## Annexure I: Daily Clinical Observations of Control Group Rats

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: 1 = Normal, 2 = Dead, 23 = Dyspnea, 24 = Abdominal Breathing, 25 = Gasping, 27 = Tachypnea, 28 = Nasal Discharge, 146 = Coughing
| Animal Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---------------|---|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|    |
| 11            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |    |
| 12            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |    |
| 13            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |    |
| 14            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |    |
| 15            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |    |
| 16            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |    |
| 17            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |    |
| 18            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |    |
| 19            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |    |
| 20            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |    |

**Note:** 1 = Normal, 2 = Dead, 23 = Dyspnea, 24 = Abdominal Breathing, 25 = Gasping, 27 = Tachypnea, 28 = Nasal Discharge, 146 = Coughing
Annexure III: Daily Clinical Observations of Quercetin Treated Rats

| Animal Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 21            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| 22            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| 23            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| 24            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| 25            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| 26            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| 27            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| 28            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| 29            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
| 30            | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |

Note: 1 = Normal, 2 = Dead, 23 = Dyspnea, 24 = Abdominal Breathing, 25 = Gasping, 27 = Tachypnea, 28 = Nasal Discharge, 146 = Coughing
Annexure IV: Daily Clinical Observations of Sulindac Treated Rats

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>34</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>35</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>36</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>37</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>38</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>39</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>40</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: 1 = Normal, 2 = Dead, 23 = Dyspnea, 24 = Abdominal Breathing, 25 = Gasping, 27 = Tachypnea, 28 = Nasal Discharge, 146 = Coughing
Annexure V: Daily Clinical Observations of Rats treated with Quercetin and Sulindac in combination

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>41</td>
<td>1</td>
</tr>
<tr>
<td>42</td>
<td>1</td>
</tr>
<tr>
<td>43</td>
<td>1</td>
</tr>
<tr>
<td>44</td>
<td>1</td>
</tr>
<tr>
<td>45</td>
<td>1</td>
</tr>
<tr>
<td>46</td>
<td>1</td>
</tr>
<tr>
<td>47</td>
<td>1</td>
</tr>
<tr>
<td>48</td>
<td>1</td>
</tr>
<tr>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: 1 = Normal, 2 = Dead, 23 = Dyspnea, 24 = Abdominal Breathing, 25 = Gasping, 27 = Tachypnea, 28 = Nasal Discharge, 146 = Coughing
Evaluating the inhibitory potential of sulindac against the bleomycin-induced pulmonary fibrosis in wistar rats

Ramesh Verma\textsuperscript{a}, Mahesh Brahmankar\textsuperscript{a}, Lokendra Kushwah\textsuperscript{a}, Balakrishnan Suresh\textsuperscript{b,*}

\textsuperscript{a} Department of Toxicology, Jai Research Foundation, Valvada 396108, Gujarat, India
\textsuperscript{b} Division of Toxicology, Department of Zoology, Faculty of Science, The M.S. University of Baroda, Vadodara 390002, Gujarat, India

\textbf{ARTICLE INFO}

Article history:
Received 30 April 2013
Accepted 15 July 2013
Available online 24 July 2013

Keywords:
Bleomycin
Lung fibrosis
Sulindac
Hydroxyproline
Tumour necrosis factor-\(\alpha\)

\textbf{ABSTRACT}

The present study examined the protective effect of sulindac on bleomycin-induced lung fibrosis in rats. Animals were divided into saline group, bleomycin group (single intratracheal instillation of bleomycin) and bleomycin+ sulindac (orally from day 1 to day 20). Bleomycin administration reduced the body weight, altered antioxidant status (such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione) while it increased the lung weight, hydroxyproline content, collagen deposition and lipid peroxidation. However, simultaneous administration of sulindac improved the body weight, antioxidant status and decreased the collagen deposition in lungs. Moreover, the levels of inflammatory cytokine tumour necrosis factor-\(\alpha\) increased in bleomycin-induced group, whereas, on treatment with sulindac the levels of tumour necrosis factor-\(\alpha\) were found reduced. Finally, histological evidence also supported the ability of sulindac to inhibit bleomycin-induced lung fibrosis. The results of the present study indicate that sulindac can be used as an agent against bleomycin-induced pulmonary fibrosis.

© 2013 Elsevier B.V. All rights reserved.

1. \textbf{Introduction}

Pulmonary fibrosis is a severe chronic disease with various causes and poor prognosis. Its main histological features include lesions of the alveolar septa, fibroblast and myofibroblast proliferation in lung parenchyma, abnormal re-epithelialization, and excessive extracellular matrix deposition. Lung fibrosis is associated with chronic inflammation and is characterized by the recruitment of macrophages, neutrophils, and lymphocytes in the airways. During lung inflammation, activated phagocytes release large amounts of reactive oxygen species, which may be involved in tissue injury and in impeding tissue repair, both of which lead to pulmonary fibrosis. Recent studies show that antioxidant compounds protect rats against tissue damage and pulmonary fibrosis because these compounds can attenuate the oxidant burden on tissue (Manoury et al., 2005).

Bleomycin is a commonly used chemotherapeutic agent that can cause dose-dependent pulmonary fibrosis (Jules-Elysee and White, 1990). Bleomycin-induced pulmonary injury and lung fibrosis has been documented in several animal species (Wang et al., 1991; Tzurel et al., 2002). This model has been widely used for studying the mechanisms involved in
the progression of human pulmonary fibrosis and the impact of various drugs on this progression (Yara et al., 2001; El-Khatib, 2002). Bleomycin is known to generate reactive oxygen species upon binding to DNA and iron, which cause DNA damage. The interaction of bleomycin with DNA appears to initiate the inflammatory and fibroproliferative changes via a concerted action of various cytokines leading to collagen accumulation in the lungs. Bleomycin also promotes the depletion of endogenous antioxidant defences thus exacerbating oxidant mediated tissue injury (Atzori et al., 2004). The lung is selectively affected by bleomycin because this tissue lacks an enzyme that hydrolyzes the ε-aminoalanine moiety of bleomycin, which prevents its metabolite from binding metals such as iron (Feldman et al., 1988).

Sulindac ([Z]-5-fluoro-2-methyl-1-[p-(methylsulfnyl)]-benzylidenejindene-3-acetic acid), a non-steroidal anti-inflammatory drug, is well known for its anti-inflammatory activity, which is due to its ability to inhibit the cyclooxygenases enzymes thereby inhibiting prostaglandin synthesis (Vane et al., 1998). Sulindac is a sulfoxide prodrug, which is converted in vivo to the metabolites sulindac sulfide and sulindac sulfoxone. In addition to their anti-inflammatory properties, sulindac and its metabolites have been shown to play an important role in the prevention of colonic carcinogenesis (Fernandes et al., 2003).

The forerunner for bleomycin-induced pulmonary fibrosis has been considered the inflammatory response induced by the anti-neoplastic agent (Arafa et al., 2007). Therefore, sulindac has been utilized in the current study primarily for its anti-inflammatory effects. Besides, the notion that the cyclooxygenases inhibitor has shown some antiradical effects prompted us to investigate its modulatory effects on bleomycin-induced lung fibrosis possibly by regulating the oxidant/anti-oxidant imbalance (Fernandes et al., 2003).

Taking all that into consideration, we have addressed in the present work whether or not sulindac can inhibit bleomycin-induced lung injury using a rat model of lung fibrosis.

2. Materials and methods

2.1. Animals

Specific pathogen-free, healthy young adult male Wistar rats (RCCHan:WIST), weighing 274–362 g, were used in this study. They were obtained from the Barrier Maintained Rodent Animal Breeding Facility, Jai Research Foundation, Vapi, India. All the animals were fed with standard Teklad Certified Global High Fibre rat feed manufactured by Harlan, USA and provided U.V. sterilized water filtered through Kent Reverse Osmosis water filtration system ad libitum. The rats were kept in a controlled environment (temperature: 22 ± 2 °C and relative humidity: 30–70%) with an alternating cycle of 12-h light and dark. The animals used in this study were handled and treated strictly in accordance with the guiding principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The test facility at Jai Research Foundation is AAALAC accredited and also complies with the National Good Laboratory Practice, India. The experimental protocols were approved by the Institutional Animal Ethics Committee (Approval no. 35/1999/CPCSEA).

2.2. Experimental design

Rats were randomized into 3 groups, each consisting of 8 animals (Gad and Weil, 1994). Briefly, after the weights were recorded, the rats were anesthetized using a combination of ketamin (80 mg/kg body weight, i.p.) and xylazine (20 mg/kg body weight, i.p.) as per standard protocol (Teixeira et al., 2008). A midline incision was made in the neck and the trachea was exposed. A tracheal cannula was inserted into the trachea under direct visualization. For induction of pulmonary fibrosis, the rats received a single dose of 6.5 U/kg body weights bleomycin sulfate dissolved in 0.5 mL of 0.9% NaCl solution by intratracheal instillation on day 0 of the experiment (Wang et al., 2002). Control group rats were given a single intratracheal dose of sterile saline. Group 1 (vehicle control) and Group II (bleomycin treated) rats were treated with 0.5% carboxymethylcellulose solution (10 ml/kg body weight) from day 1 to day 20 of the experiment. Animals from Group III were treated with sulindac within its therapeutic anti-inflammatory dose (ED50 for rats, 20 mg/kg body weight) in carboxymethylcellulose solution for day 1 to day 20 of the experiment after bleomycin instillation (Vaish and Sanyal, 2012). The drug was freshly prepared and the concentration was adjusted so that each animal received 10 ml/kg body weight. The animals were weighed at the beginning, through and at the end of experiments. The changes in body weight were recorded.

2.3. Biochemical assays

2.3.1. Preparation of lung tissue for biochemical studies

On day 21 of the experiment, five animals from each group were sacrificed using thiopentone sodium and the lung lobes were excised. Broncholaveolar lavage was performed in three animals from each group under anaesthesia with thiopentone sodium.

2.3.2. Determination of lung hydroxyproline

The hydroxyproline assay was performed as described by Edwards and O’brien (1980). Briefly, the lung was dried and hydrolysed at 120 °C in a pressure vessel for 2–4 h. The acid hydrolysates and standards were added to 1.5-ml tubes, along with the same volume of citric/acetate buffer (citric acid, sodium acetate, sodium hydroxide, glacial acetic acid and n-propanol, pH 6.0) and chloramine T solution (chloramine T dissolved in Milli-Q water). The tubes were incubated for 20 min at room temperature and Ehrlich’s solution [aldehydepchloric acid reagent (p-dimethyl-amino-benzaldehyde) perchloric acid and n-propanol] was added to the tubes, which were then incubated at 60 °C for 15 min. The absorbance (OD at 550 nm) of the reaction product was read.

2.3.3. Determination of lipid peroxidation

Malondialdehyde is the most abundant individual aldehyde resulting from lipid peroxidation breakdown in biological systems and is commonly used as an indirect method for
estimation of lipid peroxidation. Malondialdehyde content was assayed using the thiobarbituric acid test as described by Ohkawa et al. (1979). Malondialdehyde reacts with thiobarbituric acid to form a coloured complex. Absorbance was measured at 532 nm.

2.3.4. Determination of reduced glutathione

Reduced glutathione level was measured by the method of Ellman (1959). To the homogenate 5% trichloro acetic acid was added to precipitate the protein content of the homogenate. After centrifugation (at 3000 rpm for 10 min) the supernatant was taken and DTNB solution (Ellman's reagent) was added to it. The absorbance was measured at 412 nm.

2.3.5. Determination of glutathione peroxidase activity

The glutathione peroxidase activity was based on the method of Paglia and Valentine (1967). Tert-butylhydroperoxide was used as substrate. The assay measures the enzymatic reduction of H₂O₂ by glutathione peroxidase through consumption of reduced glutathione that is restored from oxidized glutathione GSSG in a coupled enzymatic reaction by glutathione reductase (GR). GR reduces GSSG to GSH using NADPH as a reducing agent. The decrease in absorbance was recorded at 340 nm was recorded.

2.3.6. Estimation of glutathione content

The concentration of glutathione in the lung was assayed by the method of Grunert and Philips (1951). Glutathione present in the tissue reacts with sodium nitroprusside to give a red coloured complex in saturated alkaline medium. The absorbance was measured at 520 nm.

2.3.7. Measurement of superoxide dismutase

The activity of superoxide dismutase was measured following the method of Kakkar et al. (1984). A known amount tissue homogenates was mixed with sodium pyrophosphate buffer, phenazine methosulphate and nitroblue tetrazolium chloride. The reaction was started by the addition of NADH. The reaction mixture was incubated at 30 °C for 90 s and stopped by the addition of 1 ml of glacial acetic acid. The absorbance of the chromogen formed was measured at 560 nm.

2.3.8. Determination of catalase activity

Catalase activity was assessed by the method of Luck (1963), wherein the breakdown of hydrogen peroxide is measured. In brief, the assay mixture consisted of 3 ml of H₂O₂-phosphate buffer and 0.05 ml of the supernatant of the tissue homogenate. The change in absorbance was recorded for a minutes at 30-s interval at 240 nm.

2.3.9. Broncholaveolar lavage

Broncholaveolar lavage fluid was obtained by the injection of 3 ml saline (three times, total 9 ml) followed by gentle aspiration of the fluid from the lung after securing an intratracheal catheter within a trachea. With this catheter, the ratio of the recovery of lavage fluid was approximately 80% and did not significantly differ among the groups. The total numbers of cells in the broncholaveolar lavage fluid were counted with a hemocytometer. For differential counts of leukocytes in the broncholaveolar lavage fluid, smear slides were prepared and stained with Giemsa solution. Differential cell counts were performed on 300 cells per smear.

2.3.10. Measurement of tumour necrosis factor-α

Tumour necrosis factor-α concentration was measured using an enzyme-linked immunosorbent assay kit (Xpressbio Life Scientific Products). The determinations were done according to the Test Kit instructions.

2.4. Histological studies

After sacrifice, each lung tissue was perfused and fixed in 10% neutral buffer formalin and routinely processed and embedded in paraffin. Serial sections (4 μm) were cut and stained with haematoxylin & eosin and Masson trichrome for light microscopic evaluation to examine the degree of fibrosis. The severity of fibrosis was individually assessed using the semi-quantitative grading system described by Szapiel et al. (1979). The scores of fibrosis in lung specimens were graded from – to +++ and correspondingly numbered as from 0 to 3. The entire lung section was reviewed at a magnification of 10×. Each of the 25 random microscopic fields per section were detected, a score ranging from 0 to 3 was assigned. All assessments were performed in blind fashion.

2.5. Materials

Sulindac, chloramine-T and hydroxyproline were procured from Sigma-Aldrich Chemie GmbH. Bleomycin hydrochloride was procured from the market and was in the form of bleomycin ampoules (15 units) manufactured by Biochem Pharmaceutical Industries Ltd., Mumbai, India. All other chemicals were of analytical grade and procured from reputed manufacturers of India, viz., Sisco Research Laboratories Pvt. Ltd., Qualigen Fine Chemicals Pvt. Ltd. and HiMedia Laboratory Pvt. Ltd.

2.6. Statistical analysis

Statistical analyses were carried out by analysis of variance (ANOVA) followed by post hoc test (Dunn’s test). All analyses of data were performed using SPSS for windows version 12.0 and probability values of 0.05 or less were considered statistically significant.

3. Results

3.1. Changes in body weight

Fig. 1 shows the effect of sulindac on the body weight of bleomycin administered groups of rats. Single intratracheal administration of bleomycin (6.5 U/kg) resulted in a marked decrease in their body weight on day 14 as compared to the saline treated control group because of severe tissue damage caused by free radicals. However, body weight of sulindac-treated rats remained comparable to the control group rats throughout the experiment.
3.3. **Hydroxyproline content**

The effect of sulindac on the hydroxyproline content of lung homogenates is presented in Table 1. It is well known that the hallmark of fibrosis is collagen deposition. Measurement of hydroxyproline is an efficient index of collagen deposition, since collagen contains significant amounts of the amino acid. After 21 days, the hydroxyproline content of the lungs in the bleomycin group increased significantly when compared to the control group. Lung hydroxyproline content in the bleomycin + sulindac group was found significantly lower than that of bleomycin group.

3.4. **Lipid peroxidation**

The result of this study showed an increase in the level of lipid peroxidation in bleomycin administered group when compared to the control group, which might be due to tissue injury and damage. Sulindac treatment however, significantly lowered the bleomycin induced lipid peroxidation in the lungs of rats as evident from the MDA levels (Table 1).

3.5. **Reduced glutathione**

Table 1 shows the changes in the level of reduced glutathione in control, bleomycin-administered and sulindac treated lung tissues. The results amply testify that bleomycin has significantly decreased the levels of reduced glutathione compared to that of control group. Sulindac treated group of rats showed significantly higher levels of reduced glutathione when compared with corresponding bleomycin treated group.

3.6. **Glutathione peroxidase activity**

Bleomycin produced a significant reduction in the glutathione peroxidase activities in lung tissue after 21 days when compared with control groups (Table 1). The depletion in glutathione peroxidase activity in the tissue reflects indirectly the generation of free radicals. However, treatment with sulindac improved the activity of glutathione peroxidase as is evident from the significantly higher values of glutathione peroxidase activity in the lungs of sulindac group compared to bleomycin group.

![Graph showing body weight changes](image)

**Fig. 1** – The mean body weight of rats during the course of experiment. *n = 8; *p < 0.05 vs. control group.

![Graph showing organ weight changes](image)

**Fig. 2** – The relative weights of lungs in rats subjected to various treatments. BLM – bleomycin; SLD – sulindac; **p < 0.01 vs. control group. *p < 0.05 vs. bleomycin Group. #p < 0.01 vs. bleomycin group.

### Table 1 – The hydroxyproline content and oxidative stress status of lung tissue of rats subjected to various treatments.

<table>
<thead>
<tr>
<th>Biochemical estimations</th>
<th>Control</th>
<th>Bleomycin</th>
<th>Bleomycin + sulindac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyproline content (mg/g dried tissue)</td>
<td>1.77 ± 0.07</td>
<td>2.54 ± 0.19</td>
<td>1.93 ± 0.14</td>
</tr>
<tr>
<td>Malondialdehyde level (nmol MDA/mg tissue/60 min)</td>
<td>44.62 ± 1.23</td>
<td>60.06 ± 1.64</td>
<td>46.60 ± 2.16</td>
</tr>
<tr>
<td>Reduced glutathione (µg/g tissue)</td>
<td>1.78 ± 0.04</td>
<td>1.07 ± 0.07</td>
<td>1.67 ± 0.05</td>
</tr>
<tr>
<td>Glutathione peroxidase (U/g tissue)</td>
<td>5.51 ± 0.13</td>
<td>2.44 ± 0.17</td>
<td>3.33 ± 0.15</td>
</tr>
<tr>
<td>Glutathione content (µg tissue)</td>
<td>38.64 ± 3.26</td>
<td>24.77 ± 1.96</td>
<td>35.38 ± 2.61</td>
</tr>
<tr>
<td>Superoxide dismutase (U/mg tissue)</td>
<td>0.34 ± 0.03</td>
<td>0.22 ± 0.01</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>Catalase (µM H₂O₂ consumed/mg tissue/min)</td>
<td>19.22 ± 1.13</td>
<td>12.58 ± 0.94</td>
<td>17.37 ± 1.01</td>
</tr>
</tbody>
</table>

Values are given as Mean ± SE for groups of six rats each.

**p < 0.01 vs. control group.

*p < 0.01 vs. bleomycin group.
3.7. **Glutathione content**

Significantly ($p < 0.01$) lower level of glutathione was observed in the lung tissues of bleomycin administered rats as compared to that of the controls. However, treatment with sulindac enhanced the glutathione content significantly by day 21 (Table 1).

3.8. **Superoxide dismutase activity**

Effect of bleomycin and bleomycin plus sulindac on lung tissue superoxide dismutase activity is presented in Table 1. The superoxide dismutase activity of bleomycin-treated rats significantly decreased as compared to the control group. Administration of sulindac was found to significantly restore the activity of antioxidant enzyme superoxide dismutase.

3.9. **Catalase activity**

The catalase activity in the lung homogenate of bleomycin-treated rats was considerably lower than that of vehicle control rats. In the sulindac-treated group, the catalase activity was significantly higher as compared to the bleomycin-treated group (Table 1).

3.10. **Total and differential cell count in bronchoalveolar lavage fluid**

Table 2 shows the effect of sulindac on bronchoalveolar lavage fluid differential and total cell count in control and experimental groups of rats. Bleomycin treatment caused a significant increase in the total cell count in the bronchoalveolar lavage fluid as compared to control rats. In rats treated with sulindac, total cell count remained similar to the levels of control rats. The differential cell count showed a significant increase in neutrophils and eosinophils in the lungs of rats exposed to bleomycin. The sulindac treatment for 21 days significantly reduced the bleomycin-induced hike in the blood cells in the bronchoalveolar lavage. While the percentage of lymphocytes and alveolar macrophages were decreased in bleomycin-induced group, treatment with sulindac upturned these changes significantly.

3.11. **Tumour necrosis factor-α concentration**

Plasma levels of tumour necrosis factor-α are presented in Table 3. The tumour necrosis factor-α protein levels in plasma from rats in bleomycin administered group remained elevated on day 21 when compared with the sham group. Treatment with sulindac was found to decrease the bleomycin-induced increase in tumour necrosis factor-α level at the end of the experiment.

### Table 2 – The total and differential blood cell count of rats from various study groups.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Total cells ($\times 10^6 \text{ ml}^{-1}$)</th>
<th>Macrophage (%)</th>
<th>Neutrophils (%)</th>
<th>Eosinophil (%)</th>
<th>Lymphocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.68 ± 0.13</td>
<td>84.22 ± 1.41</td>
<td>2.11 ± 0.37</td>
<td>0.78 ± 0.15</td>
<td>12.33 ± 1.11</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>1.63 ± 0.18</td>
<td>48.00 ± 3.33**</td>
<td>39.00 ± 1.56**</td>
<td>4.00 ± 0.67**</td>
<td>5.67 ± 0.89**</td>
</tr>
<tr>
<td>Bleomycin + sulindac</td>
<td>0.93 ± 0.05**</td>
<td>71.11 ± 1.93**</td>
<td>11.44 ± 1.41**</td>
<td>3.89 ± 0.59**</td>
<td>12.89 ± 0.15**</td>
</tr>
</tbody>
</table>

Values are given as Mean ± SE for groups of four rats each.

** $p < 0.01$ vs. control group.

### Table 3 – The tumour necrosis factor-α and grade of fibrosis in rats subjected to various treatments.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Tumour necrosis factor-α (pg/mL)</th>
<th>Grade of fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.82 ± 2.10</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>53.89 ± 3.04**</td>
<td>2.50 ± 0.07**</td>
</tr>
<tr>
<td>Bleomycin + sulindac</td>
<td>26.69 ± 2.42**</td>
<td>1.58 ± 0.15**</td>
</tr>
</tbody>
</table>

Values are given as Mean ± SE for groups of six rats each.

** $p < 0.01$ vs. control group.

### Table 3 – The tumour necrosis factor-α and grade of fibrosis in rats subjected to various treatments.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Tumour necrosis factor-α (pg/mL)</th>
<th>Grade of fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.82 ± 2.10</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>53.89 ± 3.04**</td>
<td>2.50 ± 0.07**</td>
</tr>
<tr>
<td>Bleomycin + sulindac</td>
<td>26.69 ± 2.42**</td>
<td>1.58 ± 0.15**</td>
</tr>
</tbody>
</table>

Values are given as Mean ± SE for groups of six rats each.

** $p < 0.01$ vs. control group.

### Table 3 – The tumour necrosis factor-α and grade of fibrosis in rats subjected to various treatments.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Tumour necrosis factor-α (pg/mL)</th>
<th>Grade of fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.82 ± 2.10</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>53.89 ± 3.04**</td>
<td>2.50 ± 0.07**</td>
</tr>
<tr>
<td>Bleomycin + sulindac</td>
<td>26.69 ± 2.42**</td>
<td>1.58 ± 0.15**</td>
</tr>
</tbody>
</table>

Values are given as Mean ± SE for groups of six rats each.

** $p < 0.01$ vs. control group.

** $p < 0.01$ vs. bleomycin group.

Normal lung tissues showed typical open alveoli, inter-alveolar spaces with customary terminal bronchi, normal appearance of bronchiolar epithelium, thin interalveolar septa, lack of inflammatory cells and fibrosis.

![Fig. 3 – Control lung tissue showing normal open alveoli and interalveolar space (Haematoxylin and Eosin staining, 10×).](image-url)
Bleomycin-administered group rats showed distorted architecture of the tissue i.e. moderate to severe haemorrhages, congestion, emphysema, sloughing of bronchial epithelium from basement membrane, areas of increased alveolar thickening, leukocytes accumulation in alveolar walls and increased fibrosis. However, lungs from sulindac-treated rats showed decreased amount of leukocytes and less thickening of the alveoli compared to the bleomycin-treated group.

Masson staining is regarded as a reliable method for localizing collagen as defined area in a histological preparation. Bleomycin-treated group displayed an increased grade of collagen deposition and large fibrotic areas, compared to the control group. However, collagen accumulation was remarkably decreased in sulindac-treated groups when compared to the bleomycin group. Furthermore, the semi-quantitative assessment of lung sections was performed to number pathology score as per the Szapiel examination (Table 3). The Szapiel score of bleomycin-induced group was found significantly higher on day 21 when compared with control group. However, Szapiel scores on day 21 of sulindac-treated group showed remarkable decrease when compared to the bleomycin-treated group.
4. Discussion

The clinical usefulness of bleomycin, an anti-cancer drug for human malignancies including germ-cell tumours, lymphomas, Kaposi’s sarcoma, cervical cancer and squamous cell carcinomas of the head and neck has been hampered due to its detrimental effects (Siejfer, 2001). The major side-effect of this drug is the induction of lung fibrosis in patients treated with bleomycin. Pulmonary fibrosis is commonly progressive and essentially an untreated disease with an increasingly fatal outcome (Coker and Laurent, 1998). The bleomycin animal model of lung fibrosis is an established and widely used surrogate model of human lung fibrosis. There have been a number of studies employing bleomycin in different animal models including mice, rats, hamsters and dogs (Keane et al., 2001). The use of these animal models has helped in partly establishing the pathways of lung damage leading to fibrosis and by comparison studies of patients with lung pneumopathy, have validated many of these animal studies (Cooper, 2000).

In the current study, we have used wistar rat model of lung fibrosis by challenging the rats with a single dose of bleomycin sulfate by intratracheal instillation. A marked reduction in the body weight was observed in the bleomycin treated group, which might be due to the progression of the fibrosis (Zhou et al., 2007). Moreover there was increase in the relative organ weight of the lungs of the bleomycin-challenged rats when compared to the control rats, which may be due to the excessive deposition of collagen. This is in accordance with the finding of Soumyakrishnan and Sudhandiran (2011). However, the body weight and relative organ weight of the lungs of the sulindac treated group remained comparable to the control group rats.

Lung injury was quantitatively assessed biochemically (hydroxyproline, an index of collagen deposition; malondialdehyde, as a measure of lipid peroxidation; lung contents of reduced glutathione, glutathione peroxidase activity, glutathione content; superoxide dismutase and catalase) and cytologically (total and differential cell counts in bronchovascular lavage fluid). Further, histochemical localization of collagen in lung tissue was done to confirm the assessment of collagen deposition. Lung histopathology was also done to confirm the model and to unravel the possible inhibitory activity of sulindac.

Deposition of excess or abnormal collagen is a characteristic of lung fibrosis as reported by many previous studies (Daba et al., 2002; Pardo et al., 2003; Serrano-Molar et al., 2003). Since, the amino acid hydroxyproline is the precursor for collagen, the estimation of the amino acid following acid digestion of collagen is a good biochemical index of collagen content. Our result is in accordance with previous findings, which too demonstrated remarkable increase in lung hydroxyproline content as an index of collagen accumulation and deposition (Gazdhar et al., 2007; El-Medany et al., 2005; Zhao et al., 2010). This finding was further confirmed by collagen-specific staining using Masson’s trichrome staining of lung sections for collagen deposition. Bleomycin, in the present work, induced collagen accumulation and deposition in peribronchial and perivascular tissues that obliterated alveolar spaces as tiny fibrils. However, intensity of collagen deposition was considerably reduced in sulindac-treated group, which might be due to the inhibitory effect of sulindac.

Further, it is known that reactive oxygen species play an important role in the development of fibrotic responses in the lung, especially in those induced due to bleomycin challenge. Bleomycin binds to iron (Fe²⁺), undergoes redox cycling and catalyzes the formation of reactive-oxygen species, ultimately increasing lipid peroxidation and resulting in lung damage (Liang et al., 2011). Sulindac, a non-steroidal anti-inflammatory drug, is effective in scavenging reactive oxygen and nitrogen species free radicals (Dairam et al., 2007). In our findings, elevated level of reactive oxygen species was observed in bleomycin treated group but this was considerably reduced in sulindac-treated rats thus signifying its antioxidant potential.

Lipid peroxidation, a marker of oxidative stress is an autocatalytic, free radical mediated, destructive process, wherein polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (Kalayarasan et al., 2009). In our study, the observed high levels of lipid peroxidation in bleomycin-injured rats can be attributed to free radical-mediated membrane damage. However, treatment with sulindac significantly decreased the observed levels of malondialdehyde in bleomycin-treated rats.

Imbalances in the expression of glutathione and associated enzymes have been implicated in a variety of pathological conditions. Beside enzymatic antioxidants, the level of glutathione, a nonenzymatic reducing agent that traps free radicals and prevents oxidative stress, was also decreased in bleomycin-treated group. It has been well documented that decrease in glutathione reductase activity often leads to decrease in reduced glutathione levels (Dairam et al., 2007). A notable descent in the activity of glutathione peroxidase was observed in bleomycin-challenged rats, which might be due to overproduction of reactive oxygen species that exerts inhibitory effect on this enzyme (Sogut et al., 2004; Blum and Fridovich, 1985). Administration of sulindac restored the activities of these enzymatic antioxidants close to normal values. This might be due to the inhibitory action of sulindac on reactive oxygen species, consequently decreasing the oxidative stress produced during pulmonary fibrosis.

Superoxide dismutase catalyzes the dismutation of superoxide into oxygen and hydroperoxides, thereby acting as a potent antioxidant. A decline in the activity of superoxide dismutase was evident in bleomycin-treated rats, which is in concordance with previous studies (Ozyurt et al., 2004). Catalase is another antioxidant enzyme found in peroxisomes. This enzyme functions as the catalyst for the conversion of hydrogen peroxide, formed previously by the dismutation of superoxide dismutase, into water and molecular oxygen (Sogut et al., 2004). Decrease in the activity of this enzyme was also observed in bleomycin-treated group. Treatment with sulindac brought the levels of these enzymes too, close to that of control group.

Similarly, glutathione peroxidase is also a powerful endogenous antioxidant enzyme, which contains the non-metallic element selenium. This enzyme protects the system from the harmful effect of free radicals by reducing these into alcohol and water (Soumyakrishnan and Sudhandiran, 2011).
Treatment with sulindac significantly increased the level of this enzyme when compared to the bleomycin treated group. This may be due to the antioxidant property of sulindac which may inhibit reactive oxygen radical production in the lungs.

In addition to the oxidative stress mentioned earlier, intrachal administration of bleomycin leads to interstitial inflammation, with the marked increase in the recruitment of leukocytes. The leukocytes such as macrophages, neutrophils and lymphocytes play a key role in inflammation and tissue remodelling (Xin et al., 2010). A significant increase in the total number of cells, neutrophils and lymphocytes while significant decrease in macrophages in broncholaveolar lavage fluid was seen in bleomycin treated group. This is in accordance with previous studies of Gong et al. (2005) and Sriram et al. (2009). However, the total cell count, neutrophils, lymphocyte and macrophages count in sulindac treated rats remained comparable to the control group rats. Inhibited leukocytes recruitment, which directly impacted inflammation and tissue repair, might partly account for the preventive effect of sulindac on bleomycin-induced pulmonary fibrosis, which may be due to its ability to interfere with free radical-mediated reactions.

Moreover, tumour necrosis factor-α, a potent pro-inflammatory cytokine acts as one major molecule among the multifaceted networks of cellular and molecular interactions that regulate the fibrotic process (Razzaque and Taguchi, 2003). In this study, a significant elevation in the tumour necrosis factor-α expression was observed in the bleomycin-treated group, which is in accordance with the findings of El-Medany et al. (2005). The tissue injury caused by bleomycin is found to be inflammation-mediated, which might be due to the production of free radicals, possibly leading to activation of nuclear factor kappa-B and increase in synthesis of tumour necrosis factor-α (Ortiz et al., 2002; Kalayarasan et al., 2008). Sulindac has an inhibitory effect on nuclear factor kappa-B activity (Berman et al., 2002). Sulindac substantially reduced the expression of tumour necrosis factor-α, perhaps by inhibitory effect on nuclear factor kappa-B activity.

The subsequent corroboratory histopathological observation showed marked structural distortion of the alveolar space with collapsed alveolae, interalveolar inflammation, thickened alveolar wall and abnormal collagen deposition in bleomycin-induced rats. Similar histopathological changes reported by others give credence to the present observation (Liang et al., 2011; Teixeira et al., 2008). Moreover, in the present study it was observed that sulindac could hinder the structural distortion caused by bleomycin as indicated by the improvement in lung fibrosis scores that might be due to its antioxidant potency of the former.

Increase in the number of fibroblasts leads to excessive deposition of collagen content in the lung interstitium. One of the strategies to attenuate fibrosis is to inhibit the overproduction of collagen and proliferation in fibroblasts (Gong et al., 2005). Decrease in the deposition of collagen was observed in the sulindac-treated group as observed in Masson’s trichrome stained section of the lungs.

Nitric oxide plays an important role in pathogenesis of lung fibrosis and idiopathic pulmonary fibrosis (Yildirim et al., 2004). Although we have not undertaken the estimation of the nitric oxide in the present study, a report does state that sulindac has an inhibitory effect on the production of nitric oxide (Fernandes et al., 2003).

In the present study, the pulmonary response to bleomycin challenge includes a rapid development of oxidative stress in combination with reduction of antioxidant capacity in lung. Inhibitory effect of sulindac reduced oxidative stress by anti-inflammatory and reactive oxygen species scavenging capacity. Additional studies are required to specify the protective mechanism of sulindac on this model, as well as to investigate the effect of sulindac on other animal model of lung fibrosis.

5. Conclusion

Pulmonary fibrosis is generally non-responsive to conventional corticosteroid therapy (Green, 2002). While instillation of bleomycin resulted in reduction of antioxidant capacity, elevation of inflammatory cytokines, fibrotic changes and collagen accumulation in lung tissue, Sulindac displayed pneumoprotective property through enhancement of antioxidant defence, decrease in the level of inflammatory cytokines and collagen accumulation. Thus, the present results suggest that sulindac effectively prevents the pulmonary injury induced by bleomycin challenge.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors are very grateful to Dr. A. Deshpande and Dr. T.G. Marvania for their kind support as well as for providing the necessary facility to conduct the study in the experimental premises. This work was financially supported by Jai Research Foundation, Vapi, India.

References


Research Article

Evaluating the Ameliorative Potential of Quercetin against the Bleomycin-Induced Pulmonary Fibrosis in Wistar Rats

Ramesh Verma, Lokendra Kushwah, Darpesh Gohel, Manish Patel, Tulsi Marvania, and Suresh Balakrishnan

1 Department of Toxicology, Jai Research Foundation, Valvada, Gujarat 396108, India
2 Division of Toxicology, Department of Zoology, Faculty of Science, The M.S. University of Baroda, Vadodara, Gujarat 390002, India

Correspondence should be addressed to Suresh Balakrishnan; suved9@hotmail.com

Received 11 June 2013; Revised 15 August 2013; Accepted 10 October 2013

Academic Editor: Charlie Strange

Copyright © 2013 Ramesh Verma et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The current study deals with the effect of a dietary flavonoid quercetin on fibrotic lung tissue in rats. Bleomycin was administered by single intratracheal instillation to Wistar rats to induce lung fibrosis. The pathologies associated with this included significantly reduced antioxidant capacity, ultimately leading to protracted inflammation of the lung tissue. The hallmark of this induced fibrosis condition was an excessive collagen deposition in peribronchial and perialveolar regions of the lung. Oral quercetin treatment over a period of twenty days resulted in significant reversal of the pathologies. The antioxidant defense in lung tissue was revived. Moreover, activity of the collagenase MMP-7, which was high in fibrotic tissue, was seen restored after quercetin administration. Trichrome staining of lung tissue sections showed high collagen deposition in fibrotic rats, which may be a direct result of increased mobilization of collagen by MMP-7. This was appreciably reduced in quercetin treated animals. These results point towards an important protective role of quercetin against idiopathic lung fibrosis, which remains a widely prevalent yet incurable condition in the present times.

1. Introduction

Bleomycin is a commonly used chemotherapeutic agent which, however, induces dose-dependent pulmonary fibrosis upon long-term administration [1]. Interstitial pulmonary fibrosis is characterized by an altered cellular composition of the alveolar region with excessive deposition of collagen. However, lung inflammation is considered to be a major underlying factor for the induction of pulmonary fibrosis [2]. Reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radical are reported as major mediators of lung inflammatory processes [3]. Nevertheless, the direct linkage between reactive oxygen species formation and pulmonary fibrosis has not been established conclusively. Bleomycin-induced pulmonary injury and lung fibrosis have been documented in studies using several animal models [4, 5]. These models have been widely used for studying the mechanisms involved in the progression of human pulmonary fibrosis and the impact of various drugs on this progression [6, 7]. The bleomycin induces the genesis of reactive oxygen species upon binding to DNA and iron, which in turn causes DNA damage [8]. The interaction of bleomycin with DNA is postulated to initiate the inflammatory and fibroproliferative changes through a concerted action of various cytokines leading to collagen accumulation in the lung [5]. Further, it is reported that bleomycin promotes the depletion of endogenous antioxidant defences, thus exacerbating oxidant mediated tissue injury [8]. The lung is selectively affected by bleomycin as it lacks an enzyme that hydrolyzes the $\beta$-aminoalanine moiety of bleomycin, which prevents its metabolite from binding to metals such as iron [9]. Strategies aimed at reducing oxidative stress have been found to be successful in decreasing bleomycin-induced lung injury and fibrosis [10–12].

The detrimental role of reactive oxygen species in many disease states has led to the development of new antioxidants. One such group of compounds with potential antioxidant property is the flavonoids present in fruits and vegetables, of which quercetin (3,3',4',5,7-pentahydroxyflavone) has attracted much attention for its beneficial health effects.
It has been suggested that daily intake of these substances may reduce the risk of various chronic health disorders such as cardiovascular disease, diabetes, tumor development, stroke, and neurodegenerative disease [13–16]. The beneficial effects of quercetin have been attributed to multiple mechanisms including antioxidant activity, anti-inflammation, modification of signal transduction pathways and interactions with receptors and other proteins [17]. The antioxidant activity of quercetin is primarily credited to its phenolic hydroxyl groups [18]. Beneficial health effects of quercetin against various oxidative stress-related diseases have been documented [19]. However, studies examining its potential pneumoprotective effects are limited. Although experimental evidence for the role of a redox imbalance in lung fibrosis is substantial, this chronic disease is generally nonresponsive to conventional anti-inflammatory and immunomodulatory therapy [20]. Therefore, it was of interest to determine the ameliorative role, if any, of quercetin against bleomycin-induced lung injury because of the former's potent antioxidant activity.

2. Materials and Methods

2.1. Animals. Specific pathogen-free, healthy young adult male Wistar rats (RCCHan:WIST) of 10 to 12 weeks were used in this study. They were obtained from the Barrier Maintained Rodent Animal Breeding Facility, Jai Research Foundation, Vapi, India. All the animals were fed with Teklad certified global rat feed manufactured by Harlan, USA and UV sterilised water filtered through Kent Reverse Osmosis water filtration system was provided ad libitum. The rats were kept in a controlled environment (temperature: 22 ± 2°C and relative humidity: 30 to 70%) with an alternating cycle of 12 h light and dark. The animals used in this study were handled and treated in accordance with the strict guiding principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The test facility at Jai Research Foundation is AAALAC accredited and also complies with the National Good Laboratory Practice, India. The experimental protocols were approved by the Institutional Animal Ethics Committee (approval no. 35/1999/CPCSEA).

2.2. Experimental Design. Rats were randomized into three groups each consisting of 10 animals [21]. Briefly, the rats were anesthetized using a combination of ketamine (80 mg/kg body weight, i.p.) and xylazine (20 mg/kg body weight, i.p.) as per standard protocol [22]. A midline incision was made in the neck and the exposed trachea was intubated with tracheal cannula under direct visualization. For induction of pulmonary fibrosis, the rats received a single dose of 6.5 U/kg body weight bleomycin sulfate dissolved in 0.5 mL of 0.9% NaCl solution on day 0 of the experiment [23]. Control rats received a single intratracheal dose of sterile saline alone. Group 1 (vehicle control) and group II (bleomycin treated) rats were treated with 0.5% carboxymethylcellulose solution orally from day 1 to day 20 of the experiment. Animals from group III were orally administered with quercetin (100 mg/kg body weight/day) in 0.5% carboxymethylcellulose solution from day 1 to day 20 of the experiment after bleomycin instillation [24]. The drug was freshly prepared in 0.5% carboxymethylcellulose solution and the concentration was adjusted so that each animal received 10 mL/kg body weight. The animals were weighed at the beginning, through and at the end of experiment. The changes in body weight were recorded.

2.3. Biochemical Assays

2.3.1. Preparation of Lung Tissue for Biochemical Studies. On day 21 of the experiment, six animals from each group were sacrificed with thiopentone sodium and the lung lobes were excised and the tissue was used for the biochemical estimations. BAL was performed in four animals from each group under anaesthesia with thiopentone sodium and the tissue was used for the preparation of the slides to be used for histological examinations.

2.3.2. Determination of Lung Hydroxyproline. The hydroxyproline assay was performed as described by Edwards and O’Brien [25]. The lung was dried and then hydrolysed at 120°C in a pressure vessel for 2–4 h. The acid hydrolysates and standards were added to 1.5 mL tubes, along with the same volume of citric/acetate buffer (citric acid, sodium acetate, sodium hydroxide, glacial acetic acid, and n-propanol pH 6.0) and chloramine T solution (chloramine T dissolved in Milli Q water). The tubes were incubated for 20 min at room temperature and Ehrlich’s solution (p-dimethyl-amino-benzaldehyde, perchloric acid and n-propanol) was added to the tubes which were then incubated at 60°C for 15 min. The absorbance of the reaction product was read at 550 nm.

2.3.3. Determination of Lipid Peroxidation. Malondialdehyde (MDA) is the most abundant individual aldehyde resulting from lipid peroxidation breakdown in biological systems and is commonly used as an indirect method for the estimation of lipid peroxidation. MDA content was assayed using the thiobarbituric acid test as described by Ohkawa et al. [26]. MDA reacts with thiobarbituric acid to form a coloured complex. Known amount of tissue homogenate was added to the tubes containing dodecyl sulphate, acetic acid, and thiobarbituric acid solution and heated in a water bath at 95°C for 60 minutes. Trichloroacetic acid was added to the tubes and absorbance was measured at 532 nm to determine the malondialdehyde content.

2.3.4. Measurement of Superoxide Dismutase Activity. The activity of superoxide dismutase was measured following the method of Kakkar et al. [27]. A known amount of tissue homogenates was mixed with sodium pyrophosphate buffer, phenazine methosulphate, and nitroblue tetrazolium chloride. The reaction was started by the addition of NADH. The reaction mixture was incubated at 30°C for 90 seconds and stopped by the addition of glacial acetic acid. The absorbance of the chromogen formed was measured at 560 nm.
2.3.5. Determination of Catalase Activity. Catalase activity was assessed by the method of Luck [28], wherein the breakdown of hydrogen peroxide is measured. Briefly, a known amount of tissue homogenate is added to 0.1% chilled digitonin and was centrifuged at 4°C and 0.05 mL of the supernatant of the tissue homogenate is mixed with 3 mL of H$_2$O$_2$ phosphate buffer. The change in absorbance was recorded at 30-second interval at 240 nm.

2.3.6. Bronchoalveolar Lavage (BAL). BAL fluid was obtained by the injection of 3 mL saline (three times, total 9 mL) followed by gentle aspiration of the fluid from the lungs after securing an intratracheal catheter within a trachea. With this catheter, the ratio of the recovery of lavage fluid was approximately 80% and did not significantly differ among the groups. The total numbers of cells in the bronchoalveolar lavage fluid were counted with a hemocytometer. For differential counts of leukocytes in the bronchoalveolar lavage fluid, smear slides were prepared and stained with Giemsa solution. Differential cell counts were obtained from a count of 300 cells per smear.

2.3.7. Measurement of Tumor Necrosis Factor-α (TNF-α). TNF-α in plasma was assayed by specific enzyme-linked immunosorbent assay using commercially available ELISA test kits (XpressBio Life Science Products, USA). The kit contains a TNF-α monoclonal antibody coat for a 96-well microtiter plate and polyclonal antibody to TNF-α. The representative standard curve was generated using the TNF-α standard supplied with the kit.

2.3.8. Estimation of MMP-7. MMP-7 levels in plasma and BAL were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA). The samples were added in duplicate to 96-well plates coated with the MMP-7 antibody and incubated for 2 h. After washing three times with washing buffer, the conjugated secondary antibody was added and the plate was further incubated for 2 h. Plates were washed again prior to incubation with the substrate solution for 1 h. The amplifier solution was then added, and the plate was incubated for additional 30 min. All incubation cycles were performed at room temperature. Following termination of the reaction with the stop solution; the optical density was measured at 490 nm using a microplate reader. The concentration of MMP-7 in each sample was calculated from a standard curve.

2.4. Histological Studies. After sacrifice, each lung tissue was perfused and fixed in 10% neutral buffer formalin and routinely processed and embedded in paraffin. Serial sections (4 mm) were cut and stained with hematoxylin and eosin and Masson's trichrome for light microscopic evaluation to examine the degree of fibrosis. The severity of fibrosis was individually assessed using the semiquantitative grading system described by Szapiel [29]. According to this system, the fibrosis in lung specimens was graded as none (0) when no alveolitis was observed; mild (+) —when focal lesions occupying less than 25% of the lung was noticed in alveolar septum; moderate (++)—when widespread alveolitis involving 25–50% of the lung was observed; and severe (+++)—when a diffused alveolitis spanning more than 50% of the lung, with occasional consolidation of air spaces and patches of hemorrhagic areas within the interstitium, was observed. The entire lung section was reviewed at a magnification of 10X. Each of the 20 random microscopic fields per section was detected; a score ranging from 0 to 3 was assigned. All assessments were performed in double-blind fashion.

2.5. Material. Quercetin, Chloramine-T and Hydroxyproline were procured from Sigma-Aldrich Chemie GmbH. Bleomycin hydrochloride was procured from the local market and was in the form of bleomycin ampoules (15 units) manufactured by Biochem Pharmaceutical Industries Ltd., Mumbai, India. All other chemicals were of analytical grade and procured from reputed manufacturers of India, namely, Sisco Research Laboratories Pvt. Ltd., Qualigens Fine Chemicals Pvt. Ltd., and HiMedia Laboratories Pvt. Ltd.

2.6. Statistical Analysis. Statistical analyses were carried out by analysis of variance (ANOVA) followed by Dunn's post hoc test. All analyses of data were performed using SPSS for windows version 12.0 and a probability value of 0.05 or less was considered as statistically significant.

3. Results

3.1. Changes in Body Weight. Figure 1 shows the effect of quercetin on the body weight of bleomycin administered groups of rats. Single intratracheal administration of bleomycin (6.5 U/kg) resulted in a marked decrease in their body weight on days 14 and 21 as compared to the saline-treated control group possibly because of severe tissue damage caused by free radicals. However, body weight of quercetin-treated rats remained comparable to the control group rats throughout the period of experiment.

3.2. Change in Percent Relative Lung Weight. As evident from Figure 2, the percent relative lung weight of bleomycin-treated animals remained significantly high compared to the other two groups of animals. However, twenty-day quercetin treatment protected the lung tissue from bleomycin-induced fibrotic response as evident from the statistically comparable mean percent relative lung weight in both quercetin-treated as well as control animals.

3.3. Hydroxyproline Content. The effect of quercetin on the hydroxyproline content of lung homogenate is presented in Figure 3. Measurement of hydroxyproline is an efficient index of collagen deposition since collagen contains significant amount of this amino acid. After 20 days, the hydroxyproline content in the lungs of bleomycin group increased significantly compared to that in the control group. However, an apparent reduction ($P \leq 0.05$) in lung hydroxyproline content was observed in the bleomycin plus quercetin group compared to the bleomycin group, indicating the possible protective role of quercetin against lung collagen deposition.
3.4. Lipid Peroxidation. The result of this study showed an increase in the level of lipid peroxidation in bleomycin-administered group over that of control group, which could be a manifestation of bleomycin induced tissue injury and damage. Nevertheless, quercetin treatment significantly lowered the bleomycin-induced lipid peroxidation in the lung of rats as evident from the near normal MDA levels (Table I).

3.5. Superoxide Dismutase Activity. Effect of bleomycin and bleomycin plus quercetin on lung tissue superoxide dismutase activity is presented in Table I. The superoxide dismutase activity in the lung of bleomycin-treated rat was found significantly reduced as compared to the control group. Administration of quercetin proved to be effective in restoring the altered activity of this antioxidant enzyme.

3.6. Catalase Activity. The catalase activity in the lung homogenate of bleomycin-treated rats was found considerably lower than that of vehicle control rats. The quercetin administration which improved the catalase activity on day 21 of treatment of the animals in this group showed comparable catalase activity with that of controls (Table I).

3.7. Total and Differential Leukocyte Count in BAL. The total leukocyte count and the subset proportion in the bronchoalveolar lavage of control and experimental group of rats are given in Table 2. Bleomycin induction caused a statistically significant ($P \leq 0.05$) increase in the total leukocyte count in the BAL as compared to that in control animals. However, in rats treated with quercetin, total leukocyte count remained similar to that observed in control rats at the end of experimental regime. The differential cell count showed a significant increase in proportion of neutrophils and eosinophils in the lungs of rats exposed to bleomycin. It was observed that the quercetin treatment for 20 days significantly reduced the bleomycin induced hike in neutrophils in the BAL. While the percentage of lymphocytes and alveolar macrophages was decreased in bleomycin-induced group, treatment with quercetin reversed these changes significantly.

3.8. Tumor Necrosis Factor- (TNF-)α Concentration. Figure 4 shows the effect of quercetin on plasma levels of TNF-α. The plasma TNF-α level of bleomycin-administered rats remained elevated on day 21 of experiment when compared with the sham-treated group. Treatment with quercetin, however, decreased bleomycin-induced increase in plasma TNF-α level by the end of the experiment.
Table 1: Levels or activities of various markers of oxidative stress in the lung tissue of the rats from control and treated groups.

<table>
<thead>
<tr>
<th>Biochemical estimations</th>
<th>Control</th>
<th>Bleomycin</th>
<th>Bleomycin + quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malondialdehyde level (nmol MDA/mg tissue)</td>
<td>44.34 ± 1.28</td>
<td>60.63 ± 1.87*</td>
<td>46.64 ± 1.13#</td>
</tr>
<tr>
<td>Superoxide dismutase (U/mg tissue)</td>
<td>0.34 ± 0.03</td>
<td>0.21 ± 0.01*</td>
<td>0.31 ± 0.02#</td>
</tr>
<tr>
<td>Catalase (µM H₂O₂ consumed/mg tissue/min)</td>
<td>18.94 ± 1.22</td>
<td>12.68 ± 0.82*</td>
<td>17.32 ± 1.46#</td>
</tr>
</tbody>
</table>

Values are given as mean ± SE for groups of six rats each, *P ≤ 0.05 versus control group, #P ≤ 0.05 versus bleomycin group.

3.9. Estimation of MMP-7. The levels of MMP-7 were estimated in blood plasma and bronchoalveolar lavage fluid in the various stated experimental groups with the help of an ELISA-based method. Our results (Table 3) showed a significant increase in the levels of MMP-7 in both plasma and bronchoalveolar lavage fluid in response to bleomycin challenge. The level of this extracellular matrix digesting enzyme in animals that received quercetin treatment after bleomycin administration was found to be markedly lower than that in the bleomycin-treated group, and, at the same time, comparable to that in the control group.

3.10. Histopathological Examination of Lung Tissue. Histopathological abnormalities in lungs were studied at the end of the experiment using hematoxylin and eosin staining as well as Masson's trichrome staining.

Bleomycin administered rats showed distorted architecture of the lung tissue which included moderate to severe hemorrhages, emphysema, areas of increased thickening of alveolar septa, leukocytic infiltration in alveolar walls, and fibroplasia (Figures 5(b) and 5(c)) when compared with control group (Figure 5(a)). Nevertheless, quercetin showed to have ameliorative effect on the inflammatory lesions developed by bleomycin treatment. Pulmonary histoarchitectural changes in animals treated with quercetin showed mild to moderate degree of septal thickening with few inflammatory cells. Emphysematous changes and alveolar hemorrhages were remarkably reduced in quercetin-treated group of animals (Figures 5(d) and 5(e)).

In order to understand the degree of lung fibrosis in various treatment groups, the extent of collagen deposition was studied as a marker of fibrosis through Masson's trichrome staining. As expected, bleomycin-treated group displayed an increased grade of collagen deposition, compressed alveoli and large fibrotic areas, compared to that in the control group.
Table 2: Total leukocyte count and percentage leukocyte subset in the bronchoalveolar lavage fluid of rats from control and treatment groups.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Total cells ($\times 10^6$ mL$^{-1}$)</th>
<th>Macrophage (%)</th>
<th>Neutrophils (%)</th>
<th>Eosinophil (%)</th>
<th>Lymphocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.65 ± 0.12</td>
<td>85.17 ± 2.00</td>
<td>1.83 ± 0.50</td>
<td>0.83 ± 0.17</td>
<td>11.67 ± 1.50</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>1.64 ± 0.15$^*$</td>
<td>49.33 ± 3.83$^*$</td>
<td>38.33 ± 1.83$^*$</td>
<td>4.08 ± 0.58$^*$</td>
<td>5.42 ± 0.92$^*$</td>
</tr>
<tr>
<td>Bleomycin + quercetin</td>
<td>0.87 ± 0.14$^z$</td>
<td>71.00 ± 3.00$^z$</td>
<td>13.00 ± 2.00$^z$</td>
<td>4.08 ± 0.96$^z$</td>
<td>10.50 ± 1.25$^z$</td>
</tr>
</tbody>
</table>

Values are given as mean ± SE for groups of four rats each, $^*P \leq 0.05$ versus control group, $^zP \leq 0.05$ versus bleomycin group.

Table 3: Level of MMP-7 in blood plasma and bronchoalveolar lavage fluid.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>MMP-7 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAL</td>
</tr>
<tr>
<td>Control</td>
<td>7.65 ± 0.15</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>10.58 ± 0.56$^*$</td>
</tr>
<tr>
<td>Bleomycin + quercetin</td>
<td>8.16 ± 0.25$^z$</td>
</tr>
</tbody>
</table>

Values are given as mean ± SE for groups of four rats each, $^*P \leq 0.05$ versus control group, $^zP \leq 0.05$ versus bleomycin group.

Table 4: Szapiel examination scores of lung tissue from rats subjected to various treatments.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Grade of fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>2.51 ± 0.06$^*$</td>
</tr>
<tr>
<td>Bleomycin + quercetin</td>
<td>1.48 ± 0.15$^z$</td>
</tr>
</tbody>
</table>

Values are given as mean ± SE for groups of six rats each, $^*P \leq 0.05$ versus control group, $^zP \leq 0.05$ versus bleomycin group.

(Figures 6(a) and 6(b)). However, collagen accumulation was remarkably decreased in rats from quercetin-treated group (Figure 6(c)) when compared to the bleomycin group.

Furthermore, the semiquantitative assessment of fibrosis in lung sections was performed by scoring pathological lesions as per the Szapiel method of examination (Table 4). The Szapiel score of bleomycin-induced group was found significantly higher on day 21 when compared with control group. However, Szapiel scores on day 21 of quercetin treated group showed marked decrease compared to the bleomycin-treated group reaffirming protective role of quercetin against bleomycin-induced lung fibrosis.

4. Discussion

The clinical use of bleomycin, as an anticancer drug for a myriad of human malignancies, has been hampered due to its detrimental effects [30]. The major side effect is the induction of lung fibrosis in patients treated with bleomycin. Pulmonary fibrosis is commonly progressive and essentially an untreatable disease with an increasingly fatal outcome [31]. In order to understand the finer mechanisms of development of pulmonary fibrosis as well as to screen the efficacy of various compounds as potential therapeutic agent against this pathological manifestation, animal experiments are inevitable. The bleomycin-rodent animal model of lung fibrosis is an established and widely used surrogate model of human lung fibrosis [32]. Comparison studies of patients with lung pneumopathy and experimentally induced lung fibrosis animal model have validated effectiveness of this experimental system as an acceptable model [33]. Subsequently, the use of this animal model has helped in partly establishing the pathways of lung damage leading to fibrosis [33].

In the current study, we have used Wistar rat model of lung fibrosis created by challenging the rats with a single dose of bleomycin sulfate by intratracheal instillation. A marked reduction in the body weight was observed in the bleomycin-treated group, which could be attributed to the progression of the fibrosis [34]. Twenty-one days after single intratracheal installation of bleomycin, an obvious increase in the relative weight of lungs was observed in the experimental animals compared to that of control rats. Soumyakrishnan and Sudhandiran [12] have also reported a similar increase in lung weight in bleomycin-treated animals. This increase in lung weight is a clear indication of lung fibrosis that is characterised by excessive deposition of collagen as evident from the increased hydroxyproline levels observed in the lungs of bleomycin-treated rats. However, it was noticed that administration of quercetin effectively thwarted the progression of bleomycin-induced lung fibrosis, and, hence, body weight and relative weight of the lungs in the animals.
of quercetin-treated group was in concurrence with that of vehicle control and remained within 95% confidence limits.

Further, the local tissue response to bleomycin-induced lung injury was evaluated by assessing various biochemical markers, namely, hydroxyproline—an index of collagen deposition, malondialdehyde—as a measure of lipid peroxidation and superoxide dismutase, as well as catalase—two key antioxidant enzymes in the lungs of rats from various study groups. In order to complement the biochemical response of bleomycin-challenged lung tissue, cytological analyses such as total and differential leukocyte counts in bronchoalveolar lavage fluid were also conducted. In addition, histochemical localization of collagen in lung tissue was done to confirm the extent of lung fibrosis in various groups of animals. Lung histopathology also was done to confirm the progression of lung fibrosis in bleomycin-instilled rats and also to unravel the possible inhibitory potential of quercetin against the bleomycin-induced lung fibrosis.

Since the amino acid hydroxyproline is the precursor for collagen, the estimation of this amino acid following acid digestion of collagen is considered a good biochemical index of collagen content. Bleomycin-treated group exhibited statistically significant increase in lung hydroxyproline content as compared to control group. This result is in accordance with previous reports, which also demonstrated remarkable increase in lung hydroxyproline content in bleomycin-instilled pulmonary fibrosis models [2, 35, 36]. Deposition of excess or abnormal collagen is a characteristic of lung fibrosis as stated by many while studying the mechanisms behind xenobiotic-induced lung fibrosis [37–39]. The present biochemical indication of bleomycin-induced lung fibrosis was further confirmed visually by collagen-specific Masson’s trichrome staining of lung sections. The microscopical observation of the trichrome stained histological sections revealed that bleomycin treatment induced collagen accumulation and deposition in peribronchial and perialveolar tissues that obliterated alveolar spaces as tiny fibrils. However, the lung tissues from quercetin-treated rats showed much improved histological profile with near normal collagen deposition and a basal level of hydroxyproline content indicating quercetin’s ameliorative role against bleomycin-induced pulmonary fibrosis.

Further, it is known that reactive oxygen species play an important role in the development of fibrotic responses in the lung of the subject upon bleomycin challenge. Bleomycin binds to iron (FeII), undergoes redox cycling, and catalyzes the formation of reactive oxygen species (11). It is well documented that these free radicals, once produced, target biomacromolecules such as DNA, protein, and lipid, with the ultimate progression of lipid peroxidation, resulting in damage to the lung [40]. In the current study too, we observed signs of oxidative stress as exemplified by heightened MDA activity in the lungs of animals subjected to bleomycin. Moreover, the drug-treated rats also showed compromised antioxidant response as evident from subdued activity of antioxidant enzymes such as catalase and superoxide dismutase. Similar observations made by others give credence to the present notion [41, 42]. However, it was observed that the extent of oxidative damage was well within the normal level in the lungs of quercetin-treated rats signifying this flavonoid’s antioxidant potential. Flavonoids are reported to be powerful antioxidants providing remarkable protection against oxidative stress and free radical damage [13]. Moreover, quercetin, a member of the flavonoid family, is cited as one of the most prominent dietary antioxidants [13]. A study regarding the tissue distribution of quercetin in rats has shown that, upon quercetin treatment, the highest accumulation of this flavonoid and its metabolites was observed in rat lungs [43]. Hence, it is prudent to presume that quercetin could emerge as a pneumoprotective agent against local oxidative stress inducers like the one that is addressed in the current study.

In addition to the oxidative stress mentioned earlier, intratracheal administration of bleomycin might also results in interstitial inflammation characterised by an increase in the recruitment of leukocytes [44]. It is known that the recruitment of inflammatory cells to the site of inflammation plays a pivotal role in the pathogenesis of several inflammatory conditions. Further, it is also reported that the leukocytes such as macrophages, neutrophils, and lymphocytes play a key role in inflammation and tissue remodelling [44]. In the current study, a significant increase in the total leukocytes count with a marked increase in the proportion of neutrophils and eosinophils was apparent in the bronchoalveolar lavage of bleomycin treated animals. At the same time, the proportion of macrophages and lymphocytes was found low in the bronchoalveolar lavage fluid of bleomycin-challenged rats. The observed trend in the count of inflammatory cells in the bronchoalveolar lavage fluid during bleomycin induced pulmonary injury is in accordance with the previous reports of experimental pulmonary fibrosis [11, 45]. However, the anti-inflammatory property of quercetin gained further consolidation from our observation that twenty-day repeated treatment of this flavonoid after intratracheal instillation of bleomycin significantly reduced the recruitment of leukocytes into the alveolar air space. Analysis of extravasated cells from lung revealed that the recruitment of neutrophils was significantly reduced in the quercetin-treated rats compared to bleomycin-administered ones. The obvious reduction in the extravasation of leukocytes upon quercetin treatment indicates substantial recovery from bleomycin-induced lung inflammation, and hence, underlining the ameliorative property of this flavonoid against the drug-induced lung injury.

Moreover, it is reported that TNF-α, a potent proinflammatory cytokine, acts as a major component of a multifaceted network of cellular and molecular interactions that regulate the progression of fibrotic process [46]. In this study, a significant elevation in the expression of TNF-α was observed in the bleomycin-treated group, which is in accordance with previous findings [2, 47]. Furthermore, it has been reported that the reactive oxygen species generated as a result of bleomycin treatment might induce nuclear factor-κB and increase the synthesis of TNF-α [48, 49]. However, we noticed that oral administration of quercetin for twenty-days after local instillation of bleomycin reduced the expression of proinflammatory cytokine TNF-α to the basal level indicating marked recovery from the induced lung injury caused by the drug in question.
Further, it is well documented that fibrosis is a consequence of repair of damaged tissue and is known to be closely associated with the remodelling of extracellular matrix tissue [50]. Also, as has been reported by others, pulmonary fibrosis patients show elevated levels of MMP-7 [51–53]. Moreover, it has been reported that mice lacking MMP-7 are protected from bleomycin-induced lung injury suggesting a key role of MMP-7 in the induction of pulmonary fibrosis by bleomycin [53]. On the line of these reports, we took up the estimation of MMP-7 to throw light on a possible role of this enzyme as a direct or indirect target of quercetin. Our results indeed do suggest an association of MMP-7 levels with induction of fibrosis by bleomycin. This increase in expression was found to be prevented by quercetin treatment. Whether MMP-7 is a direct target of quercetin, however, needs to be assessed.

The subsequent corroboratory histopathological observation showed abnormal histological profile of the lung tissue in bleomycin-administered rats. Notable deviant histarchitecture of the lung from the drug-treated animals includes collapsed alveolae, thickened alveolar wall, and abnormal collagen deposition. Similar structural changes reported by others from bleomycin-induced lung fibrosis rat models consolidate the credence of the present experimental model [22, 40, 54]. Moreover, in the present study, it was observed that quercetin treatment decelerated the progression of bleomycin-induced structural deformation as exemplified by the low Szapiel scores in this group of animals compared to bleomycin-treated ones. It is well documented that quercetin is a strong free radical scavenger and also a good metal chelator [55]. It has also been reported that quercetin acts through various mechanisms including the antioxidative activity, the inhibition of enzymes that activate carcinogens, the modification of signal transduction pathways, and interactions with receptors and other proteins [17]. In vitro studies have demonstrated that quercetin, in fact, inhibited the production of reactive oxygen species in lipopolysaccharide-stimulated Kupffer cells [56]. Moreover, reports have also shown that quercetin treatment effectively reduced superoxide anions [57] and could inhibit lipid peroxidation, and hence, alterations in lung morphology during pulmonary injury or infection [57, 58].

MAPK pathways such as ERK1/2 and JNK 6 are activated in response to a high oxidative status, leading to increase in the levels of MMP-7 [59]. The MAPK pathway is a known target for potential fibrosis therapy as several fibrogenic cytokines signal through MEK/ERK, including noncanonical TGF-β, PDGF, IL-13, and TNF-α [60]. We, therefore, suggest that quercetin, due to its antioxidative property and inhibitory effect on TNF-α, targets the above mentioned pathway to ultimately decrease the MMP-7 levels in quercetin-treated rats.

In the light of the current observations as well as the above cited reports, it is pertinent to presume that quercetin, being an antioxidant, effectively reduces the formation of bleomycin-induced free radicals which are known to trigger a cascade of inflammatory responses culminating in moderate to severe lung injury in treated subjects, and hence, could be used as an effective inhibitor of the bleomycin-induced pulmonary fibrosis.

5. Conclusion

Pulmonary fibrosis is generally nonresponsive to conventional therapy. The bleomycin-induced fibrosis model displayed reduced antioxidant capacity, elevation of inflammatory cytokines, increased MMP-7 expression, fibrotic changes, and collagen accumulation in lung tissue. Quercetin appeared to have a pneumoprotective effect through enhancement of antioxidant status, decrease in the level of inflammatory cytokines, reduction of MMP-7 expression, and minimisation of collagen accumulation. We, therefore, suggest that quercetin effectively attenuates the pulmonary injury induced by bleomycin challenge. Additional studies, however, will be required to understand the comprehensive role of quercetin in the pathogenesis of pulmonary fibrosis.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

The authors are very grateful to Dr. A. Deshpande for his kind support in providing the facility to conduct the study at the experimental premises. This work was financially supported by Jai Research Foundation, Vapi, India. The authors thank the anonymous reviewers for their constructive comments that helped to improve the paper.

References


Annexure VI and VII


