxy resin). Ultrathin sections were cut, stained with uranyl acetate and lead nitrate, mounted on copper grids and examined under a transmission electron microscope (Philips EM201C).

2.9. Statistical analysis

All the grouped data was evaluated using SPSS/10.0 software. Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. \( p < 0.05 \) was considered to indicate statistical significance. All the results were expressed as mean ± S.D. for six rats in each group.

3. Results

3.1. EGCG attenuates fibrotic markers

The level of hydroxyproline in lung tissues of control and experimental groups of rats is presented in Fig. 1(A). A notable increase in the level of hydroxyproline was observed in bleomycin-challenged rats that were significantly reduced upon EGCG treatment. Fig. 1 (B) represents the histopathological analysis performed in the lung tissue sections of control and experimental groups of rats. Bleomycin-induced rats exhibited significant increase in histopathological parameters such as inflammation, alveolar damage and vascular congestion when compared with control rats. Treatment with EGCG to bleomycin-induced rats considerably reduced the parameters suggesting the anti-fibrotic effect of EGCG.

3.2. EGCG normalizes bleomycin-induced hyperactivities of marker enzymes

The activities of pathophysiological enzymes such as AST, ALT, LDH and ALP in serum of control and experimental groups of rats are presented in Table 1. A notable increase in the activities of these enzymes was observed in bleomycin-induced animals when compared to control group of rats. Administration of EGCG to bleomycin-induced rats normalized the marker enzyme activities.

\[ \text{LDH} \]

\[ \text{ALP} \]

Fig. 1. Effect of EGCG and bleomycin on the level of hydroxyproline and histopathological parameters in control and experimental groups of rats. (A) Level of hydroxyproline; (B) the histopathological parameters were scored semiquantitatively as follows: no injury = 0, mild injury = 1, moderate injury = 2, and severe injury = 3. Values are given statistically significant at \( p < 0.05 \): a bleomycin vs control; b bleomycin + EGCG vs bleomycin; c bleomycin + EGCG vs control.

Fig. 2. Activities of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) in BALF of control and experimental groups of rats. Values are given as mean ± S.D for groups of 6 rats each. Values are given statistically significant at \( p < 0.05 \): a bleomycin vs control; b bleomycin + EGCG vs bleomycin; c bleomycin + EGCG vs control.

\[ \text{LDH} \]

\[ \text{ALP} \]

The above results were published in Sriram et al. [20].

\[ \text{LDH} \]

\[ \text{ALP} \]

\[ \text{LDH} \]

\[ \text{ALP} \]

\[ \text{LDH} \]

\[ \text{ALP} \]

1 The data represented here was taken in part from our published report [20].
Table 1
Activities of pathophysiological enzymes in serum of control and experimental groups of rats.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>LDH (IU/L)</th>
<th>ALP (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>44.27 ± 3.63</td>
<td>74.17 ± 6.15</td>
<td>164.48 ± 13.32</td>
<td>128.83 ± 10.56</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>79.06 ± 7.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>126.53 ± 12.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>284.71 ± 28.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>203.68 ± 20.97&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bleomycin + EGCG</td>
<td>54.19 ± 4.82&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>89.53 ± 8.14&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>194.37 ± 17.88&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>144.89 ± 13.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>EGCG</td>
<td>45.09 ± 3.69</td>
<td>72.41 ± 5.86</td>
<td>161.82 ± 13.43</td>
<td>131.64 ± 10.79</td>
</tr>
</tbody>
</table>

Units—activity is expressed as μmol pyruvate liberated/mg of protein/h for AST, ALT and LDH; IU/L for ALP.
Values are given as mean ± S.D. for groups of 6 rats each. Values are given statistically significant at p < 0.05.

<sup>a</sup> Bleomycin vs control.
<sup>b</sup> Bleomycin + EGCG vs bleomycin.
<sup>c</sup> Bleomycin + EGCG vs control.

Table 2
Levels of glycoconjugates in lungs of control and experimental groups of rats.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Hexose (mg/g defatted tissue)</th>
<th>Hexosamine (mg/g defatted tissue)</th>
<th>Sialic acid (mg/g defatted tissue)</th>
<th>Fucose (mg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.52 ± 0.70</td>
<td>7.48 ± 0.46</td>
<td>0.41 ± 0.01</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>26.19 ± 2.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.08 ± 0.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.57 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.38 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bleomycin + EGCG</td>
<td>16.13 ± 1.12&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>9.58 ± 0.66&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.48 ± 0.02&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0.30 ± 0.01&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>EGCG</td>
<td>12.06 ± 0.75</td>
<td>7.22 ± 0.44</td>
<td>0.39 ± 0.01</td>
<td>0.23 ± 0.01</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.D. for groups of 6 rats each. Values are given statistically significant at p < 0.05.

<sup>a</sup> Bleomycin vs control.
<sup>b</sup> Bleomycin + EGCG vs bleomycin.
<sup>c</sup> Bleomycin + EGCG vs control.

Table 3
Levels of glycoconjugates in serum of control and experimental groups of rats.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Hexose (mg/dl)</th>
<th>Hexosamine (mg/dl)</th>
<th>Sialic acid (mg/dl)</th>
<th>Fucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>174.86 ± 14.33</td>
<td>29.68 ± 2.40</td>
<td>38.20 ± 3.13</td>
<td>31.85 ± 2.64</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>313.18 ± 32.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.59 ± 4.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.39 ± 5.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.30 ± 4.77&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bleomycin + EGCG</td>
<td>207.49 ± 18.88&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>34.07 ± 2.99&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>44.17 ± 3.93&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>38.45 ± 3.47&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>EGCG</td>
<td>172.04 ± 13.93</td>
<td>28.87 ± 2.39</td>
<td>39.26 ± 3.21</td>
<td>32.17 ± 2.60</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.D. for groups of 6 rats each. Values are given statistically significant at p < 0.05.

<sup>a</sup> Bleomycin vs control.
<sup>b</sup> Bleomycin + EGCG vs bleomycin.
<sup>c</sup> Bleomycin + EGCG vs control.
Table 4
Activities of matrix degrading lysosomal enzymes in serum of control and experimental groups of rats.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>β-β-D-Glucuronidase</th>
<th>β-β-D-Galactosidase</th>
<th>N-acetyl-β-β-D-glucosaminidase</th>
<th>Cathepsin-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.23 ± 0.69</td>
<td>15.06 ± 0.91</td>
<td>20.54 ± 1.29</td>
<td>14.54 ± 0.90</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>17.39 ± 1.41</td>
<td>27.74 ± 2.19</td>
<td>28.63 ± 2.23</td>
<td>21.18 ± 1.65</td>
</tr>
<tr>
<td>Bleomycin + EGCG</td>
<td>14.07 ± 0.99</td>
<td>20.62 ± 1.42</td>
<td>24.29 ± 1.74</td>
<td>18.37 ± 1.26</td>
</tr>
<tr>
<td>EGCG</td>
<td>10.49 ± 0.67</td>
<td>15.89 ± 1.01</td>
<td>21.03 ± 1.28</td>
<td>14.17 ± 0.87</td>
</tr>
</tbody>
</table>

Units: μmol of p-nitrophenol liberated/h/100 mg protein for β-β-D-glucuronidase, N-acetyl-β-β-D-glucosaminidase, β-β-D-galactosidase; μmol of tyrosine liberated/h/100 mg protein for cathepsin-D.

Values are given as mean ± S.D. for groups of 6 rats each. Values are given statistically significant at p < 0.05.

a Bleomycin vs control.
b Bleomycin + EGCG vs bleomycin.
c Bleomycin + EGCG vs control.

activities by significantly bringing back the values closer to normal.

The activities of LDH and ALP in BALF of control and experimental groups of animals are shown in Fig. 2. Bleomycin induction to rats increased the activities of these enzymes as compared to control. EGCG supplementation to bleomycin-induced rats decreased the activities of these marker enzymes when compared to bleomycin-induced group.

Table 5
Activities of matrix degrading lysosomal enzymes in lungs of control and experimental groups of rats.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>β-β-D-Glucuronidase</th>
<th>β-β-D-Galactosidase</th>
<th>N-acetyl-β-β-D-glucosaminidase</th>
<th>Cathepsin-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33.28 ± 2.72</td>
<td>36.27 ± 2.97</td>
<td>41.74 ± 3.46</td>
<td>20.39 ± 1.67</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>52.18 ± 5.06</td>
<td>49.66 ± 4.91</td>
<td>64.42 ± 6.24</td>
<td>33.71 ± 3.40</td>
</tr>
<tr>
<td>Bleomycin + EGCG</td>
<td>39.31 ± 3.53</td>
<td>42.51 ± 3.78</td>
<td>48.93 ± 4.30</td>
<td>27.17 ± 2.40</td>
</tr>
<tr>
<td>EGCG</td>
<td>32.57 ± 2.73</td>
<td>36.83 ± 3.05</td>
<td>42.07 ± 3.40</td>
<td>19.83 ± 1.64</td>
</tr>
</tbody>
</table>

Units: μmol of p-nitrophenol liberated/h/100 mg protein for β-β-D-glucuronidase, N-acetyl-β-β-D-glucosaminidase, β-β-D-galactosidase; μmol of tyrosine liberated/h/100 mg protein for cathepsin-D.

Values are given as mean ± S.D. for groups of 6 rats each. Values are given statistically significant at p < 0.05.

a Bleomycin vs control.
b Bleomycin + EGCG vs bleomycin.
c Bleomycin + EGCG vs control.
Fig. 3. Transmission electron microscopic analysis of lung sections of control and experimental groups of rats. 
(a) Normal structure of nuclei and cytoplasm in lungs of control animals (x10,000); (b) Normal architecture of type II pneumocyte observed in lungs of control rats (7000×); (c) Irregular shrunken nuclei with abnormal cytoplasm observed in bleomycin-induced group (15,000×); (d) Excess accumulation of collagen fibrils in lungs of bleomycin-induced group (15,000×); (e) Type II epithelium showing ballooning of the endothelial cell in the capillary lumen with lamellar bodies in lungs of bleomycin-induced group (7000×); (f) Restoration of normal architecture of nuclei observed in bleomycin + EGCG group (10,000×); (g) Reduced collagen fibers accumulation in lungs of bleomycin + EGCG group (30,000×); (h) Less endothelial cell ballooning in type II epithelium observed in bleomycin + EGCG group (7000×); (i) Normal architecture of nuclei observed in EGCG alone treated group (15,000×); (j) Normal architecture of type II epithelium in lungs of EGCG treated group (15,000×).
in LDH activity of BALF were characterized by increased iso-enzymes LDH-3, LDH-4 and LDH-5 as compared to serum. The induced changes in the LDH isoenzyme pattern suggest that the increased LDH activity of BALF arises from lung tissue. Inflammatory cells in the lungs and leakage of plasma into the interstitium and alveolar spaces may slightly contribute to the increase of cell-free BALF LDH activity. All the abnormal variations in the isomorphic pattern of LDH isoenzymes in bleomycin challenged rats were resolved upon EGCG treatment that signifies the protective effect of the tea catechin against lung injury and subsequent pulmonary fibrosis.

Glycoproteins are predominantly protein in nature with one or more heterosaccharide chains that contains hexose, hexosamine, sialic acid and fucose. Many substances of biologic importance, including enzymes, hormones, antibodies and membranes, represent these conjugated proteins. The elevation in the levels of glycoprotein components of serum is due to the secretion of cell membrane glycoconjugates into the circulation [48]. The observed increase in the levels of glycoprotein moieties in bleomycin-induced rats may also be due to increased deposition of macromolecular components, which is a physiological adjustment to the pathological process. Alterations in glycoprotein concentration during inflammatory conditions, liver fibrosis, malignancy, and in tumorous human lung tissue are reported earlier [49–51]. In addition, abnormal increase of glycoprotein components has been reported during pulmonary fibrosis [52]. Evidence has also been presented that pulmonary elastin and hexosamine contents are increased in bleomycin-injured lung tissue [53]. The observed increase in the sugar moieties of the glycoproteins can also be related to the augmentation in the concentration and synthesis of corresponding glycoprotein synthesizing enzymes. Elevated levels of glycoprotein synthesizing enzymes are associated to the inflamed state and increased activities of sialyl and galactosyl transferase were observed in the serum and liver of inflamed rats during earlier studies [54]. In another study it was reported that the activity of glucosyl transferase and the rate of transport of N-acetylglucosamine, galactose and sialic acid to an appropriate sugar depleted acceptor glycopeptide were increased during the process of inflammation [55]. Earlier reports suggest that during bleomycin administration, a variety of cytokines elaborated by the inflammatory cells may contribute significantly to the lung inflammation and may initiate the fibrotic process [56,57]. The above reports substantiate our study where administration of bleomycin increased the levels of glycoprotein moieties in both lungs and serum. Supplementation with EGCG reduced the levels of glycoprotein moieties in lungs and serum of bleomycin-induced rats, indicating its potential anti-inflammatory and immunosuppressive activity [58,59] that might have inhibited the inflammation and synthesis of glycoprotein synthesizing enzymes.

Lysosomes are essential for controlled intracellular digestion of cellular components by different pathways such as autophagy, heterophagy and endocytosis. Elevated lysosomal enzymes in the extracellular fluid occur as a result of decreased lysosomal membrane stability [60]. This eventually affects the metabolism of different connective tissue constituents, viz. glycosaminoglycans (GAGs), glycoproteins, collagen and results in irreversible tissue damage. The primary in vivo action of lysosomal enzyme on ECM results in the initial breakdown of proteoglycans and thereby exposing collagen fibers to the collagenolytic enzyme [61]. The elevation in the activities of lysosomal enzymes might result in the increased liberation of glycoconjugates from the tissue. β-Glucuronidase activity is associated with cell proliferation, and thus, an index of high tissue turnover. The increased β-glucuronidase activity reflects catabolic events, concerning with the degradation of GAGs. Proteases of the cathepsin family are among the most studied lysosomal hydrolases. Certain crucial functions of cathepsins outside the lysosomal compartment includes, degradation of the ECM or induction of fibroblast invasive growth when secreted into the extracellular space [62,63]. The increased activities of glycohydrolases and cathepsin-D observed in our study indicate excess ECM deposition and the possible infiltration of inflammatory cells during the progression of pulmonary fibrosis, which might be due to the consequence of unmitigated lipid peroxidation. Similar increases in the activities of the lysosomal enzymes during pulmonary fibrosis were reported earlier that further strengthen our study [64]. Administration of EGCG attenuated the bleomycin-induced alterations of matrix degrading lysosomal hydrolases probably by limiting the levels of lipid peroxidation and intensity of inflammation.

Intratracheal administration of bleomycin to rodents is considered to reproduce the histologic and cellular alterations that are found in human pulmonary fibrosis [12]. In addition, another report suggests that a variety of cells of the lung are affected by intratracheal or intravenous injection of bleomycin that can be visualized using a transmission electron microscope [65]. The report also suggests that, although intratracheal injection of bleomycin does damage some part of lower lobe, it does not damage the entire lower respiratory tract of rodents. Thus, the induction model adopted in the present study can be considered as a representative for TEM analysis. The ultrastructural findings in bleomycin-induced rats shows physical distortion of type II alveolar epithelium, shrunken cell nucleus and excess collagen deposition, which reflects the effect of lipid peroxidation on membrane phospholipids. This assumption is further supported by the presence of lamellar body residues and ballooning of endothelial cells within the alveolar lumen, which is in agreement with data reported in other experimental models of lung injury [66,67]. Regardless of the inciting injurious trigger, lung injury is accompanied by capillary endothelial cell injury and hemorrhage, suggesting that the microvasculature and endothelium are involved [68]. The concept of endothelial cell injury as a mechanism eventuating in pulmonary fibrosis has been reported earlier [69]. Collagen fibers are clearly the connective tissue elements responsible for fibrosis observed in lungs of bleomycin-induced rats. In certain areas of the fibrotic lung, direct penetration of respiratory areas of the septal units by collagen fibrils were found, which were positioned between the basement membrane and the surface of endothelial cells. This invasion of the air–blood space would increase the distance between the air and blood compartments and thus may hamper gas exchange in the alveoli. Treatment with EGCG to bleomycin-induced rats ameliorated all the observed ultrastructural changes thus establishing its protective efficacy against bleomycin-induced pulmonary fibrosis.

The present study addresses the beneficial influence of EGCG on the levels of glycoprotein moieties and the activities of lysosomal hydrolases during bleomycin-induced experimental pulmonary fibrosis. EGCG also possesses free radical scavenging and anti-inflammatory property as witnessed by the ultrastructural studies. Thus the observed protective effect can be attributed to the membrane stabilizing and antioxidant properties of EGCG that contributes its anti-fibrotic property.

**Conflict of interests**

The authors declare no conflict of interests exist.

**References**


