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# Erdosteine prevents bleomycin-induced pulmonary fibrosis in rats

Sadik Sogut[a], Huseyin Ozyurt[b], Ferah Armutcu[c], Levent Kart[d], Mustafa Iraz[e], Omer Akyol[f], Suleyman Ozen[g], Suleyman Kaplan[h], Ismail Temel[f], Zeki Yildirim[i,*]

[a] *Department of Biochemistry, Medical Faculty, Mustafa Kemal University, Hatay, Turkey*
[b] *Department of Biochemistry, Medical Faculty, Gaziosmanpasa University, Tokat, Turkey*
[c] *Department of Biochemistry, Medical Faculty, Karaelmas University, Zonguldak, Turkey*
[d] *Department of Pulmonary Medicine, Medical Faculty, Karaelmas University, Zonguldak, Turkey*
[e] *Department of Pharmacology, Medical Faculty, Inonu University, Malatya, Turkey*
[f] *Department of Biochemistry, Medical Faculty, Inonu University, Malatya, Turkey*
[g] *Department of Pathology, Medical Faculty, Yuzuncuyil University, Van, Turkey*
[h] *Department of Histology, Medical Faculty, Ondokuz Mayis University, Samsun, Turkey*
[i] *Department of Pulmonary Medicine, Turgut Ozal Medical Center, Medical Faculty, Inonu University, 44069 Malatya, Turkey*

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**Abstract**

Oxidative stress plays an important role in the pathogenesis of idiopathic pulmonary fibrosis. Therefore, erdosteine, an antioxidant, is expected to have an inhibitor potential against the disease. Rats were given one dose of bleomycin in pulmonary fibrosis groups and saline in controls. The first dose of oral erdosteine (10 mg/kg/day) was given 2 days before the bleomycin injection to achieve the plateau level in blood and continued until killing. At day 14, fibrotic changes were evaluated, using Aschoft's criteria and lung hydroxyproline content. Bleomycin produced a fivefold increase in fibrosis score that was decreased by 87% by erdosteine ($P>0.001$) and almost twofold increases in hydroxyproline content which were completely prevented by erdosteine. Myeloperoxidase activities and MDA levels, which were significantly higher in the bleomycin group, were then significantly attenuated by erdosteine. These results revealed that oral erdosteine may prevent the development of acute pulmonary inflammation caused by bleomycin injection via the repression of neutrophil accumulation and lipid peroxidation, resulting in the inhibition of subsequent lung fibrosis.


*Keywords:* Erdosteine; Bleomycin; Lung fibrosis; Myeloperoxidase; Hydroxyproline

## 1. Introduction

Idiopathic pulmonary fibrosis is a chronic disease of unknown etiology and its incidence has been estimated at 10.7 cases per 100,000 per year for males and 7.4 cases per 100,000 per year for females (American Thoracic Society, 2000). Treatment of the disease has failed. Many studies of the efficacy of corticosteroids plus other immunosuppressants such as cyclophosphamide or azathioprine, colchicine, and other agents have shown no more than 30% improvement in objective index (Mason et al., 1999). Therefore, novel therapeutic agents with improved efficacy are needed. Recent studies suggest that many promising agents against idiopathic pulmonary fibrosis include antioxidants (Wang et al., 2002; Borok et al., 1991; Behr et al., 1997), interferon gamma (Ziesche et al., 1999), *N*-acetylcysteine (Hagiwara et al., 2000) and blockade of tumor necrosis factor alpha and transforming growth factor beta (Feldman et al., 1999).

Bleomycin-induced pulmonary fibrosis in rodents is a popular model of human pulmonary fibrosis (Snider et al., 1978). The pathogenesis of the inflammation and subsequent fibrosis is the subject of ongoing investigations. However, bleomycin is known to generate reactive oxygen metabolites, including superoxide and hydroxyl radicals. Generation of the reactive species in the lung tissue results in DNA injury, lipid peroxidation, alteration

* Corresponding author. Tel.: +90-422-3410785; fax: +80-422-341-0728.
*E-mail address:* zyildirim@inonu.edu.tr (Z. Yildirim).

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in lung prostaglandin synthesis and degradation, and an increase in lung collagen synthesis. As a result of injury, inflammation and cytokine dysregulation that occur after bleomycin administration, fibroblasts are activated, and collagen production is stimulated while collagen degradation is inhibited (Sleijfer, 2001). This reactive species-mediated damage in the lung tissues was, at least partially, inhibited by addition of a variety of antioxidants, such as superoxide dismutase (Bowler et al., 2002), glutathione (Wang et al., 2002), and *N*-acetylcysteine (Behr et al., 1997; Cortijo et al., 2001) in many studies.

Erdosteine (CAS 84611-23-4) is a thiol derivate mucoactive drug that has recently been introduced into clinical practice (Dechant and Noble, 1996). Its molecule contains two sulfur atoms, one of which is present in an aliphatic side-chain and the other is enclosed in the heterocyclic ring (Braga et al., 2000). Erdosteine itself does not have a free thiol group, but its hepatic metabolism produces active metabolites with an SH group (Braga et al., 2000). The reducing potential of these SH groups accounts for the free radical scavenging and antioxidant properties of erdosteine (Inglesi et al., 1994).

In vivo and in vitro studies demonstrated that erdosteine has a potent free radical scavenging efficacy (Vagliasindi and Fregman, 1989; Fadillioglu et al., 2003). We showed that erdosteine attenuated cisplatin-induced nephrotoxicity in rats in which oxidative stress was implicated in the pathogenesis of the disorders as occurs in bleomycin-induced lung fibrosis (Yildirim et al., 2003). Kaise et al. (1993) suggested that erdosteine attenuated paraquat-induced respiratory failure in mice and bleomycin-induced pulmonary injury in rats; however, the issue was not discussed in detail in the literature. Given these data, we speculated that erdosteine would provide protection against bleomycin-induced lung fibrosis in rats through its antioxidant property. Therefore, we examined whether or not erdosteine at a dose of 10 mg/kg in rats inhibits the pulmonary fibrosis induced by a single intratracheal instillation of bleomycin and compared the results to the effect of alpha-tocopherol, which is a well-known antioxidant agent.

## 2. Method

### 2.1. Animals

A total of 42 male Sprague–Dawley rats, 12 weeks old, weighing 280–300 g, were obtained from the animal laboratory of Inonu University. All procedures involving animals were approved by the health institutional committee for animal care in Inonu University. Rats were housed in a special cage. A 12-h light/dark cycle was maintained and the rats had free access to water and food ad libitum.

### 2.2. Animal model of bleomycin-induced pulmonary fibrosis

An animal model of bleomycin-induced pulmonary fibrosis was induced in rats as described by Wang et al. (2002) with a minor modification. Briefly, after the weight was recorded, the rats were anesthetized via i.p. injection of 0.5 mg/kg of ketamine and 1 mg/kg of xylazine. A midline incision was made in the neck and the trachea was exposed by blunt dissection. A tracheal cannula (i.d. = 2 mm, length = 2.5 cm) was inserted into the trachea under direct visualization. Bleomycin hydrochloride (Nippon Kayaku, Tokyo, Japan) was dissolved in 250 μl of phosphate-buffered saline solution and instilled into the animal's lungs via the tracheal cannula at a dose of 7.5 mg/kg body weight (wt) ($n = 28$). The rats were killed 14 days after bleomycin injection. Sterile saline solution ($n = 10$) and erdosteine ($n = 10$) were administered to the control groups. The animals were shaken to facilitate distribution of the bleomycin and saline. Pulmonary fibrosis was assessed based on lung hydroxyproline content as well as lung histology. Lung histology was performed as described below.

### 2.3. Administration of erdosteine and alpha-tocopherol in an animal model of belomycin-induced pulmonary fibrosis

Erdosteine was obtained from a drug company (Ilsan, Turkey), dissolved in distilled water, and administered orally once a day at a dose of 10 mg/kg body wt via plastic disposable syringes ($n = 10$). Alpha-tocopherol (Epynal-Roche, Turkey) was injected into the peritoneum at a dose of 10 mg/kg body wt daily ($n = 9$). The first doses of erdosteine and alpha-tocopherol were given 2 days before the bleomycin injection to achieve the plateau level in blood and continued until killing.

### 2.4. Lung histology

Four animals from each treatment group were randomly chosen for histologic evaluation of their lungs at the end of the experiment. After death, the lung tissue was fixed by inflation with a buffered 10% formalin solution for 24 h and embedded in paraffin. The tissues were then sectioned at 3 μm, stained with hematoxylin and eosin, and examined for pulmonary fibrosis. Each successive field was individually assessed for severity of interstitial fibrosis, using the semiquantitative grading system described by Ashcroft et al. (1988).

### 2.5. Preparation of lung tissues for biochemical assessments

After removal, lung tissues were washed twice with cold saline solution, weighed, placed into glass bottles, labeled, and stored in a deep freeze (−30 °C) until processing (maximum 5 h). The tissues were homogenized in four volumes of ice-cold Tris–HCl buffer (50

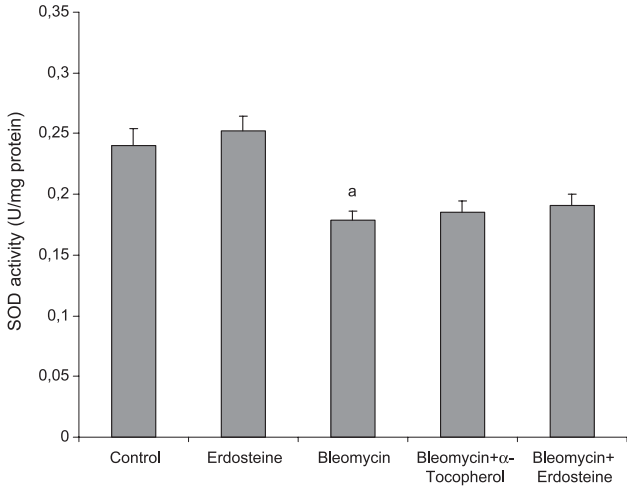


Fig. 1. Saline- and erdosteine-treated rats had similar SOD activities after 14 days of treatment. Bleomycin administration resulted in a significant decrease in SOD activity in lung tissue of rats when compared with control rats. Erdosteine and alpha-tocopherol did not attenuate the depletion of SOD activity produced by bleomycin after 14 days of treatment ($P>0.05$). Values are expressed as means ± S.E.M.: [a]significantly lower ($P<0.05$) than control rats.

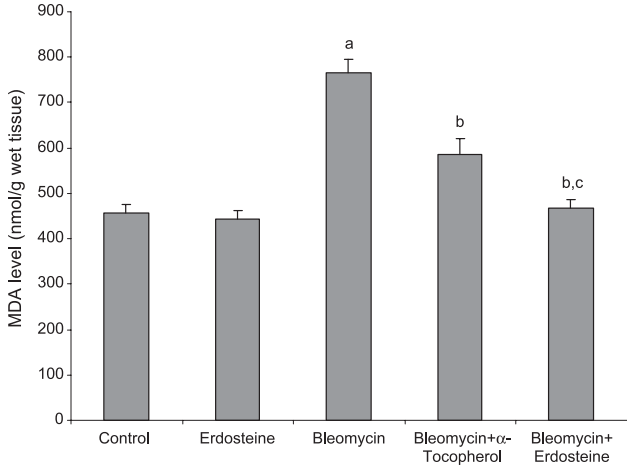


Fig. 3. Saline- and erdosteine-treated rats had a similar MDA level after 14 days of treatment. Bleomycin caused an increase in the lung tissue MDA content which demonstrates lipid peroxidation in tissue. Erdosteine and alpha-tocopherol significantly prevented the lipid peroxidation in this model. Values are expressed as means ± S.E.M.: [a]significantly higher than all the other groups ($P<0.0001$); [b]significantly lower than bleomycin-treated rats ($P<0.0001$); [c]significantly lower than alpha-tocopherol plus bleomycin-treated rats ($P<0.002$).

mM, pH 7.4) using a glass-Teflon homogenizer (Tempest Virtishear, Model 278069; The Virtis, Gardiner, NY) after cutting of the lung into small pieces with scissors (for 2 min at 5000 rpm). The malondialdehyde (MDA) level was measured in the homogenate. The homogenate was then centrifuged at 5000 × g for 60 min to remove debris. Clear upper supernatant fluid was taken and glutathione peroxidase (GSH-Px) activity and protein concentration were estimated at this stage. The supernatant solution was extracted with an equal volume of an ethanol/chloroform mixture (5:3, volume per volume [v/v]). After centrifugation at 5000 × $g$ for 30 min, the clear upper layer (the ethanol phase) was taken and used in the superoxide dismutase (SOD) activity and protein

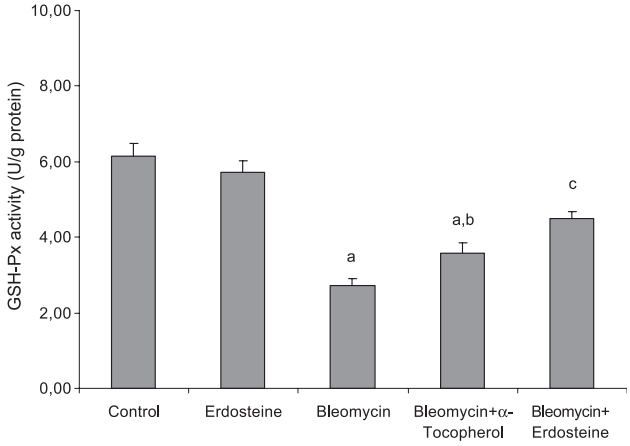


Fig. 2. Saline- and erdosteine-treated rats had similar GSH-Px activities after 14 days of treatment. Bleomycin administration significantly decreased the GSH-Px activity in the lung tissue of rats compared with lung of control rats. Erdosteine and alpha-tocopherol significantly prevented the depletion of GSH-Px activity produced by bleomycin after 14 days. Values are expressed as means ± S.E.M.: [a]significantly lower than control rats ($P<0.0001$); [b]significantly higher than bleomycin group ($P<0.019$); [c]significantly higher than bleomycin and alpha-tocopherol group ($P<0.009$).

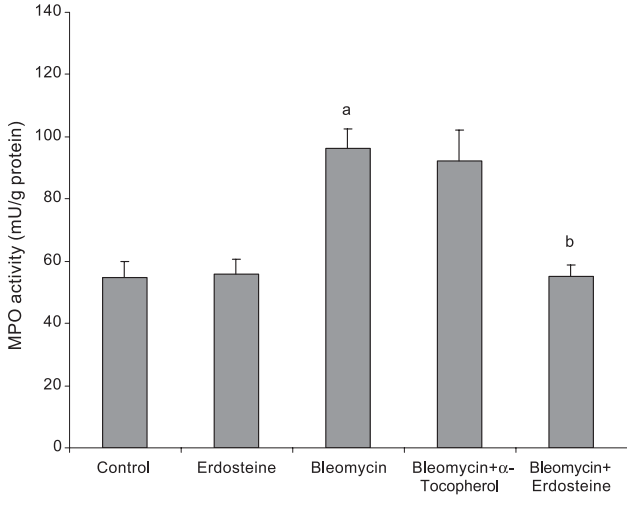


Fig. 4. Saline- and erdosteine-treated rats had similar MPO activities after 14 days of treatment. Bleomycin administration significantly increased the MPO activity as an index of neutrophil accumulation into the lung tissue as compared to the control rats. Erdosteine treatment significantly inhibited this increase but alpha-tocopherol did not. Values are expressed as means ± S.E.M.: [a]significantly higher than the other groups except the group treated with bleomycin plus alpha-tocopherol ($P<0.0001$); [b]significantly lower than bleomycin and bleomycin plus alpha-tocopherol-treated rats ($P<0.0001$).

assays. All preparation procedures were performed at +4 °C.

### 2.6. Lung tissue antioxidant enzyme analysis

Total (Cu–Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al. (1988). SOD activity was also expressed as units per milligram protein.

GSH-Px activity was measured by the method of Pagia and Valentine (1967). Activity is given in units per gram protein.

All samples were assayed in duplicate.

### 2.7. Lung tissue malondialdehyde and myeloperoxidase analysis

MDA levels were determined by Wasowicz's method (Wasowicz et al., 1993) based on the reaction of MDA with thiobarbituric acid at 95–100 °C. The results were expressed as nanomoles per gram wet tissue protein of lung (nmol/g protein tissue).

Myeloperoxidase (MPO) activity was determined using a 4-aminoantipyrine/phenol solution as the substrate for MPO-mediated oxidation by $H_2O_2$ and changes in absorbance at 510 nm ($A_{510}$) were recorded (Wei and Frenkel, 1993). Data

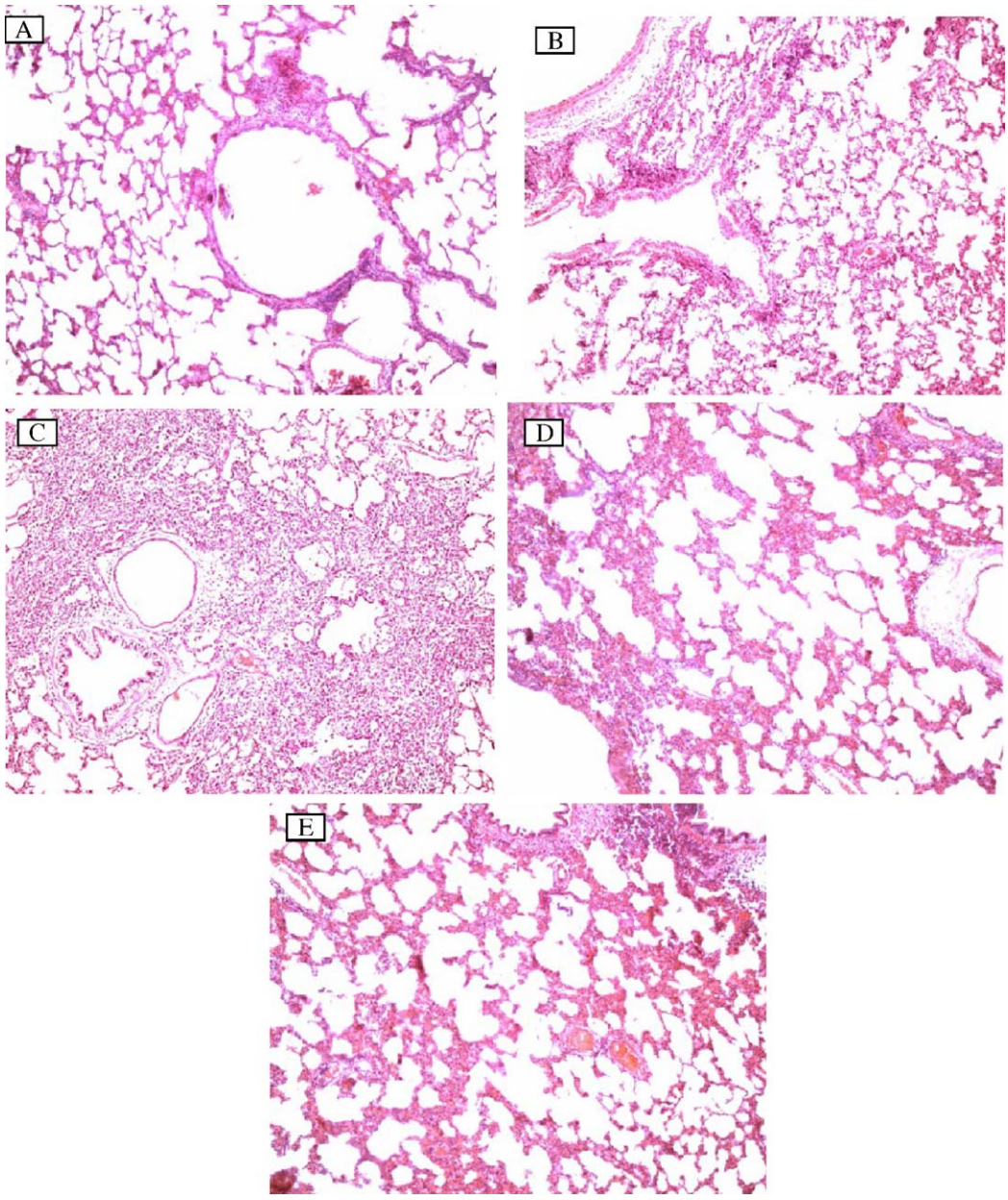


Fig. 5. Histologic analysis of lung after 14 days administration of drugs. A (Control): Normal lung parenchyma from saline-treated rats. B (Erdosteine): Normal lung parenchyma from erdosteine-treated rats. C (Bleomycin): Marked thickening in alveolar septa, collapse of alveolar spaces, a large number of leukocytes accumulated in alveolar walls and proliferation of fibroblasts after 14 days bleomycin instillation. D (Bleomycin + erdosteine) and E (Bleomycin + alpha-tocopherol): Marked prevention of the bleomycin-induced histological changes was seen in the rat lung.

are presented as U/g protein. All samples were assayed in duplicate.

### 2.8. Hydroxyproline determination in lung tissue

The tissue samples taken for hydroxyproline determination were washed with physiologic serum and dried in an oven set at 100 °C for 72 h. Hydroxyproline levels were determined spectrophotometrically with Woessner's method (Woessner, 1961).

### 2.9. Statistical analysis

Data were expressed as means ± S.E.M. One-way analysis of variance, followed by Fisher's least significant difference test, was used to evaluate differences among groups, and a value of $P < 0.005$ was considered significant.

## 3. Results

### 3.1. Analysis of oxidant stress markers

The depletion of SOD and GSH-Px activities in the tissue reflects indirectly the generation of free radical produced by bleomycin administration. As shown in Figs. 1 and 2, SOD activity was significantly decreased from the control value of 0.24 ± 0.01 to 0.18 ± 0.01 U/mg protein ($n = 9$, $P < 0.0001$), and GSH-Px activity decreased from the control value of 6.16 ± 0.31 to 2.71 ± 0.20 U/g protein ($P < 0.0001$), respectively. Erdosteine and alpha-tocopherol significantly prevented the depletion of GSH-Px activity; however, they did not attenuate the depletion of SOD activity. The mean GSH-Px and SOD activities remained 4.50 ± 0.18 U/g protein and 0.19 ± 0.009 U/mg protein in erdosteine, and 3.57 ± 0.28 U/g protein and 0.18 ± 0.01 U/mg protein in alpha-tocopherol-treated rats, respectively. Erdosteine treatment did not cause any changes in the antioxidant enzymes activities in the lung tissues when compared with the saline control group. Bleomycin treatment produced a significant increase in the lung tissue MDA level (766.3 ± 2.3 mmol/g wet tissues), an index for lipid peroxidation when compared with control groups (456.0 ± 19.3 mmol/g wet tissues), ($P < 0.0001$). Erdosteine alone did not affect the lipid peroxidation marker but caused a significant attenuation in the increase of MDA level produced by bleomycin treatment (466.2 ± 19.8 versus 766.3 ± 29.6 mmol/g wet tissue, $P < 0.0001$). Similar results were obtained for the treatment with alpha-tocopherol (585.3 ± 34.8 versus 766.3 ± 29.6 mmol/g wet tissue, $P < 0.0001$) (Fig. 3).

### 3.2. Myeloperoxidase analysis

As shown in Fig. 4, MPO, a marker of neutrophil influx in tissue, was significantly increased in rats treated with bleomycin alone (96.22 ± 6.23 mU/g protein) as compared to control (54.82 ± 5.19), ($P < 0.0001$). Erdosteine alone did not affect the lung tissue MPO activity but prevented the increase in MPO activity observed with bleomycin treatment (55.15 ± 3.49 versus 96.22 ± 6.23 mU/g protein, $P < 0.0001$). Conversely, alpha-tocopherol did not affect the lung tissue MPO activity (92.27 ± 9.94 versus 96.22 ± 6.23 mU/g protein, $P > 0.05$) (Fig. 5).

### 3.3. Effect of erdosteine and alpha-tocopherol on pulmonary fibrosis

All animals survived until the time of killing but the rats treated with only bleomycin had weight loss and were in a worse condition than rats treated with prophylactic erdosteine and alpha-tocopherol. Lung fibrosis was assessed by measuring hydroxyproline content in lungs as an index of collagen accumulation. A comparison of hydroxyproline contents among the five groups is presented in Table 1. Bleomycin treatment produced a significant increase in the hydroxyproline levels (22.68 ± 0.53 mg/g dry tissue) as compared to the controls (13.27 ± 0.46 mg/g dry tissue) after 14 days. Erdosteine treatment had little effect on the hydroxyproline content of lungs but completely prevented the increase in hydroxyproline content caused by bleomycin treatment. Although alpha-tocopherol treatment led to remarkable reductions of total lung hydroxyproline content compared to that in the rats treated with bleomycin alone, erdosteine strongly inhibited the increase of hydroxyproline level in the lung of rats treated with bleomycin when compared with alpha-tocopherol. The level of hydroxyproline in lungs of rats treated with erdosteine was reduced from 22.68 ± 0.53 to 10.06 ± 0.42, but to 17.76 ± 0.47 with alpha-tocopherol treatment.

The grades of fibrosis in five groups are presented in Table 1. In the semiquantitative assessment of lung sections, no inflammatory or fibrotic changes were observed in lungs of rats that had received normal saline and erdosteine (Fig. 5A and B). In agreement with numerous previous studies (Wang et al., 2002; Mollar et al., 2002; Giri et al., 1986), the bleomycin treatment produced an almost fivefold increase in the pathology score (Fig. 5C) as compared to the control group. Erdosteine and alpha-tocopherol pretreatment atten-

Table 1
Grade of lung fibrosis and lung hydroxyproline content

| Groups | Grade of fibrosis | Hydroxyproline (mg/g dried tissue) |
|---|---|---|
| Saline | 0.63 ± 0.06 | 13.27 ± 0.46 |
| Erdosteine | 0.55 ± 0.27 | 10.88 ± 1.64 |
| Bleomycin | 5.5 ± 0.25[a] | 22.68 ± 0.53[a] |
| Bleomycin + erdosteine | 1.5 ± 0.24[b] | 10.08 ± 0.42[b] |
| Bleomycin + alpha-tocopherol | 1.78 ± 0.23[b] | 17.76 ± 0.47[b] |

Values are expressed as means ± S.E.M.
[a] $P < 0.0001$ compared with the saline and erdosteine control groups.
[b] $P < 0.0001$ compared with bleomycin group.

uated the bleomycin pathology score by 73% and 68%, respectively (Fig. 5D and E).

## 4. Discussion

In the present study, we demonstrated the inhibitory effect of erdosteine on bleomycin-induced early lung injury and fibrosis as assessed from semiquantitative morphological indices of lung fibrosis and lung hydroxyproline content. Three possible explanations for this finding are inhibition of cellular infiltration, scavenging of the reactive oxygen species produced by inflammatory and lung cells, and direct protective detoxification of bleomycin-generated radicals before they damage the lung tissue.

To determine the role of inhibition of cellular infiltration, we examined the effect of erdosteine on bleomycin-induced cellular accumulation in the lungs, using tissue MPO activity and histological analysis. The tissue MPO activity has been used as a biochemical marker for the tissue content of polymorphonuclear leukocytes in previous studies (Mollar et al., 2002). In this study, bleomycin treatment produced a significant increase in the lung tissue MPO activity as compared to the control animals. The histological examination supported this finding. We observed that prophylactic erdosteine administration produced a marked inhibition of this increase of MPO activity due to the bleomycin administration. The inhibitory effect of erdosteine may reflect the reduction in the migration of neutrophils and other inflammatory cells in the inflamed region, thereby mitigating tissue damage in the lung. The possible mechanism might be one whereby erdosteine may repress reactive oxygen radicals through scavenging them, thereby decreasing neutrophil recruitment into the lungs. Previous studies demonstrated that antioxidant agents ameliorated the accumulation of leukocytes in bronchial lavage fluid and lung tissue which agrees with our findings (Hagiwara et al., 2000; Mollar et al., 2002).

The ideal time for measurements of neutrophil influx in this model of lung injury is still not clear. Recently, it was (Gurujeyalakshmi et al., 2000) reported that an increased number of neutrophils in bronchoalveolar lavage fluids in rats treated with intratracheal bleomycin occurred on day 1 and peaked on day 14, in agreement with our data, and remained significantly increased until day 21. Similar findings were reported by Hagiwara et al. (2000). They found that the total cell count in bronchoalveolar lavage fluids began to increase from day 3 and reached a plateau on day 14. The increased cellularity remained almost unchanged until day 28, and the macrophage count showed a similar course to the total cell count in their study. Also, they reported that the neutrophils showed peak levels between days 7 and 14 and gradually decreased after day 14.

Inflammation is a major component in the pathogenesis of interstitial lung disease that is orchestrated in part by endogenous and migrating leukocytes. These leukocytes together with epithelial and endothelial lung cells produce a feedback circle where stimuli from injury responses can activate alveolar and interstitial macrophages. As shown in Fig. 6, activated leukocytes can release further reactive oxygen and nitrogen species and proteases that sustain the injury/repair processes that are considered to contribute to the fibrotic processes induced by bleomycin (Sleijfer, 2001). However, the role of neutrophils in pulmonary fibrosis is still controversial. Thrall et al. (1981) reported that bleomycin-induced lung injury does take place in neutrophil-depleted rats.

The second and third possible explanations for the effect of erdosteine are based on its antioxidant effect. Substantial data indicate an important role of reactive oxygen in bleomycin-induced lung injury (Mason et al., 1999; Borok et al., 1991; Behr et al., 1991). Oxygen free radicals can injure lipids, protein and DNA, and therefore may contribute to the loss of enzymatic activity, structural integrity and to activate the inflammatory reactions. A previous study (Yildirim et al., 2003) with a cisplatin-induced acute renal failure model in rats and other studies (Dechant and Noble, 1996; Braga et al., 2000; Inglesi et al., 1994) demonstrated that erdosteine has a remarkable free radical scavenging activity. Our previous study also showed that erdosteine had a protective potential in cisplatin-induced renal failure in which free radicals are involved in the pathogenesis of the disorders (Yildirim et al., 2003). The present study also showed that bleomycin exposure caused significant decreases in the lung tissue antioxidant enzyme activities and lipid peroxidation which was ameliorated by erdosteine. These findings suggest that this the mechanism of the

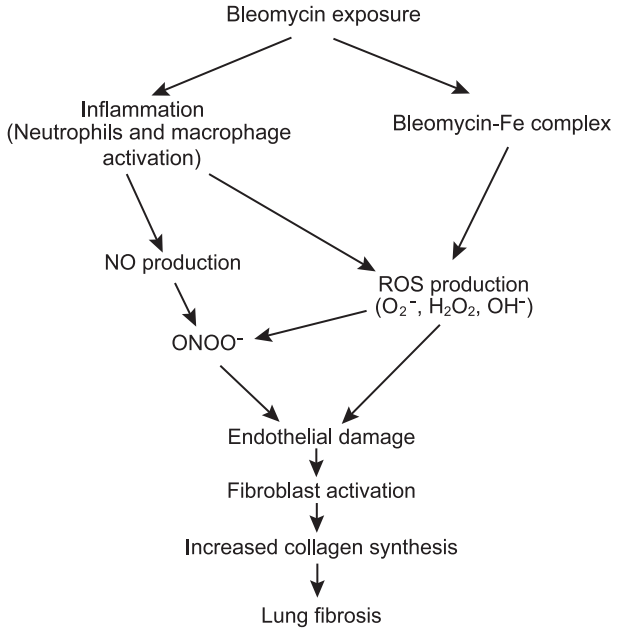


Fig. 6. Schematic presentation of bleomycin-induced oxidative stress and free radical production. ROS: Reactive oxygen species; $O_2^-$: Superoxide radical; $H_2O_2$: Hydrogen peroxide; $OH^•$: Hydroxyl radical; NO: Nitric oxide; $ONOO^-$: Peroxynitrite.

protective effect of erdosteine on bleomycin-induced lung injury may be its free radical scavenging and antioxidant activity. This may be regarded as an additional protection mechanism of erdosteine in this model.

The antioxidant effect of erdosteine was compared to that of alpha-tocopherol, which is a well-known potent antioxidant substance in this experiment. A previous study showed that pretreatment of rats with alpha-tocopherol liposomes 30 min prior to bleomycin administration resulted in little or no histological changes; however, it produced significant protection against bleomycin-induced changes in edema, lipid peroxidation, hydroxyproline content, and MPO activities (Suntres and Shek, 1997). Similar results were obtained in the present study. Our data indicated that although alpha-tocopherol has antioxidant activity, and therefore a reducing effect on the development of bleomycin-induced lung fibrosis, erdosteine has a much greater free radical scavenging activity as well as antifibrotic activity than alpha-tocopherol.

In the present study, although erdosteine could not completely prevent the increases in MPO and MDA, and the decreases in GSH-Px content of lung tissue, it totally prevented the increase in hydroxyproline content of the lung caused by bleomycin. This finding suggests that the protective effect of erdosteine we found may be another additional mechanism rather than as mentioned above. Based on these findings, one could speculate that erdosteine may have direct inhibitor activity on fibroblasts or collagen synthesis, although neither of these aspects was investigated in this study.

In conclusion, we first demonstrated the protective effect of erdosteine on the development of bleomycin-induced pulmonary fibrosis, using pathological evaluation and measurement of lung hydroxyproline content in rats. This effect may be due to the inhibition of leukocyte accumulation into the lungs or to scavenging of reactive oxygen radicals by erdosteine itself. Our data suggests that erdosteine may be a promising new therapeutic agent for idiopathic pulmonary fibrosis or at least for preventing the development of bleomycin-induced lung fibrosis during antineoplastic therapy.

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