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Effects of erdosteine on bleomycin-induced lung fibrosis in rats

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Abstract

This study was designed to examine the effects of erdosteine on bleomycin (BLM)-induced lung fibrosis in rats. Thirty-three Sprague–Dawley rats were divided randomly into three groups, bleomycin alone (BLM), bleomycin + erdosteine (BLM + ERD), and saline alone (control). The BLM and BLM + ERD groups, were given 2.5 mg/kg BLM intratracheally. The first dose of oral erdosteine (10 mg/kg/day) in the BLM + ERD group was started 2 days before BLM administration and continued until animals were sacrificed. Animals were sacrificed 14 days after intratracheal instillation of BLM. The effect of erdosteine on pulmonary fibrosis was studied by analysis of bronchoalveolar lavage (BAL) fluid, histopathology, and biochemical measurements of lung tissue superoxide dismutase (SOD) and glutathione (GSH) as antioxidants, malondialdehyde (MDA) as an index for lipid peroxidation, and nitrite/nitrate levels. Bleomycin-induced lung fibrosis as determined by lung histology was prevented with erdosteine (grades of fibrosis were 4.9, 2.3, and 0.2 in BLM, BLM + ERD, and control groups, respectively). Erdosteine also prevented bleomycin-induced increase in MDA (MDA levels were 0.50 ± 0.15, 0.11 ± 0.02, and 0.087 ± 0.03 nmol/mg protein in BLM, BLM + ERD, and control groups, respectively) and nitrite/nitrate (nitrite/nitrate levels were 0.92 ± 0.06, 0.60 ± 0.09, and 0.56 ± 0.1 μmol/mg protein in BLM, BLM + ERD, and control groups respectively) levels. Bleomycin-induced decrease in GSH and SOD levels in the lung tissue also prevented by erdosteine [(GSH levels were 213.5 ± 12.4, 253.2 ± 25.2, and 287.9 ± 34.4 nmol/mg protein) (SOD levels were 1.42 ± 0.12, 1.75 ± 0.17, and 1.89 ± 0.09 U/mg protein) in BLM, BLM + ERD, and control groups respectively]. Erdosteine prevented bleomycin-induced increases in total cell number and neutrophil content of the BAL fluid. In conclusion, oral erdosteine is effective in prevention of BLM-induced lung fibrosis in rats possibly via the repression of neutrophil accumulation, inhibition of lipid peroxidation, and maintenance of antioxidant and free radical scavenger properties. (Mol Cell Biochem **281:** 129–137, 2006)

Key words: bleomycin, erdosteine, lung, nitric oxide

Introduction

The process of fibrosis is important in the lung since its progression may result in respiratory failure and a poor prognosis in patients with a heterogeneous group of disorders of known and unknown etiology. The progression of fibrosis is closely controlled by a complicated network consisting of many cytokines, chemical mediators, growth factors, and biophysical peptides derived from inflammatory immune cells, endothelial cells, and alveolar type II cells. It has generally

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been hypothesized that activated inflammatory cells which accumulate in the lower airways release a harmful amount of reactive oxygen species (ROS), including superoxide and hydroxyl radicals, that results in lung injury and proliferation of fibroblasts in alveolar walls [1, 2]. Recently it has been suggested that reactive nitrogen species (RNS), including nitric oxide (NO), peroxynitrite ($ONOO^-$) and nitrogen dioxide, which are released from neutrophils and macrophages, play an important role in the pathogenesis of lung diseases [3–5]. It is known that when the superoxide anion is released, NO is rapidly spent to produce the high reactive $ONOO^-$, a potent oxidizing agent known to initiate lipid peroxidation of biological membranes, hydroxylation, nitration of aromatic amino acid residues and sulfhydril oxidation of protein [6].

Intratracheal instillation of the antitumor agent bleomycin (BLM) is the most commonly used animal model for pulmonary fibrosis. In this model, bleomycin is known to generate reactive oxygen metabolites, including superoxide and hydroxyl radicals, which can attack DNA causing strand cleavage. This in turn can induce lipid peroxidation, carbohydrate oxidation, alteration in lung prostaglandin synthesis and degradation, and an increase in lung collagen synthesis [7, 8]. Aside from histopathological examination, BLM-induced lung injury can be shown indirectly by measurements of antioxidants such as superoxide dismutase (SOD) and glutathione (GSH) and of indices of lipid peroxidation such as malondialdehyde (MDA).

Many possible treatment protocols for pulmonary fibrosis (PF) have been investigated, but none have succeeded in clinical trials [9, 10]. Reactive species mediated damage in the lung tissues has been inhibited, at least partially, by addition of different pharmacological agents, such as IL-6, ambroxol, captoril, and niacin in many studies [11–13]. Erdosteine (ERD) is a mucoactive drug. The metabolization of the erdosteine produces an active metabolite (Met I) with a reducing SH group. In addition to its mucolytic action, Met I also has useful antioxidant activity [14]. *In vivo* and *in vitro* studies have demonstrated that ERD has a potent free radical scavenging efficacy [15–18]. Recently it is suggested that ERD attenuated BLM-induced lung fibrosis [19]. Therefore, we examined whether or not ERD at a dose of 10 mg/kg in rats inhibits the PF induced by a single intratracheal instillation of BLM.

Materials and methods

Animals and preparation of BLM-induced pulmonary fibrosis

Thirty-three male Sprague–Dawley rats weighing 200–250 g and obtained from the Experimental Medical Research Unit (DETAB, Kocaeli University Medical Faculty, Kocaeli, Turkey) were used. The Kocaeli University Ethics Committee approved the procedures. Rats were kept in individual solid bottom plastic cages on sawdust bedding in a temperature- and humidity-controlled room ($22 \pm 3\,^{\circ}C$ and $62 \pm 7\%$, respectively) in which a 12–12 h light-dark cycle was maintained (08:00–20:00 h light). The rats had free access to water and food ad libitum.

After weight was recorded, rats were divided into three groups as follows: BLM, BLM + erdosteine (BLM + ERD) and saline alone (control). In BLM and BLM + ERD groups, 2.5 mg/kg BLM (Nippon Kayaku, Tokyo, Japan) in 0.3 ml sterile saline was administered intratracheally after anesthesia. The control group received 0.3 ml of sterile saline instead of BLM. From 2 days before the BLM administration, until the end of the study rats in the BLM + ERD group received ERD once a day at a dose of 10 mg/kg [20] which was administered via orogastric cannula to achieve a plateau level in blood.

The rats were sacrificed 14 days after BLM administration. Lungs were lavaged five times with 7 ml saline and the lavage fluids were centrifuged at 250 g for 10 min to separate the cells and the supernatants. A total cell count was performed with a haemocytometer and differential cell counts were performed on cytospin preparations stained with Wright–Giemsa. Two hundred cells were counted for determination of the differential cell count.

Histopathology

After death, lung tissues of rats were fixed by inflation with a buffered 10% formalin solution. Lung tissues were embedded in paraffin, and sections were stained with hematoxylin and eosin (HE) for examination by light microscopy. Morphological changes in lung sections were graded semiquantitatively without knowledge of treatment groups using a grading system that was previously described by Ashcroft *et al*. [21]. Criteria for grading PF were as follows – Grade 0: normal lung; Grade 1: minimal fibrous thickening of alveolar or bronchial walls; Grades 2 and 3: moderate thickening of walls without obvious damage to lung architecture; Grades 4 and 5: increased fibrosis with definite damage to lung architecture and formation of fibrous bands or small fibrous mass; Grades 6 and 7: severe distortion of structure and formation of fibrous areas; and Grade 8: total fibrous obliteration of the field. The mean score of ten fields was taken as the fibrosis score of that lung section.

Preparation of lung tissue for biochemical analyses

All lung tissues obtained for the biochemical measurements were washed in 0.9% NaCl and kept in ice. Tissues were homogenized with cold 1.15% KCl to make a 10%

homogenate (w/v) and centrifuged at 400 *g* for 10 min at 4 °C. The protein concentrations of tissue homogenates were determined by the method of Lowry *et al*. [22].

Lung tissue superoxide dismutase (SOD) analysis

Total Cu-Zn and Mn SOD activity was measured kinetically by the method of Sun *et al*. [23]. The activity was expressed as units per milligram protein (U/mg protein).

Lung tissue glutathione (GSH) analysis

Tissue GSH was measured using 5,5′-dithiobis-(2-nitrobenzoate) at 412 nm according to Ellman [24], and the results were expressed as nmol/mg protein.

Lung tissue malondialdehyde (MDA) analysis

Tissue lipid peroxide levels, expressed in term of MDA, were determined according to the method of Buege and Aust [25]. The results were expressed as nmol/mg protein.

Nitric oxide (nitrite/nitrate) determination in lung tissue

The supernatant (100 μl) was added to a mixture of 400 μl of distilled water and 300 μl of 0.3 N NaOH solution for NO measurement. The solution was kept for 5 min at room temperature, and then 300 μl of 5% (w/v) $ZnSO_4$ was added. The mixture was allowed to stand for another 5 min after shaking and then centrifuged at 10,000*g* for 20 min at 4 °C. The total of NO-metabolites nitrate (after reduction to nitrite by cadmium granules) + nitrite was assayed colorimetrically using the Griess reaction as described previously [26]. The Griess reagent consists of sulfanilamide and *N*-(1-naphthyl) ethylenediamine. The method is based on a two-step process, the first of which is the conversion of nitrate to nitrite using nitrate reductase. The second step is the addition of Griess reagent, which converts nitrite into a deep-purple azo compound; photometric measurement of the absorbance at 540 nm due to this azochromophore accurately determines the nitrite concentration (sodium nitrate is used as a standard). Finally we measured nitrite + nitrate (nitrite/nitrate) levels which reflects the amount of NO. Results are expressed as μmol/mg protein.

Statistical analysis

Comparisons between groups were made using Kruskal–Wallis analysis of variance and Mann–Whitney U tests. Data were analyzed in Statistical Package for the Social Science (SPSS) Version 11.5. Significance level was accepted as 0.05.

Results

Bronchoalveolar lavage (BAL) fluid cells

Recovery rates of BAL fluid ranged from 84 to 96% and were not significantly different between the three groups. A significant difference in total cell number, neutrophils, and alveolar macrophages was seen between the BLM group and the control group. The total cell number and the percentage of neutrophils in the bronchoalveolar lavage (BAL) fluid in the BLM group were significantly higher than in the control group ($p < 0.01$). This increase was significantly blocked by ERD (Table 1).

The analysis of oxidant stress markers

The SOD and GSH levels in lung tissue were found to be significantly lower in the BLM group [SOD (mean = 1.42±0.12; median = 1.49 U/mg protein), GSH (mean = 213.5 ± 12.4; median = 215 nmol/mg protein)] than in the control group [SOD (mean = 1.89 ± 0.09; median = 1.88 U/mg protein), GSH (mean = 287.9±34.4; median = 286 nmol/mg protein)] ($p < 0.01$). Treatment with ERD significantly prevented the decrease in GSH and SOD levels produced by BLM [SOD (mean = 1.75 ± 0.17; median = 1.78 U/mg protein), GSH (mean = 253.2 ± 25.2; median = 247 nmol/mg protein)] (Figs. 1 and 2).

The lung tissue MDA content was found to be significantly higher in the BLM group (mean = 0.50 ± 0.15; median = 0.57 nmol/mg protein) when compared with the control group (mean = 0.087 ± 0.03; median = 0.07 nmol/mg protein) ($p < 0.01$). Treatment with ERD significantly prevented this increase in MDA level (mean = 0.11 ± 0.02; median = 0.11 nmol/mg protein) (Fig. 3).

Table 1. Effect of erdosteine on bleomycin-induced changes in total and differential cell counts in BAL fluids from rats

Treatment groups	Total cells N ($\times 10^3$ mL^{-1})	Lymphocytes (%)	Neutrophils (%)	Macrophages (%)
BLM + ERD	5 376 (256–468)	3 (3–4)**	4 (3–5)**	93 (91–95)**
BLM alone	5 665 (391–832)*	5 (4–5)*	10 (10–11)*	85 (84–84)*
Control	6 305 (183–316)	1 (1–2)	1 (1–2)	98 (97–98)

Data presented as median and (range) of groups ($n = 5$ or 6). BLM + ERD: bleomycin + erdosteine; BLM alone: bleomycin + vehicle; control: vehicle + vehicle.

*$p < 0.05$ vs. BLM + ERD and control groups; **$p < 0.05$ vs. control group.

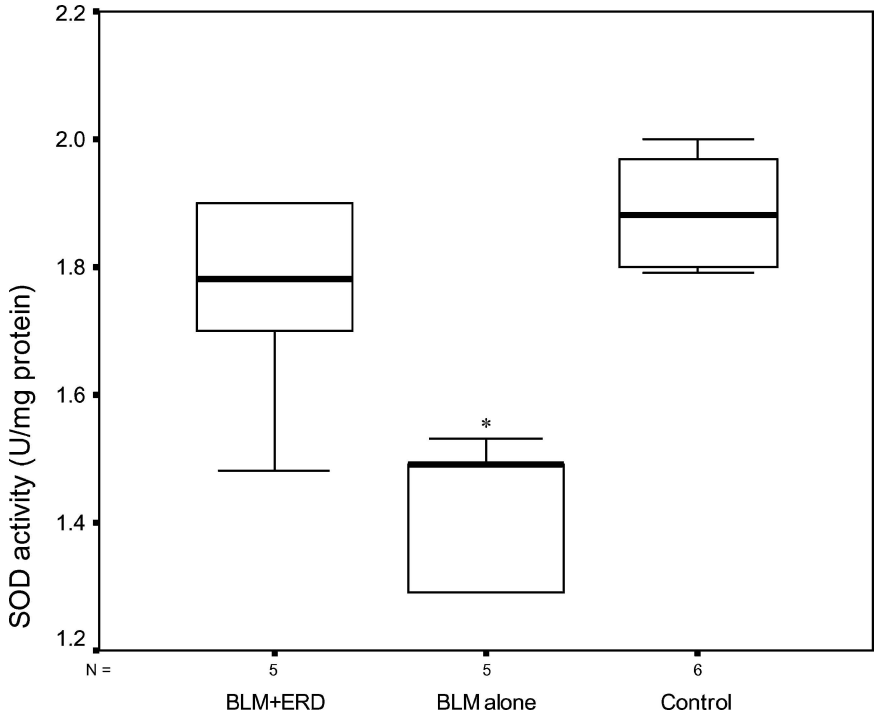

Fig. 1. Effect of erdosteine on bleomycin-induced decreases in the superoxide dismutase (SOD) activity. BLM + ERD: bleomycin + erdosteine; BLM alone: bleomycin + vehicle; control: vehicle + vehicle. Treatment with erdosteine significantly prevented the depletion of SOD activity. *Significantly lower ($p < 0.05$ when compared to the control and BLM + ERD groups).

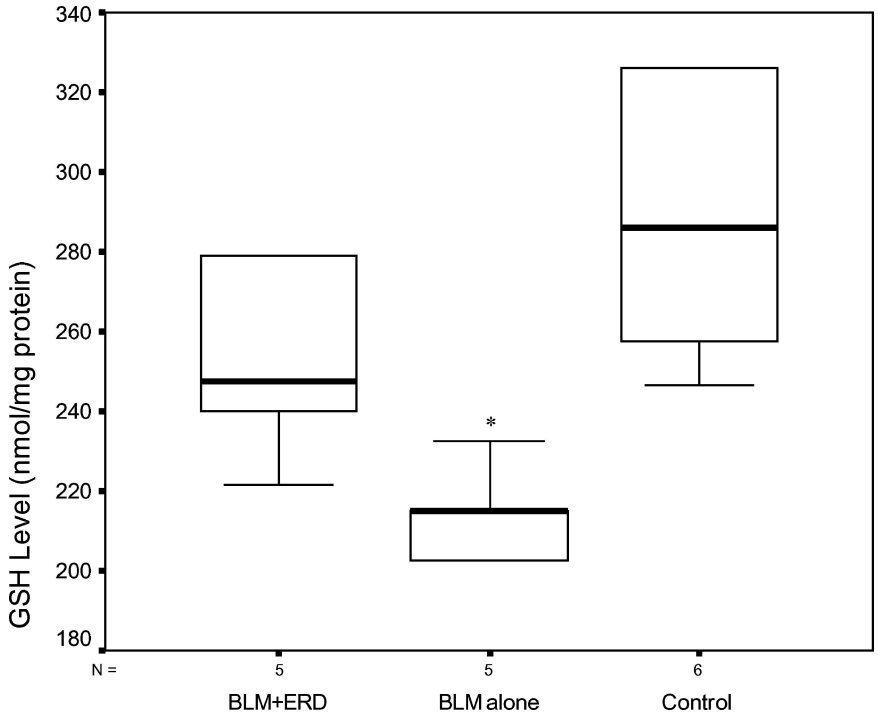

Fig. 2. The effect of erdosteine on bleomycin-induced decreases in the glutathione (GSH) level. See the legend for Fig. 1 for explanation of abbreviations. Treatment with erdosteine prevented the depletion of GSH. *Significantly lower ($p < 0.05$ when compared to the control and BLM + ERD groups).

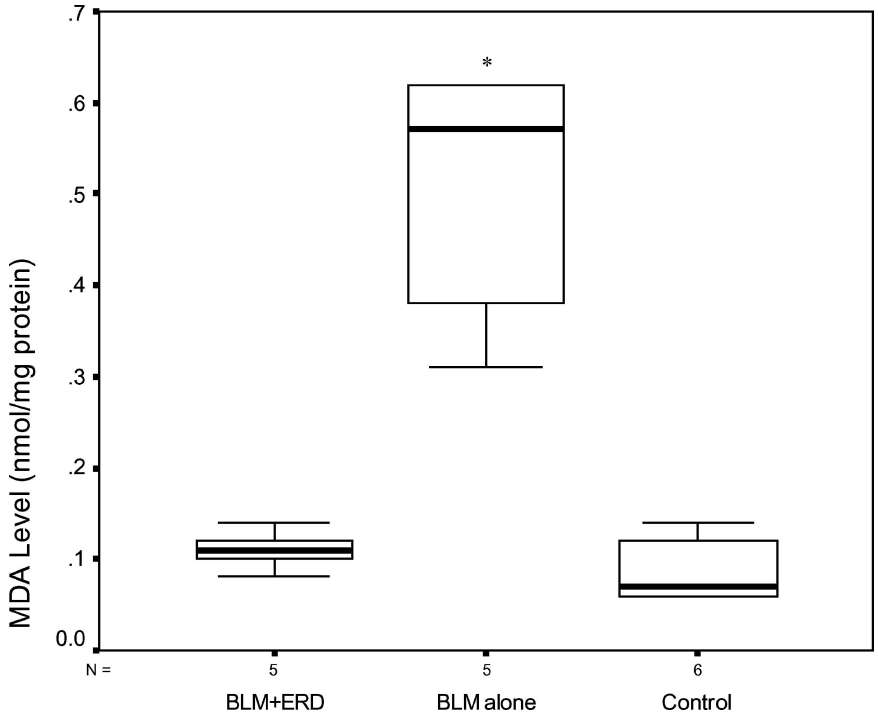

Fig. 3. The effect of erdosteine on bleomycin-induced increase in malondialdehyde (MDA) level. See the legend for Fig. 1 for explanation of abbreviations. Treatment with erdosteine significantly prevented the increase in lung tissue MDA levels. *Significantly higher ($p < 0.05$) when compared to the control and BLM + ERD groups.

Lung tissue nitric oxide (nitrite/nitrate) analysis

Bleomycin administration caused a significant increase of lung tissue nitrite/nitrate levels (mean $= 0.92 \pm 0.06$; median $= 0.91$ μmol/mg protein) when compared to control group (mean $= 0.56 \pm 0.1$; median $= 0.58$ μmol/mg protein) ($p < 0.01$). As shown in Fig. 4, erdosteine pretreatment inhibited in the increase of nitrite/nitrate level (mean $= 0.60 \pm 0.09$; median $= 0.63$ μmol/mg protein).

Assessment of pulmonary fibrosis

In the semi-quantitative assessment of lung sections, no inflammatory or fibrotic changes were observed in lungs of rats that had received normal saline (Fig. 5A). The BLM treatment produced an increase in the pathology score (Fig. 5B) as compared to the control group. This increase in fibrosis score induced by BLM was significantly reduced with ERD treatments (Fig. 5C). The grades of fibrosis in the three groups are presented in Table 2.

Discussion

In the present study, we evaluated the effect of ERD on the evolution of BLM-induced lung injury which is resulted in lung fibrosis. Our results demonstrate that ERD may prevent development of BLM-induced lung fibrosis, as evaluated by semi-quantitative morphological assessment of lung fibrosis.

Idiopathic pulmonary fibrosis (IPF) is characterized by chronic inflammation and exuberant collagen production within the lung [27]. Inflammation is a major component in the pathogenesis of interstitial lung fibrosis that is orchestrated in part by endogenous and migrating leukocytes. These leukocytes together with lung epithelial and endothelial cells produce a feedback circle where stimuli from injury responses can activate alveolar and interstitial macrophages [28]. Many chemotactic factors and adhesion molecules, including E-selectin and interleukin (IL)-8, are involved in

Table 2. Grade of lung fibrosis

Treatment groups	*N*	Grade of fibrosis
BLM + ERD	5	2.3 (2.1–3.2)**
BLM alone	5	4.9 (4.5–5.6)*
Control	5	0.2 (0.1–0.3)

Data presented as median and (range) of groups ($n = 5$). BLM + ERD, bleomycin + erdosteine; BLM alone, bleomycin + vehicle; control, vehicle + vehicle.
* $p < 0.001$ vs. BLM + ERD and control groups; ** $p < 0.001$ vs. control group.

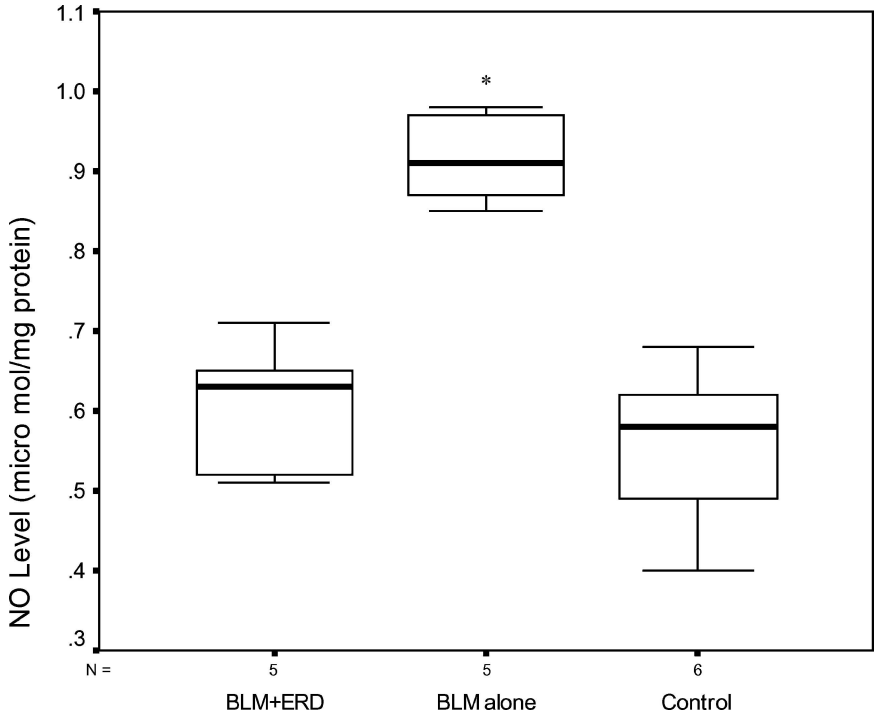

Fig. 4. The effect of erdosteine on bleomycin-induced increase in NO (nitrite/nitrate) level. See the legend for Fig. 1 for explanation of abbreviations. Treatment with erdosteine significantly prevented the increase in lung tissue nitrite/nitrate levels. *Significantly higher ($p < 0.05$) when compared to the control and BLM + ERD groups.

the migration and accumulation of neutrophils in the bronchoalveolar space. Activated neutrophils in the alveolar space release toxic radical oxygen species and various proteolytic enzymes which result in severe tissue injury [29]. BLM-induced fibrosis is the most commonly used animal model for human idiopathic pulmonary fibrosis and appears to be a significant drug-induced lung disease in the clinical setting [30]. Therefore, BLM was used in this study as a fibrogenetic agent, and BLM-induced cellular accumulation in the lungs was evaluated by determination of leukocytes in the BAL fluid and histological analysis. The exact time course of neutrophil influx to the lungs is still unclear in this animal model. Recently it was shown that after intratracheal administration of BLM in rats, BAL leukocyte counts increased significantly on day 1, reached maximum level on day 3 and were still found to be increased on day 14. This study also reported that the percentage of neutrophils in BAL increased on day 3 [31]. Similar findings were reported in other studies [29, 32]. It is also suggested that the most suitable time point for assessing lung fibrosis in this model is 14 days after intratracheal instillation of BLM, based on the observation that at 14 days the animals developed extensive fibrosis, but had less variability in the fibrotic response and lower mortality than later at 21 days [32]. We performed the BAL on the 14th day of the study according to these previous reports.

In this study, BLM administration produced a significant increase in the BAL fluid leukocytes as compared to the control animals. The histological examination supported this finding. We observed that prophylactic ERD administration produced a marked inhibition in this accumulation of leukocytes in the BAL fluid. The inhibitor effect of ERD may be reflected in a reduction in the migration of neutrophils and other inflammatory cells in the inflamed region, thereby mitigating tissue damage in the rat lung. Previous studies have demonstrated that antioxidant agents ameliorated the accumulation of leukocytes in the BAL fluid and lung tissue, which is in agreement with our finding [33–35]. Our present findings are generally in agreement with the reports of previous study on ERD [19].

It is known that neutrophil influx is not the only mechanism in the development of lung fibrosis, though their role is appreciated. Thrall *et al*. reported BLM-induced lung injury in neutrophil-depleted rats [36]. Substantial data indicates an important role of ROS in BLM-induced lung injury [37, 38]. ROS that considered initiating inflammation produced by BLM after oxidation of the BLM-Fe(II) complex and by activated polymorphonuclear leukocytes [39]. Iron deficiency blunts BLM-induced pulmonary injury and lipid peroxidation, suggesting iron-catalyzed oxygen radicals (Fenton reaction) may be responsible for the initial injury response [28].

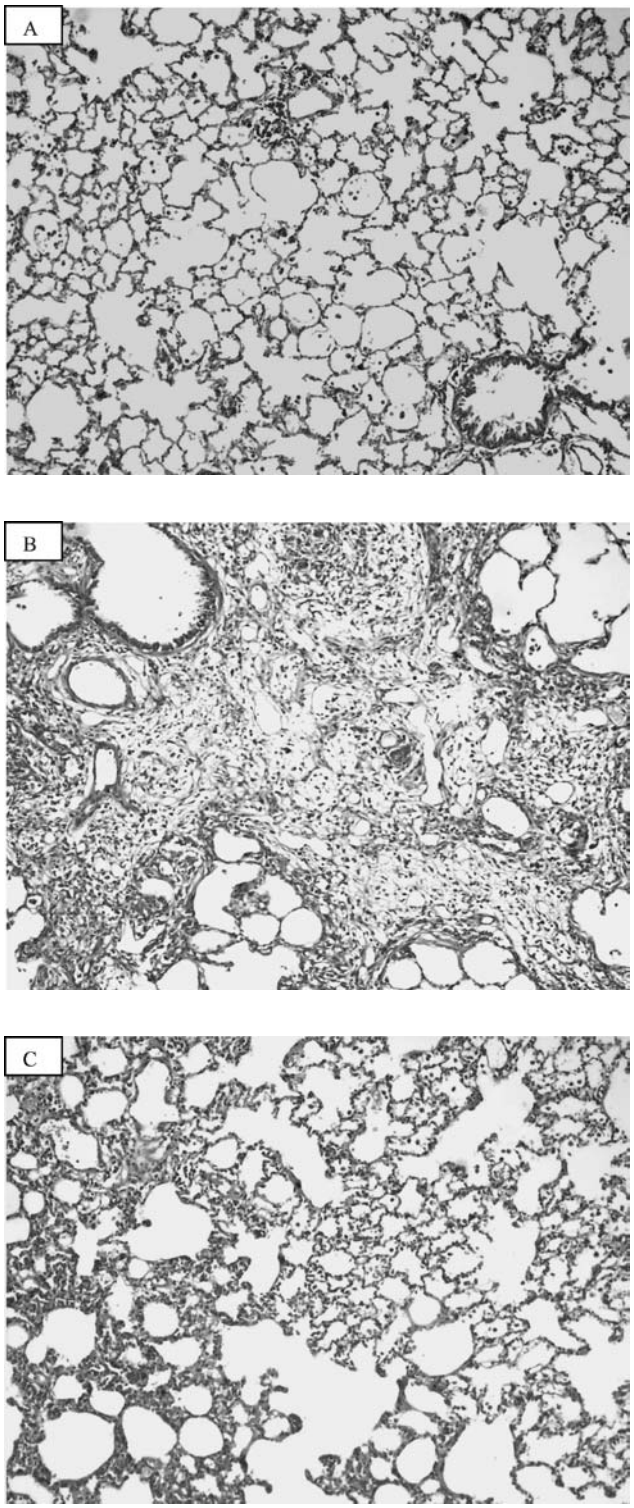

Fig. 5. Histologic analysis of lung after 14 days administration of drugs (HE × 100). ((A) control): normal lung parenchyma. ((B) bleomycin alone): collapse of alveolar spaces, proliferating fibroblasts and formation of fibrosis. ((C) bleomycin + erdosteine): slight or moderate thickening in alveolar septa, moderate amount of leukocytes in alveolar walls, prominent prevention of fibrosis.

Oxygen free radicals can damage lipids, proteins and DNA, and therefore may contribute to the loss of enzymatic activity, structural integrity of enzymes and to activation of the inflammatory reactions. Oxidant-induced lipid peroxidation causes a loss of membrane stability and integrity leading to increased transepithelial permeability. In the present study, lipid peroxidation was monitored by measuring MDA which results from free radical damage to membrane components of the cells. We observed a significant increase in the MDA concentration in the lung tissue of rats treated with BLM alone. We also monitored the SOD and GSH levels, key antioxidants. BLM produced significant decreases in both SOD and GSH levels.

Since ROS and nitrogen species have been implicated in lung injury caused by BLM, antioxidants such as Mn-SOD, *N*-acetylcysteine, and ambroxol have been used to inhibit BLM-induced fibrosis [35, 40, 41]. ERD serves as a free radical scavenger to inhibit peroxidation of membrane lipids, and may maintain cell membrane integrity and function, thus preventing protein leakage and accumulation. Similar to previous reports, ERD significantly attenuated the BLM-induced increase of MDA concentrations in lung tissue in the present study. ERD also prevented the BLM-induced decrease in SOD and GSH levels. Together, these data suggest that the protective effect of ERD from BLM-induced lung fibrosis may be due to its free radical scavenging and antioxidant activity. Reduction of lipid peroxidation may also contribute to its protective effect in lung tissue.

Previous studies have shown that tissue NO levels significantly increased in rats after BLM administration and have suggested the possible role of peroxynitrite in BLM-induced lung fibrosis which formatted from the interaction between NO and superoxide [42, 43]. Under aerobic conditions, NO react with superoxide anion and form peroxynitrite, which is demonstrated to be present in the inflammatory lesions of acute lung injury [44]. Peroxynitrite oxides cellular structures and causes lipid peroxidation. This increase in NO levels has been shown to be caused by an increase in the activity of nitric oxide synthase [45]. Several investigators have demonstrated that macrophages when activated *in vitro* by cytokines as tumor necrosis factor-α, secrete reactive nitrogen intermediate throught an L-arginine-dependent pathway [46, 47]. In the present study, erdosteine treatment markedly decreased bleomycin-induced NO (nitrite/nitrate) production thereby preventing tissue injury. Erdosteine also has been shown to prevent NO production in doxorubicin-induced cardiotoxicity and cisplatin-induced renal failure in rats [48, 49]. It is not clear how erdosteine prevents bleomycin-induced NO production. Erdosteine may exert this effect by inhibiting macrophage activation due to its free radical scavenging activity and thereby secondarily decreasing NO produced by these cells.

In conclusion, we have demonstrated a possible protective effect of ERD in BLM-induced lung fibrosis. This effect may be related to inhibition of leukocyte accumulation in the lungs or elimination of free oxygen radicals and reduction of lipid peroxidation by ERD. Our results suggest that erdosteine might be a new therapeutic agent for preventing idiopathic pulmonary fibrosis or drug-induced lung fibrosis which may occur after antineoplastic treatment.

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