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ORIGINAL ARTICLE

Pyrrolidinedithiocarbamate attenuates bleomycin-induced pulmonary fibrosis in rats: Modulation of oxidative stress, fibrosis, and inflammatory parameters

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ABSTRACT

Objective: The current study aimed to investigate the modulatory effects of pyrrolidinedithiocarbamate (PDTC; 100 mg/kg) on bleomycin-induced pulmonary fibrosis (5 mg/kg; intratracheal) in rats. **Materials and Methods:** Rats were randomly assigned to three groups: normal control, bleomycin control, and PDTC-treated groups. Lung injury was evaluated through histological examination, immunohistochemical detection of inducible nitric oxide synthase (iNOS) in lung tissue and evaluating the total and differential leucocytes count in bronchoalveolar lavage fluid. Lung tissue was used for biochemical assessment of lung content of hydroxyproline, transforming growth factor beta-1 (TGF- β 1), tumor necrosis factor-alpha (TNF- α) as well as analysis of lipid peroxides, reduced glutathione (GSH), and total nitrite contents. **Results:** PDTC attenuated bleomycin-induced pulmonary fibrosis as evidenced by histological observations, decreased iNOS expression and prevention of bleomycin-induced altered total and differential leukocytes count. Additionally, PDTC caused a significant decrease in lung contents of hydroxyproline, TGF- β 1, TNF- α , lipid peroxides, and total nitrite coupled with increase in lung GSH content as compared to bleomycin control group. **Conclusion:** PDTC attenuated bleomycin-induced pulmonary fibrosis in rats via its anti-inflammatory, antioxidant, and antifibrotic activities.

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KEYWORDS

bleomycin; nitric oxide; pyrrolidinedithiocarbamate; pulmonary fibrosis; tumor necrosis factor-alpha

Introduction

Bleomycin is an antitumor drug that used in the management of some human cancers, including lymphomas, squamous cell carcinomas, testicular tumors, and malignant pleural effusions. Its cytotoxicity occurs by induction of free radicals that cause DNA breaks leading to cell death.^[1] Pulmonary toxicity is potentially the most serious side effect, occurring in approximately 10% of treated patients.^[2] The most frequent manifestation is pneumonitis occasionally progressing to pulmonary fibrosis which may result in death.^[3] 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.^[4]

Bleomycin is known to generate reactive oxygen species upon binding to DNA and iron, which cause DNA damage.^[5] 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 lung.^[6] Bleomycin also promotes the depletion of endogenous antioxidant defenses thus exacerbating oxidant-mediated tissue injury.^[5] Bleomycin-induced lung fibrosis is a widely used animal model resembling human idiopathic pulmonary fibrosis.^[4] Strategies aimed at reducing oxidative stress and inflammatory cytokines release have been successfully tried in suppressing bleomycin-induced lung injury and fibrosis.^[1]

Pyrrolidinedithiocarbamate (PDTC) is a low-molecular weight thiol compound that functions as a metal chelator and antioxidant.^[7] In addition, PDTC has been found to inhibit nuclear factor-kappa B (NF- κ B) and inflammatory cytokines, protecting against many diseases including obstructive uropathy and neuropathic disorders.^[8,9] Moreover, PDTC attenuated the development of monocrotaline-induced pulmonary arterial hypertension through inhibition of NF- κ B.^[10] Clinical evidence relates NF- κ B to the pathogenesis of

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lung diseases like asthma and cystic fibrosis.^[10] Hence, the aim of the present study was to evaluate the efficacy of PDTC against bleomycin-induced pulmonary fibrosis by assessing various biochemical, histological, and ultrastructural changes in lungs of rats.

Materials and methods

Animals

Male Wistar albino rats weighing 150–200 g were used in the present study. They were purchased from the Egyptian Company for Production of Vaccines, Sera and Drugs (EGYVAC; Cairo, Egypt) and allowed free access to water and standard pellet chow. Rats were kept under constant conditions (temperature $25^{\circ}\text{C} \pm 3^{\circ}\text{C}$, and humidity 50%) with 12/12 hours light/dark cycles and were housed in plastic cages in the animal house at October University for Modern Science and Arts (MSA University). The study was carried out according to the guidelines of the Ethics Committee, Faculty of Pharmacy, Cairo University.

Drugs and chemicals

PDTC was purchased from Sigma-Aldrich (MO, USA); whereas bleomycin hydrochloride was obtained from Nippon Kayaku (Tokyo, Japan). All other chemicals used were of analytical grade.

Induction of pulmonary fibrosis

Briefly, after the weights were recorded, the rats were anesthetized using a combination of ketamine (80 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.). A mid-line incision was made in the neck and the trachea was exposed. A tracheal cannula was inserted into the trachea under direct visualization.^[11] For induction of pulmonary fibrosis, the rats received a single dose of 5 mg/kg bodyweights bleomycin HCl dissolved in 0.9% NaCl solution by intratracheal instillation and the skin was closed with cat gut surgical suture. The selected route and dose of bleomycin were chosen from published literature.^[12]

Experimental design

Rats were randomly allocated into three groups ($n = 12$). The first group of rats received a single intratracheal dose of sterile saline and served as normal control group. The second group (bleomycin control) received

a single intratracheal dose of bleomycin HCl solution (5 mg/kg), while the last group was treated with PDTC (100 mg/kg, p.o.) for 28 days started one week before intratracheal instillation of bleomycin.

After 21 days from bleomycin intratracheal instillation, six rats from each group were sacrificed by cervical dislocation under ketamine anesthesia, and then both lungs were rapidly dissected out and washed with ice-cold saline. In these animals, the right lung was homogenized in isotonic saline to prepare 10% homogenate that was used for biochemical assessment of lung content of hydroxyproline, transforming growth factor beta-1 (TGF- β 1) and tumor necrosis factor-alpha (TNF- α) as well as analysis of lipid peroxides, reduced glutathione (GSH) and total nitrite content, while the left lung was used for histopathological examination and immunohistochemical detection of inducible nitric oxide synthase (iNOS). The other six rats in each group were anesthetized with ketamine and their lungs were used for assessment of lung injury markers in bronchoalveolar lavage fluid (BALF) as total and differential leucocytes count.

Biochemical investigations

Hydroxyproline content was measured in lung homogenate as an indication of collagen deposition using rat specific immunoassay kit according to the method described by Yao et al.^[13] Pulmonary TNF- α and TGF- β 1 were determined by enzyme-linked immunosorbent assay (ELISA) technique using standard kits (MyBioSource, Inc., USA).

Lipid peroxidation in lung tissues was estimated by the determination of thiobarbituric acid reactive substances content that was evaluated as malondialdehyde (MDA) in lung homogenate using a standard kit purchased from Biodiagnostic (Egypt). Pulmonary GSH content was determined using a commercial kit based on Ellman's reaction (Biodiagnostic, Egypt). Pulmonary nitrite content was determined as an index of NO content in lung homogenate using a commercial kit (Assay Designs, Inc., USA).

Bronchoalveolar lavage fluid collection and analysis

After ketamine anesthesia, the lungs were prepared for lavage by inserting a cannula (size: 24G, flow rate: 16 mL/min) attached by a syringe into the trachea. BALF was obtained by washing the lung three times with 3 mL aliquots of saline through the

tracheal cannula (total 9 mL). Each time, saline remained in the lung for 30 seconds followed by gentle aspiration of the fluid from the lung. Recovery rates of BALF exceeded 80% and were not significantly different between groups. The total numbers of cells in the BALF were counted with a hemocytometer. For differential counts of leukocytes in the BALF, smear slides were prepared and stained with Giemsa solution.^[11]

Immunohistochemical expression of iNOS

Paraffin embedded tissue sections of 3 μm thickness were rehydrated in xylene and then in graded ethanol solutions. The slides were then blocked with 5% bovine serum albumin (BSA) in tris buffered saline (TBS) for 2 hours. The sections were then immunostained with primary antibody polyclonal immunoglobulin-G (IgG) to rat iNOS at a concentration of 1 $\mu\text{g}/\text{mL}$ with 5% BSA in TBS and incubated overnight at 4°C. After washing the slides with TBS, the sections were incubated with secondary antibody, diluted 1:2000 with 5% BSA in TBS and incubated for 2 hours at room temperature. Finally, sections were washed with TBS and incubated for 5–10 minutes in a solution of 0.02% diaminobenzidine containing 0.01% hydrogen peroxide. Counter staining was performed using hematoxylin, and the slides were visualized under a light microscope.^[4]

Histopathologic assessment of lung tissue damage

Autopsy samples were taken from the lung of rats in different groups and fixed in 10% formal saline for 24 hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56° in hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 μm thickness by slide microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by hematoxylin & eosin stain for routine examination as well as Masson stain for detection of the fibrous tissue and collagen through the light electric microscope.^[14] The criteria used for grading lung fibrosis were as follows. Grade 0: normal lung; Grade 1: minimal fibrous thickening of alveolar or bronchial walls; Grades 2–3: moderate thickening of walls without obvious damage to lung architecture; Grades 4–5: increased fibrosis with definite damage to lung architecture and formation of

fibrous bands or small fibrous mass; Grades 6–7: severe distortion of structure and large fibrous areas; “honeycomb lung” was placed in this category; and Grade 8: total fibrous obliteration of the field. The mean score of all the fields was taken as the fibrosis score of that lung section.^[4]

Statistical analysis

Data were expressed as mean \pm SEM. Comparisons between means of different groups were carried out using one-way analysis of variance followed by Tukey–Kramer multiple comparisons test.^[15] The level of significance was taken as $P < 0.05$. GraphPad Prism software package, version 5 (GraphPad Software, Inc., USA), was used to carry out all statistical tests.

Results

Effect of PDTC treatment on biochemical investigations

Intratracheal instillation of rats with bleomycin resulted in a marked increase in pulmonary hydroxyproline content (131.16 ± 8.7 ng/g wet tissue) as compared to that of the normal control rats (28.61 ± 1.3 ng/g wet tissue). Pretreatment with PDTC significantly suppressed the elevated hydroxyproline (38.90 ± 2.6 ng/g wet tissue) as compared to the bleomycin control group with nonsignificant difference from the normal control rats (Table 1).

In addition, bleomycin caused a marked increase in pulmonary TGF- β 1 and TNF- α contents (104.9 ± 7.75 and 39.89 ± 3.15 pg/g wet tissue, respectively) as compared to that of the normal control rats. Meanwhile, pretreatment with PDTC significantly decreased TGF- β 1 and TNF- α contents (34.83 ± 2.60 and 7.23 ± 0.32 pg/g wet tissue, respectively) as compared to

Table 1. Effect of pyrrolidinedithiocarbamate (PDTC) treatment on pulmonary contents of hydroxyproline, transforming growth factor-beta1 (TGF- β 1) and tumor necrosis factor-alpha (TNF- α) in bleomycin-induced pulmonary fibrosis in rats.

Parameter groups	Hydroxyproline (ng/g wet tissue)	TGF- β 1 (pg/g wet tissue)	TNF- α (pg/g wet tissue)
Normal control	28.61 ± 1.3	27.55 ± 1.51	6.84 ± 0.44
Bleomycin control	$131.16^a \pm 8.7$	$104.9^a \pm 7.75$	$39.89^a \pm 3.15$
PDTC (100 mg/kg)	$38.90^b \pm 2.6$	$34.83^b \pm 2.60$	$7.23^b \pm 0.32$

Each value represents mean \pm SEM ($n = 6$).

^a Significantly different from normal control group at $P < 0.05$.

^b Significantly different from bleomycin control group at $P < 0.05$.

Table 2. Effect of pyrrolidinedithiocarbamate (PDTC) treatment on pulmonary contents of malondialdehyde (MDA), reduced glutathione (GSH) and nitrite in bleomycin-induced pulmonary fibrosis in rats.

Parameter groups	MDA ($\mu\text{mol/g}$ wet tissue)	Nitrite ($\mu\text{mol/g}$ wet tissue)	GSH (mg/g wet tissue)
Normal control	31.94 \pm 2.36	10.44 \pm 0.34	27.74 \pm 0.80
Bleomycin control	192.50 ^a \pm 11.60	3.16 ^a \pm 0.13	113.73 ^a \pm 3.25
PDTC (100 mg/kg)	99.69 ^{a,b} \pm 3.58	6.09 ^{a,b} \pm 0.32	73.47 ^{a,b} \pm 2.22

Each value represents mean \pm SEM ($n = 6$).

^a Significantly different from normal control group at $P < 0.05$.

^b Significantly different from bleomycin control group at $P < 0.05$.

bleomycin control group with nonsignificant difference from normal control rats (Table 1).

Bleomycin instillation also resulted in a significant increase in pulmonary contents of MDA and nitrite (192.50 \pm 11.60 and 113.73 \pm 3.25 $\mu\text{mol/g}$ wet tissue, respectively) with a significant decrease in GSH content (3.16 \pm 0.13 mg/g wet tissue) as compared to that of the normal control rats. However, pretreatment with PDTC significantly decreased the elevated MDA and nitrite contents (99.69 \pm 3.58 and 73.47 \pm 2.22 $\mu\text{mol/g}$ wet tissue, respectively) with consequent increase in GSH content (6.09 \pm 0.32 mg/g wet tissue) as compared to that of the bleomycin control group (Table 2).

Effect of PDTC on total and differential cell counts in bronchoalveolar lavage fluid

Bleomycin administration resulted in a significant increase in the total leukocytes count in the BALF from 0.44 \pm 0.01 ($\times 10^6 \text{ mL}^{-1}$) in the normal control group to 1.44 \pm 0.07 ($\times 10^6 \text{ mL}^{-1}$) with a significant increase in the count of all different cell types of leukocytes. However, the increased leukocytes count is characterized by abnormal proportions of cell types. The differential cell count showed that the percentages of neutrophils and eosinophils were markedly increased in the lung exposed to bleomycin. On the other hand, the percentages of lymphocytes and alveolar macrophages

were significantly decreased as compared to the normal rats (Table 3).

Rats pretreated with PDTC showed a significant decrease in the total leukocytes count 0.68 \pm 0.02 ($\times 10^6 \text{ mL}^{-1}$) with marked normalization of the altered differential cell counts that revealed a significant decrease in the percentage of neutrophils and eosinophils, in addition to the significant increase in the percentages of lymphocytes and alveolar macrophages as compared to bleomycin control group (Table 3).

Effect of PDTC on the immunohistochemical expression of iNOS in lung tissue

Lung sections from normal control rats showed a small degree of immunostaining for iNOS in the peribronchiolar tissues and alveoli (Figure 1A). On the other hand intratracheal instillation of bleomycin produced severe increase in the immunohistochemical expression of iNOS in lung tissue as shown in Figure 1B.

Lung sections from rats pretreated with PDTC showed moderate immunohistochemical expression of iNOS as compared to that of bleomycin control group (Figure 1C). Comparative quantification of iNOS staining from all groups is presented in Figure 1D.

Effect of PDTC on the histological structure of the lung tissue

Hematoxylin and eosin (H&E) staining

Figure 2A showed the normal histological structure of lung tissue of a rat in normal control group in which the spongy structure of the lung appeared with thin interalveolar septa and normal clear alveoli. Bleomycin administration produced desquamation of bronchiolar lining epithelium (b) with fibrosis and inflammatory cells infiltration in collapsed air alveoli (f) while other alveoli showing emphysema (a; Figure 2B). On the other hand, pretreatment with PDTC produced almost

Table 3. Effect of pyrrolidinedithiocarbamate (PDTC) treatment on total and differential cell counts in bronchoalveolar lavage fluid (BALF) of bleomycin-induced pulmonary fibrosis in rats.

	Total leukocyte count ($\times 10^6 \text{ mL}^{-1}$)	Lymphocytes %	Macrophages %	Eosinophiles %	Neutrophiles %
Normal control	0.44 \pm 0.01	13.08 \pm 0.36	82.63 \pm 0.2	0.50 \pm 0.02	2.84 \pm 0.27
Bleomycin control	1.44 ^a \pm 0.07	6.64 ^a \pm 0.15	49.29 ^a \pm 0.97	4.07 ^a \pm 0.06	39.59 ^a \pm 0.67
PDTC (100 mg/kg)	0.68 ^{a,b} \pm 0.02	10.13 ^{a,b} \pm 0.13	71.58 ^{a,b} \pm 0.61	2.20 ^{a,b} \pm 0.1	15.11 ^{a,b} \pm 0.61

Each value represents mean \pm SEM ($n = 6$).

^a Significantly different from normal control group at $P < 0.05$.

^b Significantly different from bleomycin control group at $P < 0.05$.

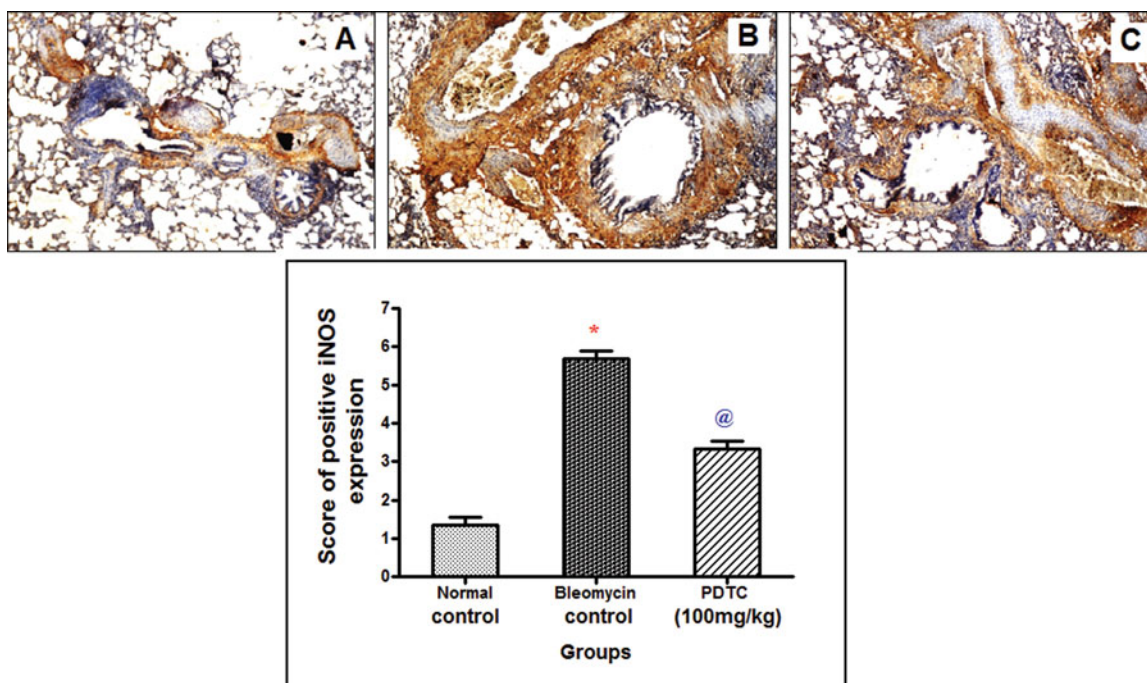


Figure 1. Effect of pyrrolidinedithiocarbamate (PDTC) treatment on the immunohistochemical expression of inducible nitric oxide synthase (iNOS) in lung tissue: (A) Section of a lung from a rat in the normal control group showed a small degree of immunostaining of iNOS in lung tissue as illustrated by the brown color ($\times 16$), (B) Section of a lung from a rat in the bleomycin control group showed severe increased expression of iNOS in peribronchiolar tissue and alveoli as illustrated by the brown color immunostaining ($\times 16$), (C) Section of a lung from a rat in pyrrolidinedithiocarbamate treated group showed moderate positive immunoreaction of iNOS in peribronchiolar tissue and bronchioles ($\times 16$), (D) Quantification of iNOS staining represents the number of stained (positive) cells per $16\times$ field was averaged across 15 field for each rat section. Each value represents mean \pm SEM ($n = 6$), *significantly different from normal control group at $P < 0.05$, @ significantly different from bleomycin control group at $P < 0.05$.

normal histological structure with marked decrease in inflammatory cells infiltration and fibrosis (Figure 2C).

Masson trichome staining

Lung tissue from normal rats showed normal histological structure with typical open alveoli, interalveolar spaces and bronchioles with lack of inflammatory cells infiltration and fibrosis (Figure 2D). Bleomycin injection produced obvious high increase in collagen deposition in lung tissue and peribronchiolar fibrosis as demonstrated by the blue color (Figure 2E). Meanwhile, lungs of the rats pretreated with PDTC showed marked decrease in collagen and fibroblastic cells proliferation in peribronchiolar tissue as compared to bleomycin control group (Figure 2F). Grading of pulmonary fibrosis, collagen deposition and inflammatory cells infiltration in histological examination of lung tissues is presented in Table 4.

Discussion

The major side effect of bleomycin that limit its use as a chemotherapeutic agent is the induction of pulmonary

Table 4. Effect of pyrrolidinedithiocarbamate (PDTC) treatment on grades of pulmonary fibrosis, collagen deposition, and inflammatory cells infiltration in histological examination of lung tissues.

Histological groups	Normal control	Bleomycin control	PDTC (100 mg/kg)
Inflammatory cells infiltration (H&E)	0.70 \pm 0.04	3.50 ^a \pm 0.18	0.92 ^b \pm 0.08
Focal fibrosis (H&E)	0.33 \pm 0.03	1.75 ^a \pm 0.13	0.60 ^b \pm 0.03
Collagen deposition (Masson trichome)	0.43 \pm 0.03	5.47 ^a \pm 0.27	0.95 ^b \pm 0.07

Each value represents mean \pm SEM ($n = 6$).

^a Significantly different from normal control group at $P < 0.05$.

^b Significantly different from bleomycin control group at $P < 0.05$.

fibrosis because of the low levels of cysteine hydrolyase that inactivates bleomycin in lung tissue leading to bleomycin accumulation.^[4] Bleomycin can be used in experimental models to cause lung injury leading to inflammatory and fibrotic lesions in the lung tissue of various animal species.^[11] This animal model of pulmonary fibrosis resembles that seen in humans and it

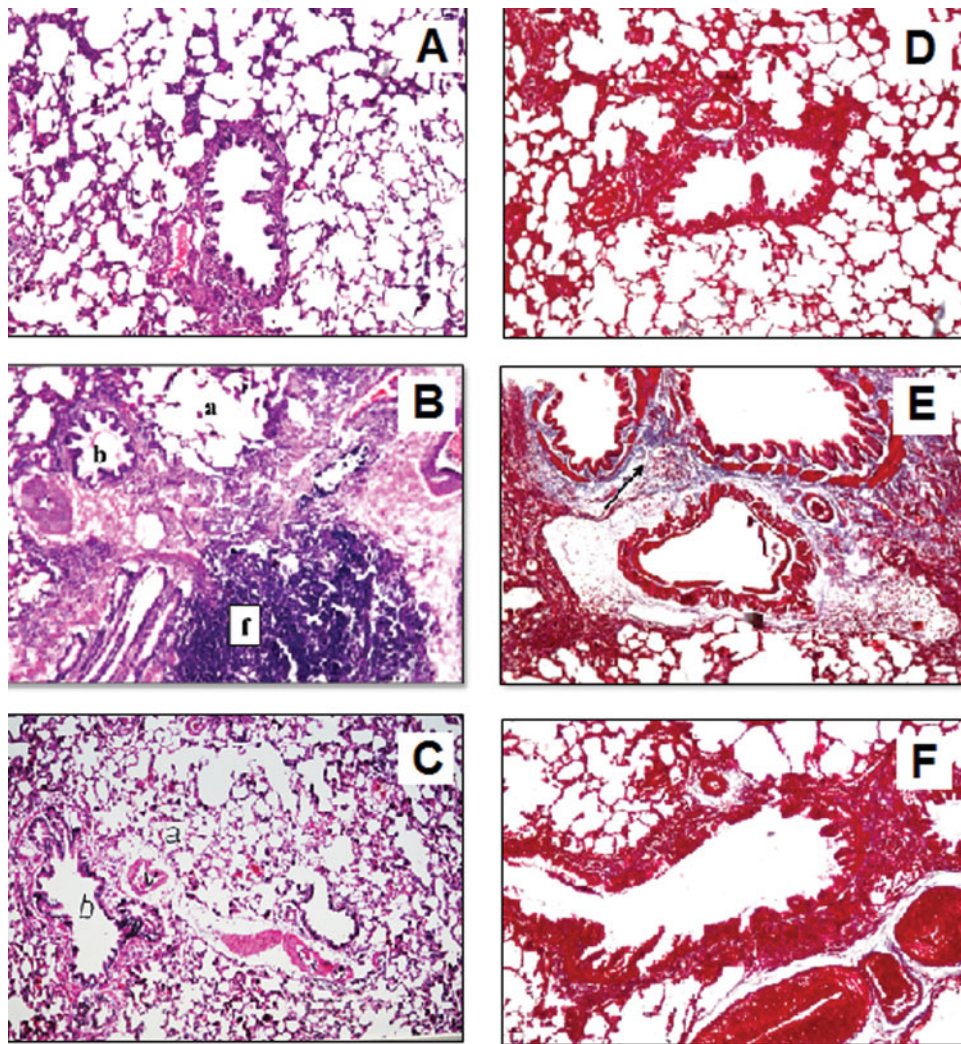


Figure 2. Effect of pyrrolidinedithiocarbamate (PDTC) treatment on the histological structure of the lung tissue in rats with bleomycin induced pulmonary fibrosis: Hematoxylin and eosin staining (H&E $\times 16$): (A) Section of a lung from a normal rat showed normal histological structure of the bronchioles and the surrounding air alveoli. (B) Section of a rat in bleomycin control group showed massive number of lymphoid cells aggregation and fibroblastic cells proliferation surrounding the bronchioles (f) with desquamation of bronchiolar lining epithelium (b) and emphysema (a), (C) Section of a lung of a rat in pyrrolidinedithiocarbamate treated group showed almost normal histological structure with marked decrease inflammatory cells infiltration and fibrosis. Masson trichome staining ($\times 16$): (D) Section of a lung from a normal rat showed normal histological structure, (E) Section of a rat in bleomycin control group showed peribronchiolar fibrosis with collagen proliferation (blue color), (F) Section of a lung of a rat in pyrrolidinedithiocarbamate treated group showed marked decrease in collagen deposition and fibroblastic cells proliferation in peribronchiolar tissue.

is useful to assess the effects of potential therapeutic agents.^[16]

Mechanisms proposed to explain bleomycin-induced lung damage are varied and include induction of a strong inflammatory response with inflammatory cells infiltration that is considered to be the initial response to injury from bleomycin.^[17] Bleomycin is also able to mediate DNA strand scission in presence of iron and oxygen, producing single or double strand breaks with consequent higher reactive oxygen species and reactive nitrogen species (RNS) production.^[18] These oxidants can activate several genes related to

cell growth, cell death, and fibroblast proliferation with multiple inflammatory mediators and cytokines release from the injured lung tissue possibly through activation of NF- κ B, and thereby promote pulmonary fibrosis.^[16]

The current study demonstrated increased levels of lipid peroxides as a marker of oxidative stress with a concomitant decrease in the antioxidant GSH level in bleomycin-injured rats that could be due to free radical-mediated membrane damage and the reflection of decrease in the antioxidants defense systems. These results are consistent with previous reports.^[11,19]

The results of this study also showed that pretreatment with PDTC significantly decreased the levels of lipid peroxides production in lung tissue with a concomitant increase in pulmonary GSH content as compared to bleomycin control group. These results are in harmony with the study of Yavuz et al.^[10] who showed the ability of PDTC to decrease lipid peroxides in lung tissue of rats with monocrotaline-induced pulmonary arterial hypertension. The antioxidant properties of PDTC were revealed via decreasing lipid peroxides and increasing GSH content in brain of rats with scopolamine-induced cognitive impairment.^[20] PDTC functions as an antioxidant due to two structural features: direct scavenging of reactive oxygen metabolites by the dithiocarboxy group and chelating activity for heavy metal ions that may catalyze formation of reactive oxygen metabolites.^[21]

NO is an endogenous short-lived free radical that freely diffuses within the cells. Extensive focus has been shifted on RNS, including NO, peroxynitrite and nitrogen dioxide in the pathogenesis of pulmonary fibrosis.^[22] Bleomycin induces generation of the reactive species in the lung tissue that results in DNA injury, lipid peroxidation, alteration in lung prostaglandins and an increase in collagen synthesis.^[23] As a result of injury, inflammation and cytokine dysregulation occur, fibroblasts are activated, and collagen production is stimulated, while collagen degradation is inhibited.^[4] The current study showed a significant increase in nitrite content in lung tissue of bleomycin control rats that could be considered an indication of increased RNS and in particular NO production. That was also proved through the increased expression of iNOS in lung tissue in the immunohistochemical reaction. The increase in nitrite content in lung tissue of bleomycin-treated rats is in harmony with the study of El-Khouly et al.,^[16] in addition Kalayarsan et al.^[4] reported increased iNOS expression with bleomycin treatment. Moreover, the current study demonstrated that pretreatment with PDTC was able to decrease iNOS expression in lung tissue with consequent suppression in lung nitrite content as compared to bleomycin control group. These results are in agreement with Wang et al.,^[8] who showed the ability of PDTC to provide neuroprotection after brain ischemia in neonatal rats through its ability to inhibit iNOS production in brain.

The current study also showed a significant increase in the total number of leukocytes count with altered proportions of each cell types through the increased

percentages of neutrophils and eosinophils within the differential cell count in BALF of bleomycin treated rats. This is in accordance with previous studies of El-Khouly et al.^[16] and Verma et al.^[11] In addition to oxidative stress, intratracheal administration of bleomycin leads to interstitial inflammation, with the marked increase in the recruitment of leukocytes.^[11] The alteration in inflammatory cell count in BALF is the hallmark of acute inflammatory response. Neutrophils have an ability to induce cellular toxicity via release of myeloperoxidase. The recruitment of leukocyte caused activation of fibroblasts that result in hyperproliferative response leading to an abnormality in the ultrastructural appearance of alveoli and thus fibrosis.^[24]

However, the total cell count, neutrophils, lymphocyte, and macrophages count in PDTC pretreated rats remained comparable to the normal control rats. Inhibited leukocytes recruitment, which directly impacted inflammation and tissue repair, might partly account for the preventive effect of PDTC on bleomycin-induced pulmonary fibrosis.

The inflammatory response is the initial response following injury and fibrosis is generally the final outcome of the inflammatory process in the lung. The response includes migration and activation of both resident and circulating inflammatory cells. Inflammatory cells release cytokines and growth factors, stimulate multiplication, migration, secretory activities, and collagen production by fibroblasts. Many cytokine interactions associated with inflammation and fibrosis have been reported in the course of pulmonary fibrosis.^[19]

TGF- β 1 is an important mediator and has a broad spectrum of activities in pulmonary inflammation, tissue repair, and fibrosis. TGF- β 1 can serve as a chemoattractant for fibroblasts and monocytes/macrophages and stimulate these cells to synthesize a number of proinflammatory and fibrogenic cytokines such as TNF- α , IL-6, and TGF- β 1 itself.^[25] Furthermore, TGF- β 1 is one of the most potent inducers of extracellular matrix production. At the same time, TGF- β 1 reduces the breakdown of collagen and other matrix proteins by inhibiting the generation of plasminogen activators, matrix metalloproteinase, and elastase, as well as by enhancing the expression of tissue inhibitors of metalloproteinases, plasminogen activator inhibitor-1 and -2. In this study, TGF- β 1 production in lung tissue was elevated after bleomycin treatment, as was consistent with previous investigation.^[18]

Moreover, TNF- α , a potent proinflammatory cytokine acts as one major molecule among the multifaceted networks of cellular and molecular interactions that regulate the fibrotic process.^[24] In this study, a significant elevation in lung TNF- α content expression was observed in the bleomycin-treated group, which is in accordance with the findings of Nikbakht et al.^[11] The tissue injury caused by bleomycin is inflammatory mediated and might be due to the production of free radicals, possibly leading to activation of NF- κ B and increased synthesis of TNF- α .^[11] In the current experiments, pretreatment of rats with PDTC prevented bleomycin-induced increases in lung content of TGF- β 1 and TNF- α . PDTC was reported to bear numbers of beneficial properties plus its antioxidant activity including anti-inflammatory and immunomodulatory effects,^[26] all of which could account for the observed effects of PDTC on lung cytokines contents. PDTC blocks NF- κ B activation and finally inhibit NF- κ B mediated increased production of proinflammatory cytokines as TNF- α .^[27] Hagar^[28] showed the ability of PDTC to decrease TNF- α concentration in serum of rats subjected to lipopolysaccharide-induced oxidative stress and acute hepatic injury rats, while Abd-El-Fattah et al.^[20] demonstrated decrease TNF- α content in brain of demented rats.

Hydroxyproline is a hallmark of collagen synthesis. Furthermore, collagen which is an interstitial matrix component also have been reported to play the important role in the pathogenesis of pulmonary fibrosis.^[24] In the present study, elevated hydroxyproline content in lung after bleomycin administration was correlated with the accumulated collagen in the alveolar space. These results are in agreement with Verma et al.^[11] 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 have been reported by other studies.^[16,29] The current study showed that PDTC was successfully able to inhibit the bleomycin-induced pulmonary fibrosis through the previously mentioned mechanisms. These were demonstrated through the significant inhibition of hydroxyproline content in lung tissue as an indication of decreased collagen deposition. Moreover, it was observed that PDTC could

hinder the structural distortion caused by bleomycin as indicated by the improvement in lung fibrosis scores.

In summary, the present study demonstrated that PDTC could significantly suppress bleomycin-induced pulmonary fibrosis in rats due to its anti-inflammatory and antioxidant properties. Based on these findings PDTC may be a promising candidate in the prevention of bleomycin-induced pulmonary fibrosis and attenuation of the exacerbation of idiopathic pulmonary fibrosis.

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Declaration of interest

The authors declared no potential conflicts of interest with respect to research, authorship, and publication of this paper.

References

- [1] Nikbakht J, Hemmati AA, Arzi A, Mansouri MT, Rezaie A, Ghafourian M. Protective effect of gallic acid against bleomycin-induced pulmonary fibrosis in rats. *Pharmacol Rep.* 2015;67(6):1061–1067.
- [2] Patil N, Paulose RM, Udupa KS, Ramakrishna N, Ahmed T. Pulmonary toxicity of bleomycin: a case series from a tertiary care center in southern India. *J Clin Diagn Res.* 2016;10(4):1–3.
- [3] Calzas Rodríguez J, Carmen Juárez Morales Md, Casero MAR. Death by bleomycin pulmonary toxicity in ovarian dysgerminoma with pathologic complete response to chemotherapy. A Case Report. *Resp Med Case Rep.* 2016;18(1):48–50.
- [4] Kalayarasan S, Sriram N, Sudhandiran G. Diallyl sulfide attenuates bleomycin-induced pulmonary fibrosis: critical role of iNOS, NF- κ B, TNF- α and IL-1 β . *Life Sci.* 2008;82(23):1142–1153.
- [5] El-Medany A, Hagar HH, Moursi M, At Muhammed R, El-Rakhawy FI, El-Medany G. Attenuation of bleomycin-induced lung fibrosis in rats by mesna. *Eur J Pharmacol.* 2005;509(1):61–70. Epub 2005/02/17.
- [6] Erdem A, Congur G. Impedimetric detection of in situ interaction between anti-cancer drug bleomycin and DNA. *Int J Biol Macromol.* 2013;61(1):295–301.
- [7] Chen YW, Chen KL, Chen CH, Wu HC, Su CC, Wu CC, et al. Pyrrolidinedithiocarbamate (PDTC)/Cu complex induces lung epithelial cell apoptosis through

- mitochondria and ER-stress pathways. *Toxicol Lett.* 2010;199(3):333–340. Epub 2010/10/06.
- [8] Wang Z, Zhao H, Peng S, Zuo Z. Intranasal pyrrolidinedithiocarbamate decreases brain inflammatory mediators and provides neuroprotection after brain hypoxia: ischemia in neonatal rats. *Exp. Neurol.* 2013;249(1):74–82.
- [9] Chuang YH, Chuang WL, Huang SP, Liu CK, Huang CH. Inhibition of nuclear factor-kappa B (NF-kappaB) activation attenuates ureteric damage in obstructive uropathy. *Pharmacol Res.* 2009;60(4):347–357. Epub 2009/05/26.
- [10] Yavuz T, Uzun O, Macit A, Comunoglu C, Yavuz O, Silan C, et al. Pyrrolidinedithiocarbamate attenuates the development of monocrotaline-induced pulmonary arterial hypertension. *Pathol Res Pract.* 2013;209(5):302–308. Epub 2013/04/16.
- [11] Verma R, Brahmanekar M, Kushwah L, Suresh B. Evaluating the inhibitory potential of sulindac against the bleomycin-induced pulmonary fibrosis in Wistar rats. *Environ Toxicol Pharmacol.* 2013;36(3):769–778. Epub 2013/08/21.
- [12] Wei X, Han J, Chen ZZ, Qi BW, Wang GC, Ma YH, et al. A phosphoinositide 3-kinase-gamma inhibitor, AS605240 prevents bleomycin-induced pulmonary fibrosis in rats. *Biochem Biophys Res Comm.* 2010;397(2):311–317. Epub 2010/06/01.
- [13] Yao C, Li A, Gao W, Pallua N, Steffens G. Improving the angiogenic potential of collagen matrices by covalent incorporation of *Astragalus* polysaccharides. *Int J Burns Trauma.* 2011;1(1):17–26.
- [14] Bancroft P, Stevens A, Turner DR. *Theory and Practice of Histological Techniques.* 4th ed. London: Churchill Livingstone; 1996.
- [15] Tukey JW. Reminder sheets for “Discussion of paper on multiple comparisons by Henry Scheffe”. In the collected work of Tukey VIII John W., ed. *Multiple Comparisons.* New York: Chapman and Hall; 1951.
- [16] El-Khouly D, El-Bakly WM, Awad AS, El-Mesallamy HO, El-Demerdash E. Thymoquinone blocks lung injury and fibrosis by attenuating bleomycin-induced oxidative stress and activation of nuclear factor Kappa-B in rats. *Toxicology.* 2012;302(2):106–113.
- [17] Takemasa A, Ishii Y, Fukuda T. A neutrophil elastase inhibitor prevents bleomycin-induced pulmonary fibrosis in mice. *Eur Respir J.* 2012;40(6):1475–1482. Epub 2012/03/24.
- [18] Della-Latta V, Cecchetti A, Del Ry S, Morales MA. Bleomycin in the setting of lung fibrosis induction: from biological mechanisms to counteractions. *Pharmacol Res.* 2015;97(1):122–130.
- [19] Liang X, Tian Q, Wei Z, Liu F, Chen J, Zhao Y, et al. Effect of Feining on bleomycin-induced pulmonary injuries in rats. *J Ethnopharmacol.* 2011;134(3):971–976. Epub 2011/02/22.
- [20] Abd-El-Fattah MA, Abdelakader NF, Zaki HF. Pyrrolidinedithiocarbamate protects against scopolamine-induced cognitive impairment in rats. *Eur J Pharmacol.* 2014;723(1):330–338.
- [21] Huang CF, Liu SH, Lin-Shiau SY. Pyrrolidinedithiocarbamate augments Hg⁽²⁺⁾-mediated induction of macrophage cell death via oxidative stress-induced apoptosis and necrosis signaling pathways. *Toxicol Lett.* 2012;214(1):33–45. Epub 2012/08/23.
- [22] Abuezz SA, Hendawy N, Osman WM. Aliskiren attenuates bleomycin-induced pulmonary fibrosis in rats: focus on oxidative stress, advanced glycation end products, and matrix metalloproteinase-9. *Naunyn Schmiedeberg Arch Pharmacol.* 2016;7(1):1–13.
- [23] Guo C, Atochina-Vasserman E, Abramova H, George B, Manoj V, Scott P, et al. Role of NOS2 in pulmonary injury and repair in response to bleomycin. *Free Radic Biol Med.* 2016;91(1):293–301.
- [24] Kandhare AD, Bodhankar SL, Mohan V, Thakurdesai PA. Effect of glycosides based standardized fenugreek seed extract in bleomycin-induced pulmonary fibrosis in rats: decisive role of Bax, Nrf2, NF- κ B, Muc5ac, TNF- α and IL-1 β . *Chem Biol Interact.* 2015;237(1):151–165.
- [25] Zhao L, Wang X, Chang Q, Xu J, Huang Y, Guo Q, et al. Neferine, a bisbenzylisoquinoline alkaloid attenuates bleomycin-induced pulmonary fibrosis. *Eur J Pharmacol.* 2010;627(3):304–312.
- [26] Cuzzocrea S, Chatterjee PK, Mazzon E, Dugo L, Serraino I, Britti D, et al. Pyrrolidinedithiocarbamate attenuates the development of acute and chronic inflammation. *Br J Pharmacol.* 2002;135(2):496–510.
- [27] Pinho-Ribeiro FA, Fattori V, Zarpelon AC, Borghi SM, Staurengo-Ferrari L, Carvalho TT, et al. Pyrrolidinedithiocarbamate inhibits superoxide anion-induced pain and inflammation in the paw skin and spinal cord by targeting NF-kappaB and oxidative stress. *Inflammopharmacology.* 2016;9(1):9.
- [28] Hagar HH. An insight into the possible protective effect of pyrrolidinedithiocarbamate against lipopolysaccharide-induced oxidative stress and acute hepatic injury in rats. *Saudi Pharm J.* 2009;17(4):259–267.
- [29] Khazri O, Charradi K, Limam F, El May MV, Aouani E. Grape seed and skin extract protects against bleomycin-induced oxidative stress in rat lung. *Biomed Pharmacotherapy.* 2016;81(1):242–249.