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Protective effect of gallic acid against bleomycin-induced pulmonary fibrosis in rats

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ABSTRACT

Background: Bleomycin (BLM), a chemotherapeutic agent is indicated in the management of some types of cancers. This drug produces a dose-dependent pulmonary fibrosis (PF) in most patients as well as experimental animals through oxidative injury. This study aimed to investigate the effect of gallic acid (GA), a polyphenolic compound, against PF-induce by BLM in rats.

Materials and methods: The rats were given GA orally at doses (50, 100, and 200 mg/kg/day) for 7 consecutive days before the administration of single intratracheal (*it*) instillation of BLM at 7.5 IU/kg. GA doses were continued for 21 days after BLM exposure. The regulatory effects of GA on BLM-induced pulmonary toxicity were determined by assaying oxidative stress biomarkers, lung and serum cytokine levels, and by histopathological examination of lung tissue.

Results: The results showed that intratracheal BLM administration significantly increased the inflammatory or fibrotic changes, collagen content, levels of malondialdehyde (MDA), and pro-inflammatory cytokines such as TNF-α and IL1β in lung. Also, it significantly decreased non-enzymatic (total thiol) and enzymatic (glutathione peroxidase (GPx)) antioxidant contents in the rats' lung tissue. However, oral administration of GA reversed all of these biochemical indices as well as histopathological alterations induced by BLM.

Conclusion: Results of the present study demonstrate that GA, by its antioxidant properties, attenuates oxidative damage and fibrosis induced by BLM. Thus, an effective supplement with GA as an adjuvant therapy may be a very promising compound in reducing the side effects of BLM.

Introduction

Idiopathic pulmonary fibrosis (IPF), featured by chronic exacerbating dyspnea and respiratory failure [1,2], which caused by environmental toxins, radiation, or chemotherapy of cancers or many chronic inflammatory diseases [3,4]. Pulmonary fibrosis leads to reduced lung function and has a high mortality rate [5]. The processes that drive fibrosis in lung tissue are complex which involved the leukocytes infiltration, proliferation of fibroblast cells, damage of the alveolar structures, and the deposition of extracellular matrix proteins. Moreover, reactive oxygen species (ROS) such as hydrogen peroxide, peroxynitrite, superoxide, and hydroxyl radical are also play a pivotal role in lung inflammatory processes that can induce fibrosis [6]. Many xenobiotics that stimulate the overproduction of ROS such as paraquat [7], butylated hydroxytoluene [8] and bleomycin [9] are able to produce lung fibrosis. There is currently no Food and Drug Administration-approved drug for the treatment of pulmonary fibrosis or other fibrosing disorders [5].

Bleomycin is an antitumor drug that used in the management of some human cancers, including lymphomas, squamous cell carcinomas and testicular tumors. Its cytotoxicity occurs by induction of free radicals that cause DNA breaks leading to cell death. This drug can be given by several routes: *iv*, *im*, or *sc*; in case of malignant effusion, it can be administered intrapleurally or intraperitoneally. Moreover, some studies suggest that the route

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by which bleomycin is administrated may affect bleomycin-induced toxicity. Bleomycin continuously infused may induce less toxicity than bolus injection. However, other studies failed to show a relation between route of administration and toxicity [10].

The application of bleomycin is featured by the occurrence of pulmonary fibrosis that limits its clinical use. Moreover, bleomycin has been extensively used to prepare animal model of lung fibrosis [11]. So, a growing body of evidence has been reported that there is a linear positive relation between intratracheal dose and severity of pulmonary fibrosis induced by bleomycin in animals. Also, several studies suggested a similar relation in humans as well [10,12]. The lung is selectively influenced because this tissue lacks an enzyme that hydrolyzes the bleomycin, which prevents its metabolite from binding to metal ions and DNA [13]. The complex metabolites can generate ROS such as superoxide and hydroxyl radicals [11]. In mice, bleomycin produces DNA strand breakages that are lung selective and can be stimulated with oxygen exposure [14]. The breakage of DNA strand by bleomycin can be inhibited *in vitro* by the supplementation with variety of antioxidants, such as superoxide dismutase [15], glutathione [16], and also some herbal constituents such as cucumin [17], epigallocatechin-3-gallate [18], l-carnitine, *Ginkgo biloba* [19], and resveratrol [20].

Gallic acid (GA; 3,4,5-trihydroxybenzoic acid) and its derivatives are considered the main polyphenolic compounds in grapes, mango, areca nut, walnut, different berries, green tea and other fruits as well as in wine [21]. Preclinical studies have shown that GA possesses different pharmacological effects including antioxidant, anticancer, antimicrobial, anti-inflammatory [22] and neuroprotective activities [21,23–27]. In animal studies, GA reduces oxidative stress damages and enhances the levels of glutathione (GSH), GSH peroxidase, GSH reductase, and GSH S-transferase in hepatic and neural tissues, as well as catalase in serum [21,23,24,28]. It can also inhibit the polyunsaturated fatty acid saturation [29] and has anti-angiogenesis activity [30]. Exposure of human stomach cancer KATO-III cells and human colon adenocarcinoma COLO-205 cells to GA led to both growth inhibition and induction of apoptosis [31]. Moreover, Hsu et al. also reported that GA induces apoptosis in preadipocyte cells [28] and also in A549, a human lung adenocarcinoma cell line [32]. Since GA has the ability to induce apoptosis in tumor and preadipocyte cells, we attempted to investigate the inhibition of lung fibrosis by this compound in an experimental model of pulmonary fibrosis using intratracheal administration of bleomycin in rats.

Materials and methods

Chemicals

DTNB (2,2′-dinitro-5,5′-dithiobis-2-nitrobenzoic acid), TBA (2-thiobarbituric acid), n-butanol, Tris base, ethylendiaminetetraacetic acid disodium, glacial acetic acid, phosphoric acid, potassium chloride, 1,1′-3,3′-tetramethoxypropane (purity 99%) were obtained from Merck Company (Darmstadt, Germany). GA and bleomycin hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Nippon Kayaku Co. (Tokyo, Japan), respectively. Glutathione peroxidase (GPx) kit was obtained from Randox (Randox Labs, Crumlin, UK). The kits of TNF-α and IL-1β were purchased from eBiosource International, Inc. (Camarillo, CA, USA). All other chemicals were of analytical grade and prepared from Merck Company (Darmstadt, Germany). All drugs were dissolved in normal saline (0.9% NaCl). Drug concentrations were freshly prepared in such a way that the necessary dose could be injected in a volume of 5 ml/kg by oral route. Doses and drug administration schedules were selected based on previous report [21,33] and on pilot experiments in our laboratory.

Animals

Adult male Wistar rats weighing 220–250 g were used throughout the study. All of them were kept in the same room under a constant temperature (22 ± 2 °C), humidity (55–60%) and illuminated 7:00 a.m. to 7:00 p.m. with free access to food pellets and water. The rats were acclimatized to the laboratory conditions one day before the experimental session. All animal experiments were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals. The Institutional Animal Ethical Committee of Jundishapur University, formed under Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Reg. No. APRC-9312) approved the pharmacologic protocols.

Induction of bleomycin-induced pulmonary fibrosis

An animal model of bleomycin induced pulmonary fibrosis was used as previously described [19,34,35]. Briefly, rats were intratracheally injected with bleomycin hydrochloride (7.5 IU/kg body weight in 0.25 mL normal saline) under ether anesthesia. The rats were killed 21 days after bleomycin injection. Control group was intratracheally given the same volume of saline instead of bleomycin. Lung fibrosis was assessed by lung hydroxyproline level as well as lung histopathological examination.

Experimental groups

Animals were randomly divided into the following five groups (eight each). Group 1 was the sham group in which normal saline (5 ml/kg) was given by oral gavage; group 2 was fibrosis group in which bleomycin was administered intratracheally and received normal saline as the same as group 1 (BLM-treated). In groups 3–5, GA (50, 100 and 200 mg/kg, *po*) was administered for 28 consecutive days started 7 day before bleomycin administration.

Lung sample collection and biochemical assays

At the end of experiments, the animals were sacrificed by decapitation. The lung tissues were removed quickly, rinsed with saline, and then kept in a freezer (−80 °C) until used. The weight of the lungs was recorded for each animal. The lung tissues were used to measure the levels of collagen, pro-inflammatory cytokines and oxidative stress parameters. A small portion of both lungs was cut and used for histopathological examination. The tissues were homogenized in a cold KCl solution (1.15%) to give a 10% homogenate suspension used for assessment of thiobarbituric acid reactive substances (TBARS) value, expressed as malondialdehyde equivalents (MDA), total thiol contents and GPx activity. Also, serum was withdrawn and kept at −80 °C to measure the pro-inflammatory cytokines levels.

Assessment of hydroxyproline content in the lung tissue

To evaluate the oxidant-induced tissue fibrosis, we measured the total tissue collagen contents using a colorimetric assay as lung hydroxyproline level as previously described [36]. Briefly, 100 mg of left lung samples was homogenized and then hydrolyzed in 100 ml of 6 N HCl for 18 h at 120 °C. The hydrolysate was then neutralized with 2.5 N NaOH. Aliquots (2 ml) were analyzed for hydroxyproline content after the addition of 1 ml chloramine-T, 1 ml perchloric acid, and 1 ml para-dimethylaminobenzaldehyde to produce colored complex. The absorbance of each sample at 550 nm was measured using spectrophotometer (Shimadzu UV-1650CT). Results are expressed as μg of hydroxyproline per gram lung tissue.

Lipid peroxidation assay

TBARS levels, an index of lipid peroxidation, produced by free radicals were measured. MDA reacts with TBA to produce a red colored complex that has peak absorbance at 532 nm. Briefly, 3 ml phosphoric acid (1%) and 1 ml TBA (0.6%) were added to 0.5 ml of homogenate in a centrifuge tube and the mixture was heated for 45 min in a boiling water bath. After cooling, 4 ml n-butanol was added to the mixture and vortex-mixed for 1 min followed by centrifugation at 2000 × *g* for 20 min. The colored layer was transferred to a fresh tube and its absorbance was measured at 532 nm. TBARS levels were determined using 1,1′,3,3′-tetramethoxypropane as standard. The standard curve of MDA was constructed over the concentration range of 0–20 μM [37].

Total thiol (–SH) groups assay

Total –SH groups were measured using DTNB (5,5′-dithiobis-2-nitrobenzoic acid) as the reagent according to the method previously described [23]. This reagent reacts with the –SH groups to produce a yellow colored complex which has a peak absorbance at 412 nm. Briefly, 1 ml Tris–EDTA buffer (pH 8.6) was added to 50 μL of the homogenate in 2 ml cuvettes and absorbance was read at 412 nm against Tris–EDTA buffer alone (A_1). Then, 20 μL DTNB reagents (10 mM in methanol) were added to the mixture and after 15 min (stored in room temperature), the sample absorbance was read again (A_2). The absorbance of DTNB reagent was also read as a blank (B). Total thiol concentration (mM) was calculated from the following equation: total thiol concentration (mM) = $(A_2 - A_1 - B) \times 1.07/0.05 \times 13.6$.

GPx assay

GPx activity was measured with GPx kit (Randox Labs, Crumlin, UK).

Cytokine measurement

Lung tissue was homogenized in 0.5 mL of buffer containing protease inhibitors, and IL-1β and TNF-α levels were determined by enzyme immunoassay kits as described previously [18]. The absorbance of the produced color was measured at 450 nm using microplate reader. Moreover, levels of IL-1β and TNF-α were also measured in serum.

Histological examination

Lung tissues were fixed in 10% formalin, then processed, embedded in paraffin block 0.5 μm thick sections and stained with Hematoxylin and Eosin (H&E). The sections were examined by light microscopy and assessed for the presence of fibrosis [34].

Statistical analysis

Data were expressed as mean ± SEM. Statistical differences were analyzed using one-way ANOVA followed by Tukey's test. The p-value <0.05 was considered statistically significant. All data calculations and analysis were done with the GraphPad Prism Version 5.01 (GraphPad Software Inc., San Diego, CA, USA).

Results

Effect of GA on weight of lung tissue

As shown in Fig. 1, compared with the sham group, normalized weight of lung tissue by the weight of body markedly increased in the bleomycin-treated group. However, chronic oral treatment with GA significantly prevented the parameters at dose 200 mg/kg ($p < 0.01$).

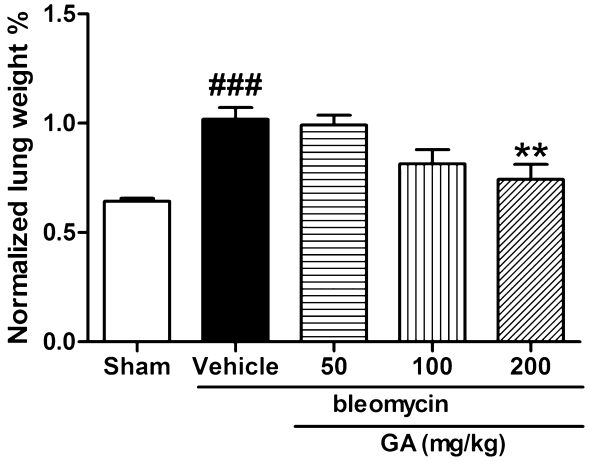

Fig. 1. Effect of gallic acid (GA) on weight of lung tissue in bleomycin-induced pulmonary fibrosis. Rat lung tissues were normalized by the weight of body. $^{###}p < 0.001$ compared with sham group, $^{**}p < 0.01$ compared with the vehicle group (one-way ANOVA followed by Tukey's *post hoc* test). Data is expressed as mean ± SEM ($n = 8$).

Effect of GA on collagen content of lung tissue

Effect of GA on collagene content in lung tissue collagen deposition was assessed by measuring the hydroxyproline content. Compared with the sham group, hydroxyproline content of the lung significantly increased in the bleomycin-treated group ($p < 0.5$). However, chronic oral administration of GA significantly inhibited the hydroxyproline accumulation at dose 200 mg/kg ($p < 0.01$, Fig. 2).

Effect of GA on lipid peroxidation of lung tissue

The degree of free radical damage following bleomycin injection was assessed using lipid peroxidation, which was measured as TBARS levels. According to Fig. 3A, there was an increase in TBARS levels of bleomycin-treated group ($p < 0.05$) as

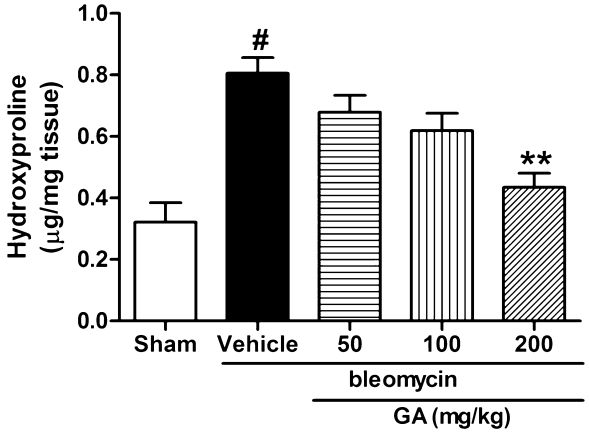

Fig. 2. Effect of gallic acid (GA) on collagen levels in bleomycin-induced pulmonary fibrosis in rats. Collagen deposition was assessed by measuring the hydroxyproline content. $^{#}p < 0.05$ compared with sham group, $^{**}p < 0.01$ compared with the vehicle group (one-way ANOVA followed by Tukey's *post hoc* test). Data is expressed as mean ± SEM ($n = 8$).

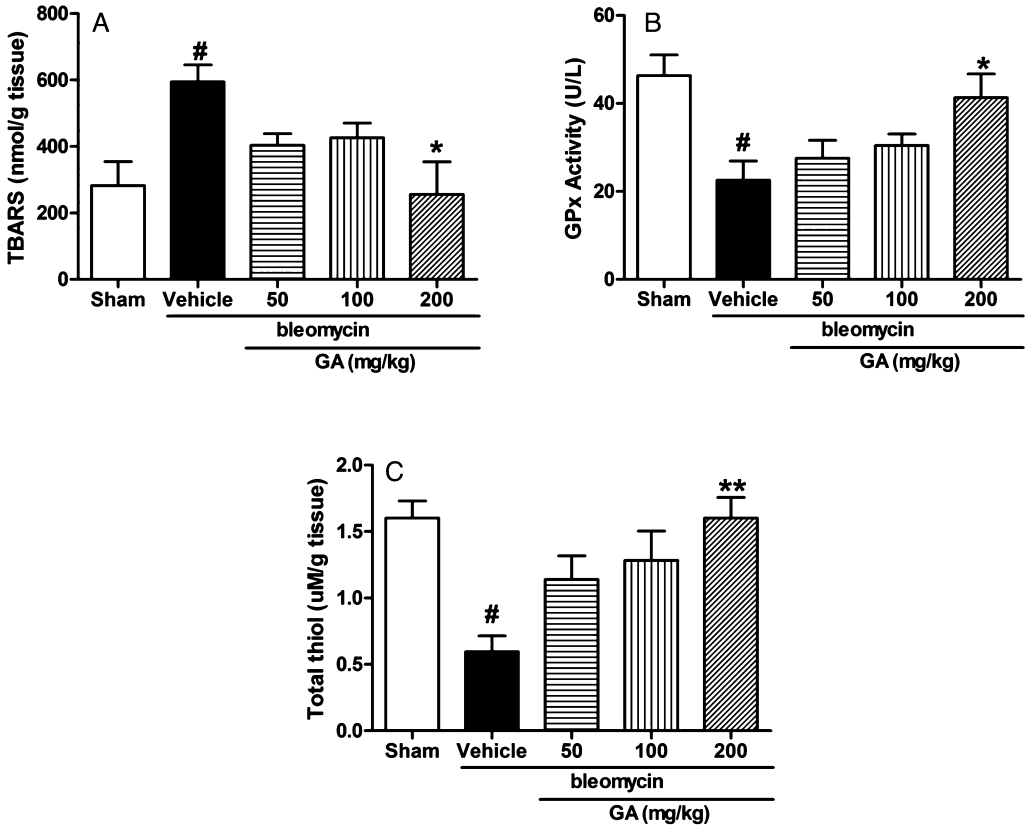

Fig. 3. Effect of gallic acid (GA) on (A) TBARS, (B) total thiol contents and (C) glutathione peroxidase (GPx) enzyme activity in lung tissue of rats exposed with bleomycin. $^{\#}p < 0.05$ compared with sham group, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle group (one-way ANOVA followed by Tukey's *post hoc* test). Data is expressed as mean ± SEM ($n = 8$).

compared to sham-operated rats in the lung tissue. Chronic oral administration of GA (200 mg/kg) resulted in a significant reduction of TBARS levels as compared to bleomycin-treated group ($p < 0.05$).

Effect of GA on total thiol levels in lung tissue

The total thiol concentration (mM) was measured to evaluate the non-enzymatic defense potential of the lung cells against the oxidative stress damage. According to Fig. 3B, total thiol levels in fibrotic animals were found to be significantly depleted as compared to sham group in the lung tissue ($p < 0.05$). Chronic treatment with GA (200 mg/kg) in bleomycin-treated rats was able to increase total thiol levels significantly as compared to bleomycin-treated group ($p < 0.05$).

Effect of GA on GPx activity in lung tissue

GPx activity (μ/l) was measured to evaluate the enzymatic defense potential of the lung cells against the oxidative stress damage. According to Fig. 3C, the GPx activity was significantly ($p < 0.05$) decreased in bleomycin-treated rats as compared to sham-operated group in the lung tissue. However, the decrease of GPx activity was significantly restored by chronic oral administration of GA at dose 200 mg/kg ($p < 0.01$).

Effect of GA on cytokine levels in serum and lung tissue

We examined whether treatment with gallic acid reverses the abnormal elevation of cytokines in serum and lung tissue following intratracheal bleomycin administration. The effects of bleomycin or gallic acid (50–200 mg/kg) on the levels of TNF-α and IL1β measured in serum and the lung homogenate are shown in Fig. 4. Treatment with BLM significantly increased the normal serum and tissue level of TNF-α and also IL1β ($p < 0.5$). However, chronic oral administration with gallic acid significantly reduced the bleomycin-induced elevation in serum and the lung tissue TNF-α level. Similarly, oral administration of GA significantly reduced the elevated serum IL1β level and tended to normalize its value.

Histopathologic examination

As shown in Fig. 5, intratracheal administration of bleomycin caused a marked alveolar thickening associated with fibroblasts and myofibroblasts proliferation and collagen production in interstitial tissue leading to pulmonary fibrosis (Fig. 5B). As observed in Fig. 5(C)–(E), pulmonary fibrosis tended to decrease in the GA-treated group at 100 mg/kg and the lesions were significantly attenuated by GA at dose 200 mg/kg.

Discussion

Pulmonary fibrosis is a chronic inflammatory interstitial lung disease with a potentially fatal prognosis and a poor response to available medical therapy. Many studies have been done to attenuate this life-threatening disorder. However, we might be at the beginning of the way to get over this disease. One of the clinically relevant agents causing pulmonary fibrosis is the anti-cancer agent, bleomycin which is widely used in animal models to produce oxidant-induced inflammatory and fibrotic lesions in lungs [11].

This model of pulmonary fibrosis is useful to assess potential therapeutic agents, including antioxidants and other drugs.

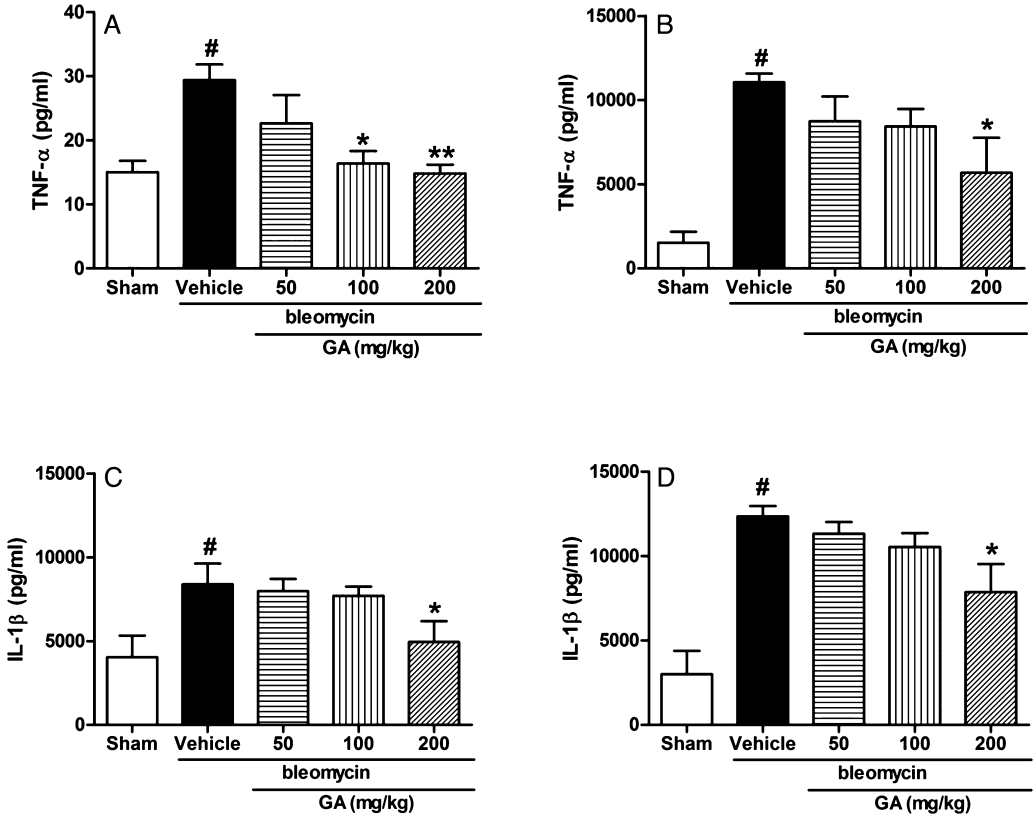

Fig. 4. Effect of gallic acid (GA) on serum (A), lung (B) levels of TNF-α and also serum (C) and lung (D) levels of IL-1β of rats exposed with bleomycin. $^{\#}p < 0.05$ compared with sham group, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle group (one-way ANOVA followed by Tukey's *post hoc* test). Data is expressed as mean ± SEM ($n = 8$).

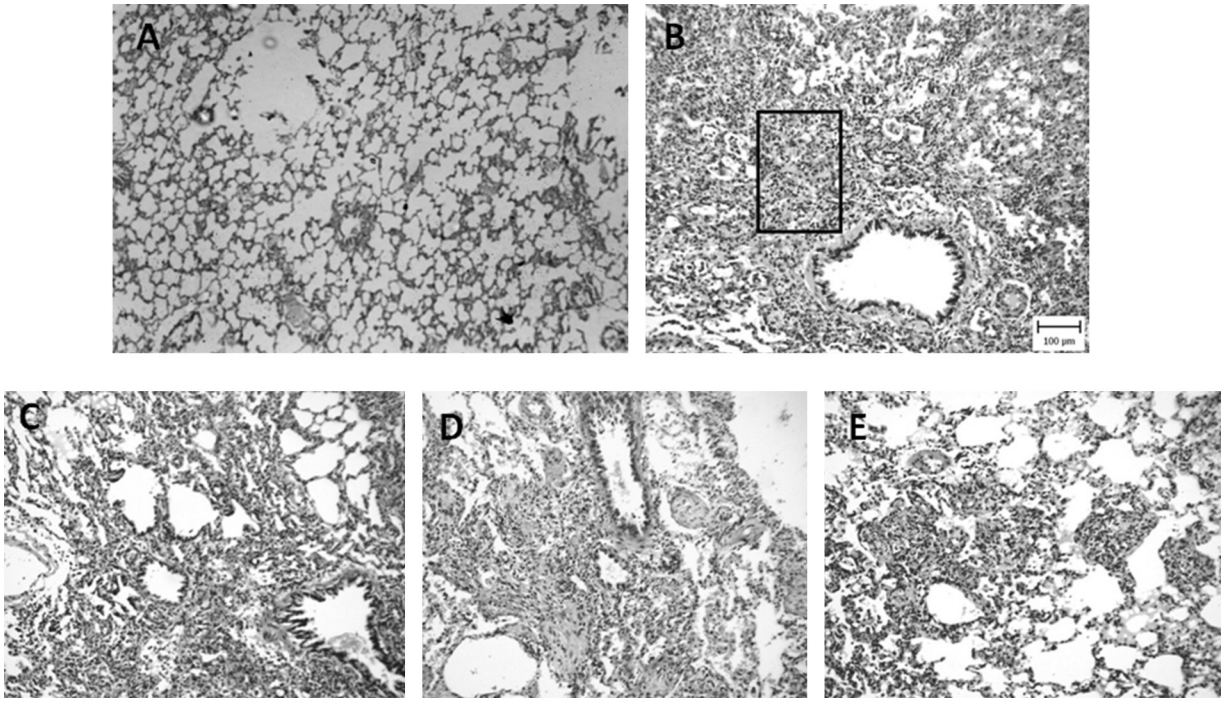

Fig. 5. Histopathologic examination of lung tissue in bleomycin-induced pulmonary fibrosis in rats (H&E ×100). (A) Normal rats showing intact alveolar spaces without pathological lesion (H&E ×100), (B) bleomycin injection induced excessive collagen deposition with infiltration of inflammatory cells in alveolar spaces and interstitial fibrosis of the alveolar wall in rats. These phenomena are most prominently shown in black rectangle, (C–E) pulmonary fibrosis tended to decline in the GA at doses 50 and 100 mg/kg and the lesions were significantly ameliorated at GA dose 200 mg/kg.

A growing body of evidences showed that when dietary flavonoids from food sources are absorbed from the gut, the circulating compounds are almost entirely conjugated and that many of these conjugated metabolites have antioxidant properties *in vitro* [17–20]. Beneficial effects of gallic acid have been studied on variety of diseases' experimental models [22,25,30]. But, protective effect of GA against lung fibrosis has not been reported yet in animal model, though some molecular mechanisms were explicated so far [38,39]. Therefore, this study might be the first report on the effect of GA on lung fibrosis in experimental model.

Results of the present study revealed that intrathecal administration of bleomycin depleted the activity of antioxidant enzyme like GPx and increased TBARS levels. Co-administration of various doses of GA ameliorated the activities of this antioxidant enzyme and restore the TBARS content to normal state, might be attributed to phenolic nature of gallic acid [40]. TBARS levels most likely determine the degree of organic lipid peroxidation, which denotes the severity of cell membranes damage [41]. Chronic oral administration of GA could increase the GPx activity which implies that this compound could improve expression of some antioxidant enzymes like GPx to improve the oxidative stress response [41]. The protective effect of GA was also found for total thiol which was applied for evaluation the non-enzymatic defense potential of the cells against the oxidative stress [38].

The development of bleomycin-induced pulmonary fibrosis is also related to the expression of inflammatory cytokines which play a key role in the chemotaxis of macrophages and neutrophils. Subsequently, these inflammatory cells support the overproduction of ROS/RNS [42]. TNF-α has a pivotal role within pro-inflammatory and immune-regulatory networks and is likely involved in the development and progression of radiation-induced pneumonitis [43]. In addition, TNF-α stimulates the proliferation of fibroblasts and the secretion of pro-inflammatory cytokines, including IL-1β and IL-6, from neutrophils and macrophages [44]. IL-1β produced by monocytes is one cytokine which could play a role in inducing peritoneal fibrosis in patients undergoing chronic peritoneal dialysis. This cytokine increases collagen synthesis associated with increased levels of pro-collagen mRNAs in fibroblasts and is rapidly expressed in response to tissue damage [45]. To investigate the effects of GA treatment on systemic and local (lung) inflammation, we measured the serum and tissue levels of the key inflammatory cytokines including IL-1β and TNF-α. These cytokines were significantly reduced in the GA-treated animals compared with the control group. The lower alveolitis score of the GA-treated rats also suggested that this compound reduces the infiltration of inflammatory immune cells induced by bleomycin. Results of this study are in agreement with those of previous studies that have shown significant anti-inflammatory effects from GA [38,45].

Taken together, gallic acid attenuates lung fibrosis as a consequence of bleomycin inhalation. This lung-protective effect is due to its anti-oxidative and anti-inflammatory, as well as down regulating of IL-1β and TNF-α cytokines. Obstruction of several key events in bleomycin-induced lung fibrosis renders GA as a promising anti-fibrotic agent for pulmonary fibrosis. We propose that gallic acid might be potentiated in the clinical setting and warrants in near future.

Conflict of interest

The authors have declared that there is no conflict of interest.

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