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Evaluating the inhibitory potential of sulindac against the bleomycin-induced pulmonary fibrosis in wistar rats

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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- α increased in bleomycin-induced group, whereas, on treatment with sulindac the levels of tumour necrosis factor- α 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.

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1. 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

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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 β -aminoalanine moiety of bleomycin, which prevents its metabolite from binding metals such as iron (Filderman et al., 1988).

Sulindac ([Z]-5-fluoro-2-methyl-1-[p-(methylsulfinyl)-benzylidene]indene-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 sulfone. 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: $22 \pm 2^\circ\text{C}$ 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 I (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 (ED_{50} 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 [aldehyde-perchloric 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 H_2O_2 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 H_2O_2 -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 μ m) 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 \times . 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 manufactures of India, viz., Sisco Research Laboratories Pvt. Ltd., Qualigens 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.

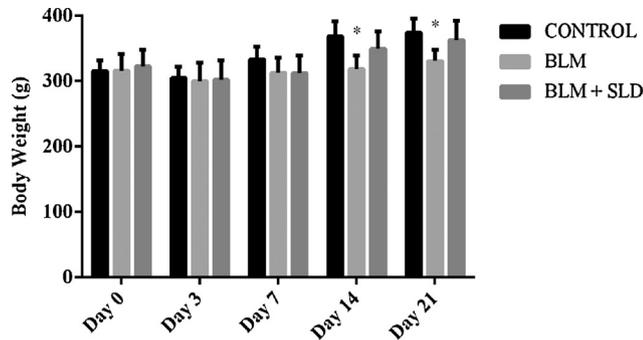


Fig. 1 – The mean body weight of rats during the course of experiment. $n = 8$; * $p < 0.05$ vs. control group.

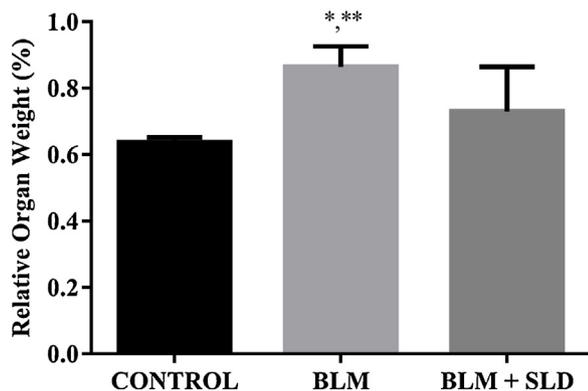


Fig. 2 – The relative weights of lungs in rats subjected to various treatments. BLM – bleomycin; SLD – sulindac; *** $p < 0.01$ vs. control group. # $p < 0.05$ vs. bleomycin group. ## $p < 0.01$ vs. bleomycin group.

3.2. Change in percent relative organ weight

As shown in Fig. 2 the percent relative organ weight of the sulindac group showed a marked decrease at the end of the experiment as compared to the bleomycin treatment group. Nevertheless, there was no significant variation in relative lung weight between sulindac treated group and control group.

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 lung 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.

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

Biochemical estimations	Control	Bleomycin	Bleomycin + sulindac
Hydroxyproline content (mg/g dried tissue)	1.77 ± 0.07	2.54 ± 0.19**	1.93 ± 0.14##
Malondialdehyde level (nmoles MDA/mg tissue/60 min)	44.62 ± 1.23	60.06 ± 1.64**	46.60 ± 2.16##
Reduced glutathione (μg/g tissue)	1.78 ± 0.04	1.07 ± 0.07**	1.67 ± 0.05##
Glutathione peroxidase (U/g tissue)	5.51 ± 0.13	2.44 ± 0.17**	3.33 ± 0.15**##
Glutathione content (μg/g tissue)	38.64 ± 3.26	24.77 ± 1.96**	35.38 ± 2.61##
Superoxide dismutase (U/mg tissue)	0.34 ± 0.03	0.22 ± 0.01**	0.31 ± 0.02##
Catalase (μM H ₂ O ₂ consumed/mg tissue/min)	19.22 ± 1.13	12.58 ± 0.94**	17.37 ± 1.01##

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

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

$p < 0.01$ vs. bleomycin group.

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

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

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

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

$p < 0.01$ vs. bleomycin group.

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

Experimental Group	Tumour necrosis factor- α (pg/mL)	Grade of fibrosis
Control	22.82 \pm 2.10	0.00 \pm 0.00
Bleomycin	53.89 \pm 3.04**	2.50 \pm 0.07**
Bleomycin + sulindac	26.69 \pm 2.42##	1.58 \pm 0.15**##

Values are given as Mean \pm 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.

3.12. Histopathological examination of lung tissue

Histopathological abnormalities in lungs were detected on day 21 using haematoxylin and eosin staining (Figs. 3–5) and Masson's trichrome staining (Figs. 6–8).

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.

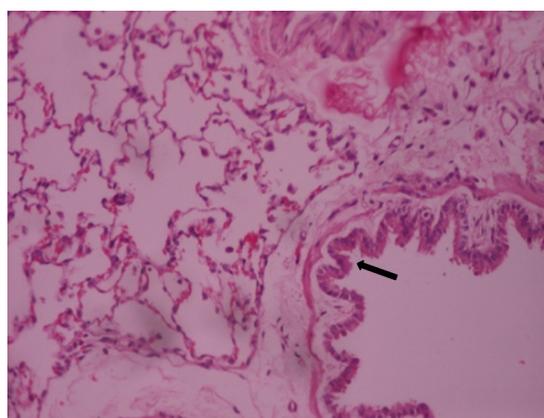


Fig. 3 – Control lung tissue showing normal open alveoli and interalveolar space (Haematoxylin and Eosin staining, 10 \times).

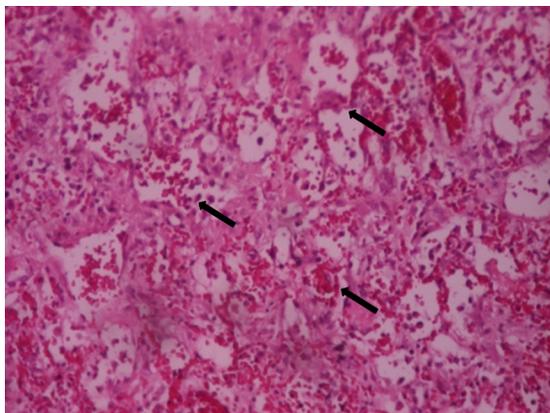


Fig. 4 – Bleomycin-treated lungs showing macrophage infiltration, haemorrhages, congestion and thickening of alveolar lining (Haematoxylin and Eosin staining – 10 \times).

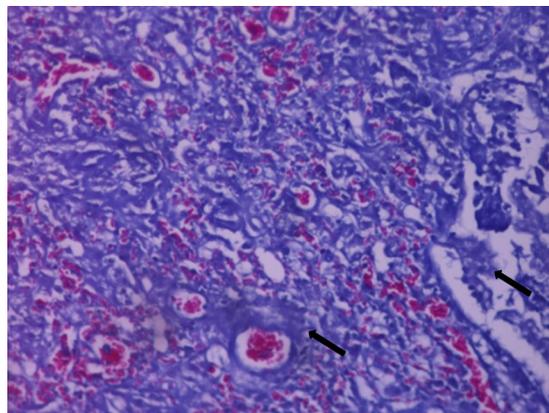


Fig. 7 – Bleomycin-treated lungs showing extensive collagen deposition as compared to the control group lungs (Masson's trichrome staining, 10 \times).

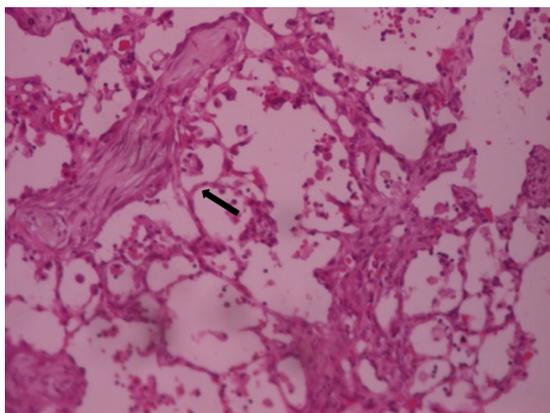


Fig. 5 – Sulindac treated lungs showing decrease in the thickening of alveolar lining and decrease in the macrophage infiltration as compared to bleomycin tissue (Haematoxylin and Eosin staining, 10 \times).

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.

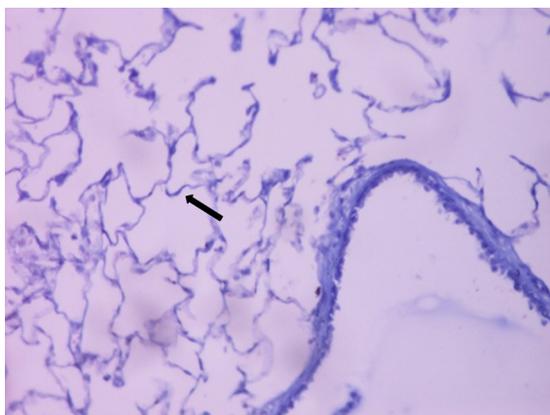


Fig. 6 – Normal lung tissue showing normal open pattern of alveoli and interalveolar space with minimal collagen deposition (Masson's trichrome staining, 10 \times).

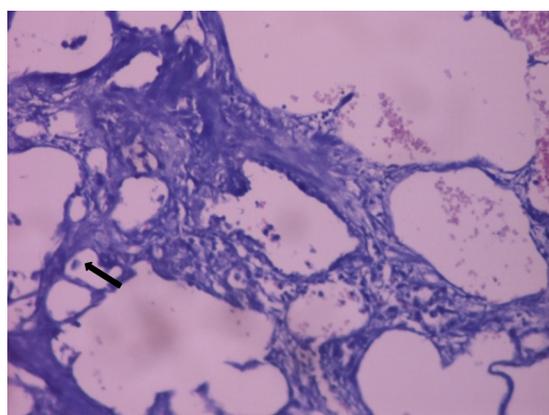


Fig. 8 – Sulindac-treated lungs showing mild collagen deposition as compared to bleomycin treated lungs (Masson's trichrome staining, 10 \times).

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 (Sleijfer, 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 untreatable 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 bronchoalveolar 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 perialveolar 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 (Fe^{2+}), 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 auto-catalytic, free radical mediated, destructive process, wherein polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides (Kalayarasan et al., 2008). 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 the antioxidant property of sulindac which may inhibit reactive oxygen radical production in the lungs.

In addition to the oxidative stress mentioned earlier, intra-tracheal 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 broncholarveolar 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.

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