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Epicatechin protective effects on bleomycin-induced pulmonary oxidative stress and fibrosis in mice

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ABSTRACT

Lung fibrosis is a chronic and intermittent pulmonary disease, caused by damage to the lung parenchyma due to inflammation and fibrosis. Epicatechin (Epi) as a flavonoid has antioxidant and anti-inflammatory properties. This study was conducted to evaluate the effect of Epi on oxidative stress, inflammation and pulmonary fibrosis induced by bleomycin (BLM) in mice. Accordingly, animals were randomly assigned into two groups of 7 and 14 days to evaluate the role of Epi in the early oxidative and late fibrotic phases of BLM-induced pulmonary injury, respectively. Each group was divided into six subgroups include control, Epi 100 mg/kg, BLM, and BLM groups pretreated with 25, 50 and 100 mg/kg Epi, respectively, from three days before until 7 or 14 days after BLM. Lung tissue oxidative stress markers including the activity of superoxide dismutase, glutathione peroxidase, catalase and the levels of malondialdehyde and glutathione were determined. Furthermore, alveolitis and inflammation were evaluated by Szapiel grading scores. In addition, fibrotic markers including lung hydroxyproline content, level of transforming growth factor beta and Ashcroft fibrotic grading of lung fibrosis were examined. Epi exerted protective effects against BLM-induced pulmonary injury in a dose-dependent manner in two early and late phases of lung injury. Oxidative stress markers persisted until the late fibrotic phase, as profibrotic events were present in the early oxidative phase of BLM-induced injury. Finally, it is concluded that Epi can protect the lung against BLM-induced pulmonary oxidative stress, inflammation and fibrosis.

1. Introduction

Pulmonary fibrosis is a condition with injured lesions and scars, which cannot work properly and make it difficult to breathe due to the disability of the lungs to carry oxygen to the bloodstream. The most common symptoms are shortness of breath and dry cough. These symptoms may be mild or even in the early stages. It may be asymptomatic, or symptoms become worse when scars develop. In pulmonary fibrosis, normal lung tissue architecture is replaced by scar tissue, which is generally characterized by collagen deposition and fibroblast proliferation. Pulmonary fibrosis is a chronic inflammatory lung disease with potential lethal prognosis with an inappreciable response to available medical therapies [1,2]. Idiopathic pulmonary fibrosis is a devastating disease of unknown cause. Some drugs include BLM, methotrexate, amiodarone, nitrofurantoin, heavy metal dust in the air and mineral agents like silica, malachite and exposure to dust and radiation can induce pulmonary fibrosis [3–7]. Pulmonary fibrosis may be due to acute and chronic pulmonary disorders. Pulmonary fibrosis arises from excessive deposition and abnormal accumulation of collagen generated by fibroblasts and myofibroblasts. These events damage alveolar cells and reduce their elasticity and flexibility [8].

BLM is an important chemotherapeutic glycopeptide antibiotic, which is used for many malignancies. It was produced by the bacterium "*Streptomyces verticillus*" that discovered by Umezawa and colleagues in 1962 [9,10]. BLM plays a very important and considerable role in the treatment of various cancers such as lymphoma, carcinoma of head and

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neck, germ cell tumors, testicular carcinoma and ovarian cancer. BLM has no serious immuno- and myelosuppressive effects. The most important toxicities of BLM in humans are pulmonary injury and skin complication [11]. Pulmonary fibrosis is the most severe adverse effects of BLM in cancer patients. Thereafter, administration of a single intratracheal (i.t.) dose of BLM was introduced as the most common animal model of pulmonary injury and fibrosis in mice, rats and hamsters. Intratracheal administration of BLM causes dose-dependent damage to the lungs [12–14]. BLM is known to generate reactive oxygen species like superoxide and hydroxyl radicals. Generation of the ROS in the lung tissue is because of DNA injury, lipid peroxidation, damage of epithelial cells, and an excessive deposition of extracellular matrix and lung collagen synthesis. Administration of BLM (i.t.) leads to inflammatory and fibrotic reactions that way collagen production is stimulated by fibroblasts [12,15]. BLM pulmonary toxicity appears as pneumonia, which begins with vascular endothelial damage due to free radicals and cytokines and eventually it may progress to fibrosis [16]. In many types of research, pulmonary adverse effects in most patients are depend on BLM dose, age, the presence of pre-existing lung diseases, smoking and exposure to polluted air in industrial cities [6,12].

The chemical structure of flavonoids has the general main backbone, which consists of two phenyl rings (A and B) and heterocyclic C ring in their main structure. Hydroxyl groups on the B ring mediate the most antioxidant activity of flavonoids. There are plenty of polyphenol compounds in many fruits and vegetables, which show antioxidant properties in addition to its nutritional role [17]. Epicatechin (3, 5, 7, 3', 4'-OH) is one of the polyphenol flavonoids that belong to flavan-3-ols group. This compound is polyphenol due to numerous phenol groups in its main structure and is generally found in green tea and cacao [18]. In addition, the antioxidant properties of flavonoids are related to the chelating activity with metal ions and scavenging of free radicals [19–21]. Previous evidences have shown the protective effects of Epi on oxidative stress and fibrosis. The green tea extract, which consists of Epi, has shown protective and antifibrotic effects in the paraquat model of pulmonary toxicity and fibrosis by controlling oxidative stress and endothelin-1 expression [22]. Furthermore, it is known that tea catechins including Epi, epicatechin gallate, epigallocatechin and epigallocatechin gallate can prevent the oxidative damage due to tert-butyl hydroperoxide (t-BHP) by reducing the oxidative stress markers in diabetic rats [23]. In addition, it has been reported that cisplatin nephropathy can be prevented by Epi via decrease in mitochondrial oxidative stress and ERK activity [24]. In order to prevent pulmonary injury caused by BLM, this study was designed to evaluate the preventive effects of Epi on oxidative stress, inflammation and pulmonary fibrosis induced by BLM in mice.

2. Materials and methods

2.1. Chemicals and kits

Bleomycin was obtained from Chemex Company (Argentina). (−)-Epicatechin (≥90%, HPLC), bovine serum albumin, chloramine T, Ellman's reagent (DTNB), thiobarbituric acid and Bradford reagent were purchased from Sigma–Aldrich. Ammonium molybdate, butylated hydroxytoluene, trichloroacetic acid, buffered formalin, HCl and perchloric acid were purchased from Merck Company. Commercial glutathione peroxidase (GPX) and superoxide dismutase (SOD) kits were purchased from RANSEL, Randox Com, UK. Transforming growth factor beta (TGF-β) commercial enzyme-linked immunosorbent assay (ELISA) kit was provided by Hangzhou, Eastbiopharm.

2.2. Animals

Male NMRI mice (8 weeks of age; 20–25 g) were obtained from Ahvaz Jundishapur University of Medical Sciences (AJUMS) animal house. Upon arrival, the animals were allowed to acclimatize for 1 week. The mice were kept in cages and given standard mouse chow and drinking water ad libitum. Mice were maintained at a controlled condition of temperature (20 ± 2 °C) with a 12 h light and 12 h dark cycle. This research was performed in accordance with the Animal Ethics Committee Guidelines of AJUMS for the care and use of experimental animals (Ethics code: IR.AJUMS.REC.1396.279).

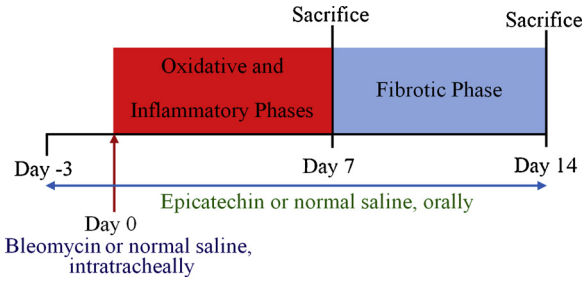

Fig. 1. Experimental protocol to study ameliorative effects of epicatechin on bleomycin-induced early (oxidative and inflammatory) and late (fibrotic) phases of the lung injury in mice. Mice were pretreated with Epi (25, 50 and 100 mg/kg) or normal saline orally 3 days before and continued 1 and 2 weeks after bleomycin administration.

Table 1
Mortality percent in groups pretreated with epicatechin (Epi) in bleomycin (BLM) model of pulmonary toxicity in two phases of 1 and 2 weeks. The number of mice in each group was 6 to 8.

Study	Groups	Mortality%
One-week study	Control	0
	Epi 100 mg/kg	0
	BLM	25
	Epi 25 mg/kg + BLM	14.3
	Epi 50 mg/kg + BLM	0
	Epi 100 mg/kg + BLM	0
Two-week study	Control	0
	Epi 100 mg/kg	0
	BLM	50
	Epi 25 mg/kg + BLM	42.9
	Epi 50 mg/kg + BLM	42.9
	Epi 100 mg/kg + BLM	28.6

2.3. Experimental design

This study was conducted on 82 mice weighing 20–25 g in two times to differentiate oxidative stress, inflammation and fibrosis [12,15]. Mice were randomly divided into six groups of 6 to 8 mice in each time of 7 and 14 days. Experimental mice groups were I. control, II. BLM 4U/kg/2 ml, III-V BLM groups were pretreated with Epi 25, 50 and 100 mg/kg/10 ml, respectively, from three days before until 7 or 14 days after BLM and VI. Epi 100 mg/kg/10 ml. Mice were anesthetized with intraperitoneal (i.p.) injection of 50 mg/kg ketamine and 5 mg/kg xylazine, and then received a single intratracheal (i.t.) dose of either saline or 0.10 U BLM in 50 μL saline per mouse, transorally [25–27]. The animal received saline or Epi at daily doses of 25, 50, and 100 mg/kg orally, three days before and 7 days or 14 days after BLM instillation (Fig. 1). The solvent of BLM and Epi was saline. BLM was diluted in saline and Epi doses were prepared in 10 ml/kg saline. The selected i.t. dose of BLM was based on previous literature [25–27] and Epi doses were selected according to the study of Shanmugam et al [28].

2.4. Sample collection

On the test day, mice were sacrificed with the injection of ketamine 100 mg/kg and xylazine 10 mg/kg cocktail and lung lobes were removed, weighed and washed with saline for biochemical and histological analysis.

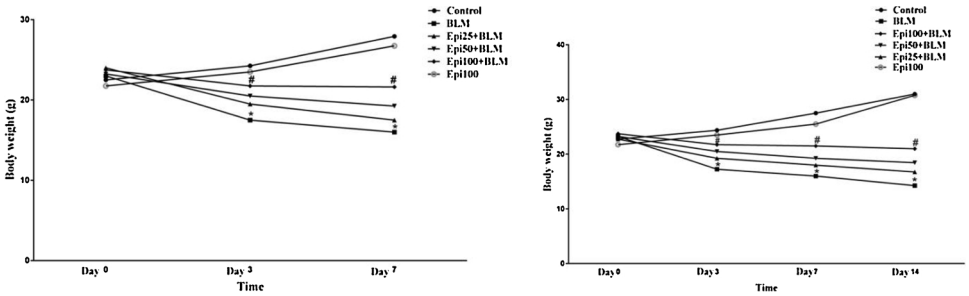

Fig. 2. Epicatechin (Epi) effect on body weight changes of mice in pulmonary damage induced by bleomycin (BLM) in two phases of the study. n = 4–8 in each group.
*P < 0.05 versus control group.
#P < 0.05 versus bleomycin group.

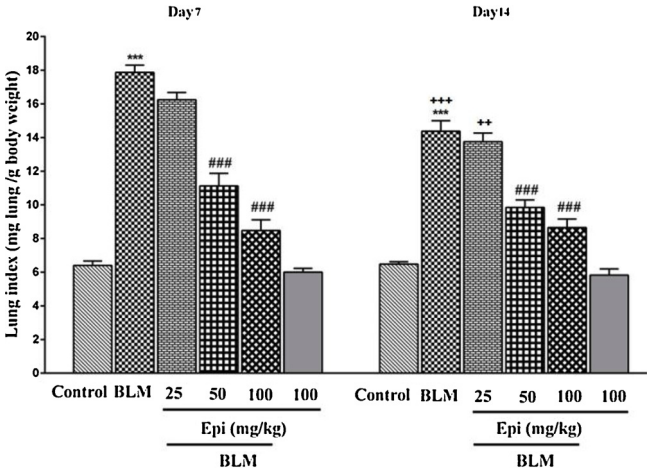

Fig. 3. Epicatechin (Epi) effect on the lung index in lung damage induced by bleomycin (BLM) in mice in both time courses. n = 4–8 in each group.
***P < 0.001 versus control group
###P < 0.001 versus bleomycin group.
+++P < 0.001, ++P < 0.01 versus respective day 7 groups.

2.5. Homogenization and determination of protein content

The right lobe was separated from the lung tissue and homogenized in PBS with a homogenizer at a ratio of 1:5 (W/V). Then samples were centrifuged in microtubes at 600 RPM and 4 °C for 15 min. The supernatant was used for biochemical assays. The protein content in homogenate was measured according to the Bradford method [29] and all the oxidative stress markers were normalized with protein content.

2.6. Oxidative stress markers

The activity of SOD was determined with a commercial kit (RANSOD kit, Randox Com, UK). Absorption was read at 412 nm for SOD and expressed as (IU/mg protein). The catalase (CAT) activity was evaluated by Goth's colorimetric method, in which the homogenized lung tissue was incubated with H_2O_2 and the reaction, stopped after 10 min by mixing with ammonium molybdate. The CAT enzyme converts H_2O_2 to H_2O and O_2 in the samples, while the ammonium molybdate is reacted with the remaining H2O2 in the sample to create a yellow complex. Absorption was measured by spectrophotometer at 410 nm. CAT activity was expressed as (μmol H_2O_2/min/mg protein) [30]. The GPX activity was measured by a commercial kit (RANSEL kit, Randox Com, UK) Based on the Paglia and Valentine method [31]. Absorption was read at 340 nm and enzyme activity expressed as (IU/mg of protein). The GSH level of lung tissue was measured by Ellman's method. Forty μl of lung tissue homogenate was mixed with 2 ml buffer phosphate, and 40 μl of Ellman's reagent added. The yellow complex was read by spectrophotometer at 410 nm and GSH content expressed as (nmol/mg protein) [32]. Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) [33]. In summary, 0.2 ml of homogenate tissue was added to 0.8 ml of solution containing 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/ v) thiobarbituric acid and 0.25 N HCl. The protein was precipitated and removed by centrifugation at 5000 RPM for 5 min. The supernatants were moved to test tubes comprising 0.02% butylated hydroxytoluene and heated for 15 min at 100 °C in a bain-marie. Thereafter, cooled samples were centrifuged at 2000 RPM for 5 min to remove the precipitant. Absorption was read at 532 nm and MDA levels expressed as (nmol/mg protein).

2.7. Fibrotic markers assay

Lung tissue hydroxyproline (HP) content was measured by Edward and O'Brien's colorimetric assay method [34]. The lung tissue was hydrolyzed with 6 N HCl for 24 h at 120 °C, afterward, the buffer was added to samples, then 1 ml chloramine T reagent was added to each sample and set for 20 min at room temperature. Afterward, 1.5 ml of perchloric acid was added and the samples then placed at 60 °C for 15 min to appear a reddish complex. After cooling, absorbance was read at 550 nm. The HP content expressed as (mg/g of lung tissue). TGF-β level was measured in lung tissue homogenate by commercial enzyme-linked immunosorbent assay (ELISA) kit (HANGZHOU EASTBIOPHARM). Absorption of samples was read at 450 nm and TGF- β levels expressed as (pg/mg protein).

2.8. Histopathological studies

2.8.1. Inflammatory and fibrotic evidences in histopathology studies

For histopathology examination, left lung tissue was placed in 10% buffered formalin and blocked in paraffin. Sections of 4 μm were taken and stained by hematoxylin and eosin (H&E), and Masson's trichrome (MT) to evaluate infiltration of inflammatory cells, alveolar thickening and collagen deposition. Alveolitis and inflammation were graded from 0 to 3 by the Szapiel score [35] and fibrotic lesions were estimated from 0 to 8 by the Ashcroft score [36]. Three slides in each mouse and 10 fields in each slide were examined for grading inflammatory and fibrotic lesions.

2.9. Statistical analysis

All values were expressed as mean ± SEM. One-way and two-way ANOVA followed by turkey's post hoc test analysis were used. Alveolitis and inflammation grades and fibrosis scores of photomicrographs were analyzed by one-way ANOVA followed by the Kruskal-Wallis test. Data were analyzed by PRISM 6 software and $p < 0.05$ was considered statistically significant.

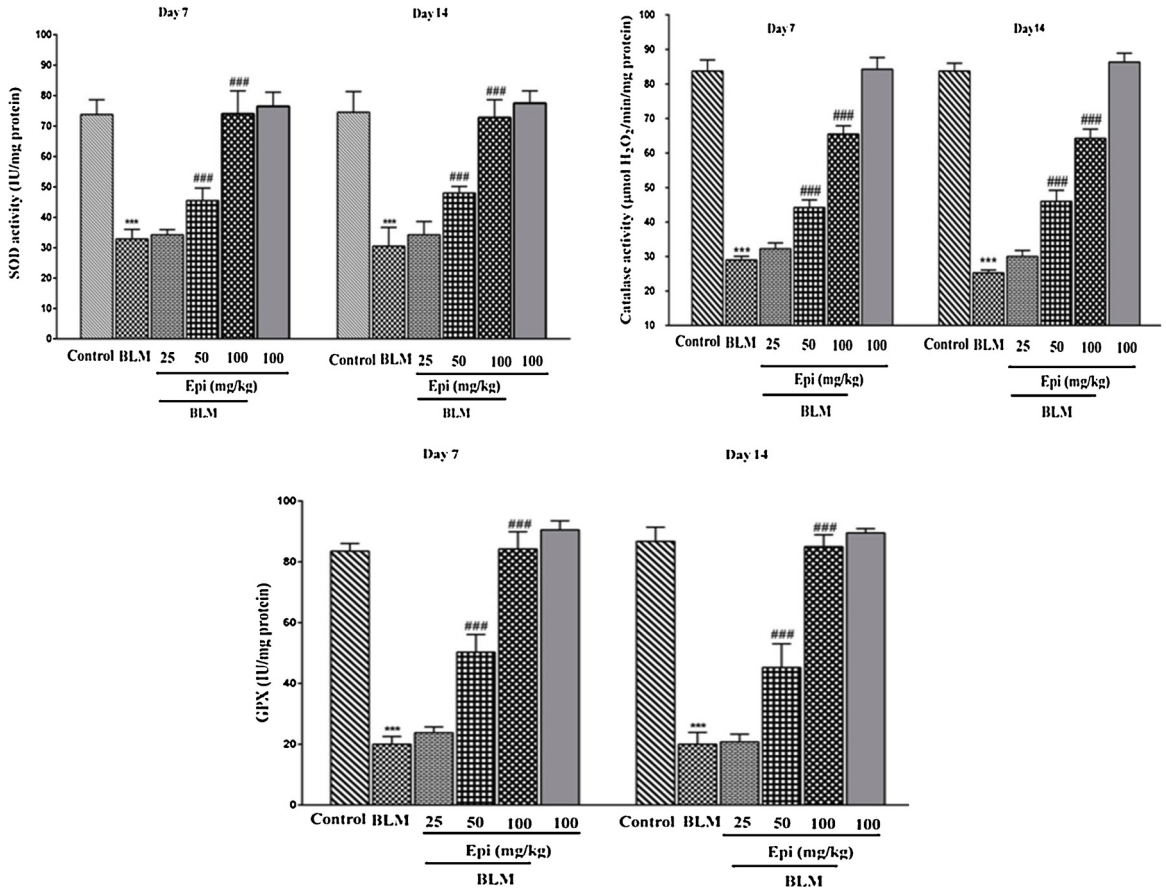

Fig. 4. Epicatechin (Epi) effects on SOD, CAT and GPX activities in both early and late phases of pulmonary damage induced by bleomycin (BLM) in mice. n = 4–8 in each group.
$^{***}P < 0.001$ versus control group.
$^{###}P < 0.001$ versus bleomycin group.

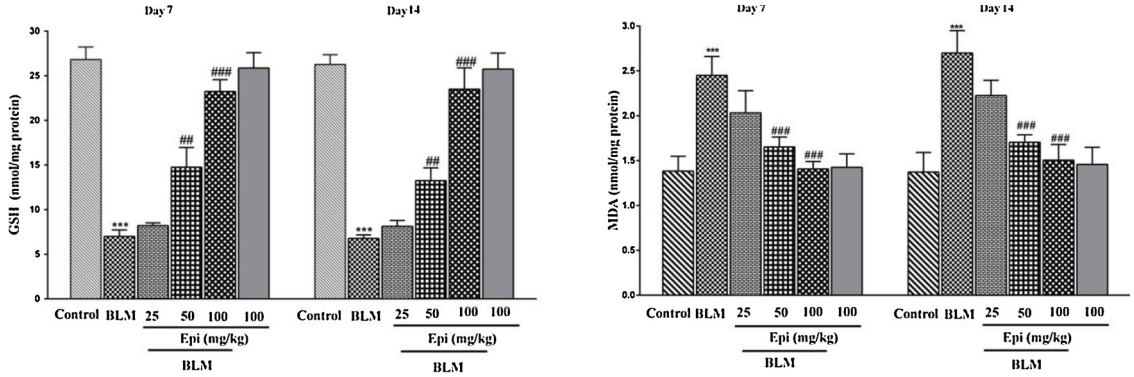

Fig. 5. Effects of epicatechin (Epi) on GSH and MDA levels in pulmonary damage of mice induced by bleomycin (BLM) in both time courses. n = 4–8 in each group.
$^{***}P < 0.001$ versus control group.
$^{###}P < 0.001$, $^{##}P < 0.01$ versus bleomycin group.

3. Results

3.1. Mortality and changes in body weight

Mice mortality in two phases of the study is presented in Table 1. Pretreatment with Epi had beneficial effects and could reduce mortality. In BLM administered groups, 25% of animals in one-week study and 50% of animals in two-week study died. However, Epi could improve survival percent of BLM treated animals. Epi alone had no effect on mortality and its effects are similar to the control group. Mice were weighed in days 0, 3, 7 and 14. The changes in body weight indicated that control and Epi 100 mg/kg groups had weight gain and other groups losing weight. BLM group obviously decreased weight in comparison with the control group ($p < 0.05$). Pretreatment with Epi in dose 100 mg/kg in days 3, 7 and 14 increased body weight compared with the BLM group ($p < 0.05$) (Fig. 2).

3.2. Lung index

The lung index was calculated as the ratio of wet lung weight to body weight (g). Epi at the doses 50 mg/kg and 100 mg/kg ($p < 0.001$) reduced lung index in both early and late phases of

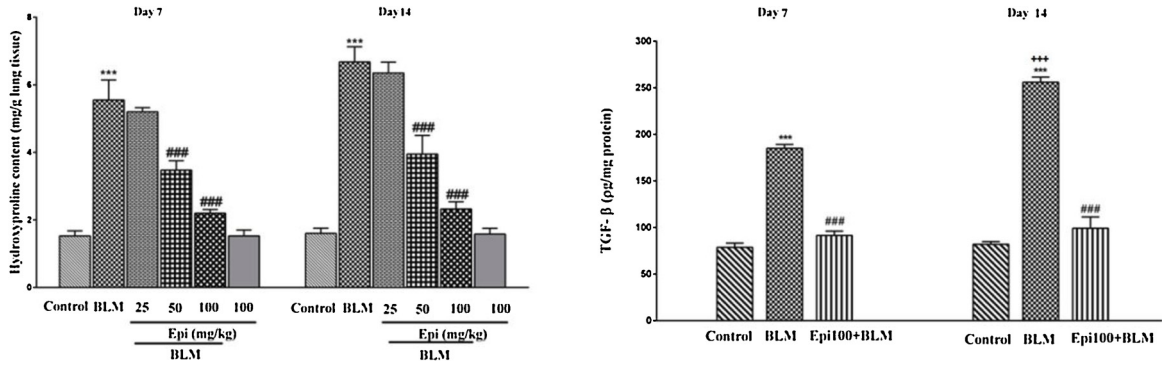

Fig. 6. Effects of epicatechin (Epi) on hydroxyproline and TFG-β content in pulmonary damage of mice induced by bleomycin (BLM) in both time courses. n = 4–8 in each group.
***P < 0.001 versus control group.
###P < 0.001 versus bleomycin group.
+++P < 0.001 versus respective day 7 group.

treatment. Epi 100 mg/kg showed a very significant recovery, whereas treatment with Epi 25 mg/kg did not change lung index. However, the lung index of BLM and Epi 25 in day 14 was significantly less than day 7 (Fig. 3).

3.3. Antioxidant defense system markers

BLM decreased the activity of SOD, CAT and GPX enzymes at the same level versus to the control group ($p < 0.001$). Pretreatment with Epi at the doses 50 and 100 mg/kg increased SOD, CAT and GPX activity in a dose-dependent manner. These effects were at the same level compared to the BLM group ($p < 0.001$) and showed significant recovery (Fig. 4).

BLM reduced lung tissue GSH levels in comparison with the control group ($p < 0.001$). Epi in doses 50 ($p < 0.01$) and 100 mg/kg ($p < 0.001$) increased GSH levels, whereas the treatment with Epi 25 mg/kg had no effect. BLM increased MDA levels ($p < 0.001$) and Epi at the doses 50 and 100 mg/kg ($p < 0.001$) decreased lung tissue MDA levels (Fig. 5).

3.4. Fibrotic markers

HP content is an important index for collagen deposition in the lung tissue. BLM obviously increased HP content ($p < 0.001$) compared with the control group. Epi in doses 50 and 100 mg/kg ($p < 0.001$) reduced lung HP but the treatment group with Epi 100 mg/kg showed significant recovery. Treatment with Epi in dose 100 mg/kg ($p < 0.001$) decreased TGF-β compared with the BLM group. TGF-β is a pro-fibrotic cytokine that is activated by ROS and triggers a cascade mechanism, which causes fibrosis in the lung tissue. The TGF-β level on day 14 was higher than day 7 (Fig. 6).

3.5. Histological changes

Histological examination shows that the treated group with BLM is damaged with the obvious lesions, cell infiltration, and decomposed tissue. In MT staining, the collagen can be seen in the form of blue strings, which is more confirmation of fibrosis. These pathological manifestations are more obvious on day 14. The treated group with Epi 25 mg/kg in both time courses is like BLM and there are no significant changes. At both early and late phases of BLM injury, treated groups with Epi 50 and 100 mg/kg showed recovery very well. Pretreated group with 100 mg/kg has similar manifestations to the control group (Figs. 7 and 8).

Grading of alveolitis and inflammation by the Szapiel method [35] on day 7, and scoring for fibrosis by the Ashcroft method on day 14, in mice model of pulmonary injury induced by BLM [36] are shown in Fig. 9. Epi in doses of 50 and 100 mg/kg has been able to attenuate inflammatory lesions on day 7 and inhibit the progression of fibrosis on day 14 (Fig. 9).

4. Discussion

In this study, we investigated possible protective effects of different doses of Epi against the harmful effect of BLM. BLM is a chemical agent for many malignancies. One of its most important adverse effects is pulmonary fibrosis; therefore, BLM is used as a model of pulmonary fibrosis. The BLM model of lung injury and fibrosis has some similarities to human lung fibrosis. Pulmonary fibrosis is a lethal lung disease that occurs when the lung tissue becomes hardly damaged by thickening of the alveolar cell walls with collagen. Alveolar wall thickening is associated with coughing, shortness of breath and dyspnea [37,38]. In this model of lung toxicity, damage can be observed as an early phase with oxidative and inflammatory events, which usually continues until day 7 and late phase with fibrotic outcomes, which will continue until 14 to 21 days after bleomycin. Thus, the days 7 and 14 were selected as endpoint days [3,39]. BLM causes apoptotic changes in the alveolar and bronchiolar epithelial cells [40]. Therapeutic agents can prevent apoptotic effects induced by BLM. BLM causes toxicity by cleaving DNA in a process dependent on the presence of molecular oxygen and iron ion as cofactors in DNA double-strand demolition. BLM binds DNA and Fe (II) and molecular oxygen and produces a complex that can attack the DNA, and then the ROS mediators cause lipid peroxidation [41]. The role of iron has been determined in the oxidative stress and damage caused by bleomycin [42,43]. Therefore, the chelating ability of flavonoids [20,44] may be responsible for attenuation of lung injury due to BLM in mice. The antioxidant activity of Epi is related to free radical scavenging and metal ion chelating ability. The antioxidant activity of Epi is due to the ortho 3',4'-dihydroxy moiety in the B ring and its chelating ability is due to the o-phenolic groups in the3',4'-dihydroxy positions in the B ring [29]. The endogenous antioxidant defense system including enzymatic (SOD, CAT and GPX activity) and non-enzymatic (GSH and MDA) was examined in this study. The oxidative stress that activates inflammatory signaling pathways causes oxidative damage [45]. In BLM model of lung injury, oxidative damage is a condition due to the lack of balance between ROS production and the antioxidant defense system [46,47]. It has been reported that oxidative stress and inflammation are early and fibrosis is a late event in BLM model of lung damage [12,15]. However, in present research it seems that oxidative stress and inflammation remain at the time of fibrosis and play a role in the development and maintenance of fibrosis. In addition, the fibrotic lesions are noticeably visible on Day 7. In

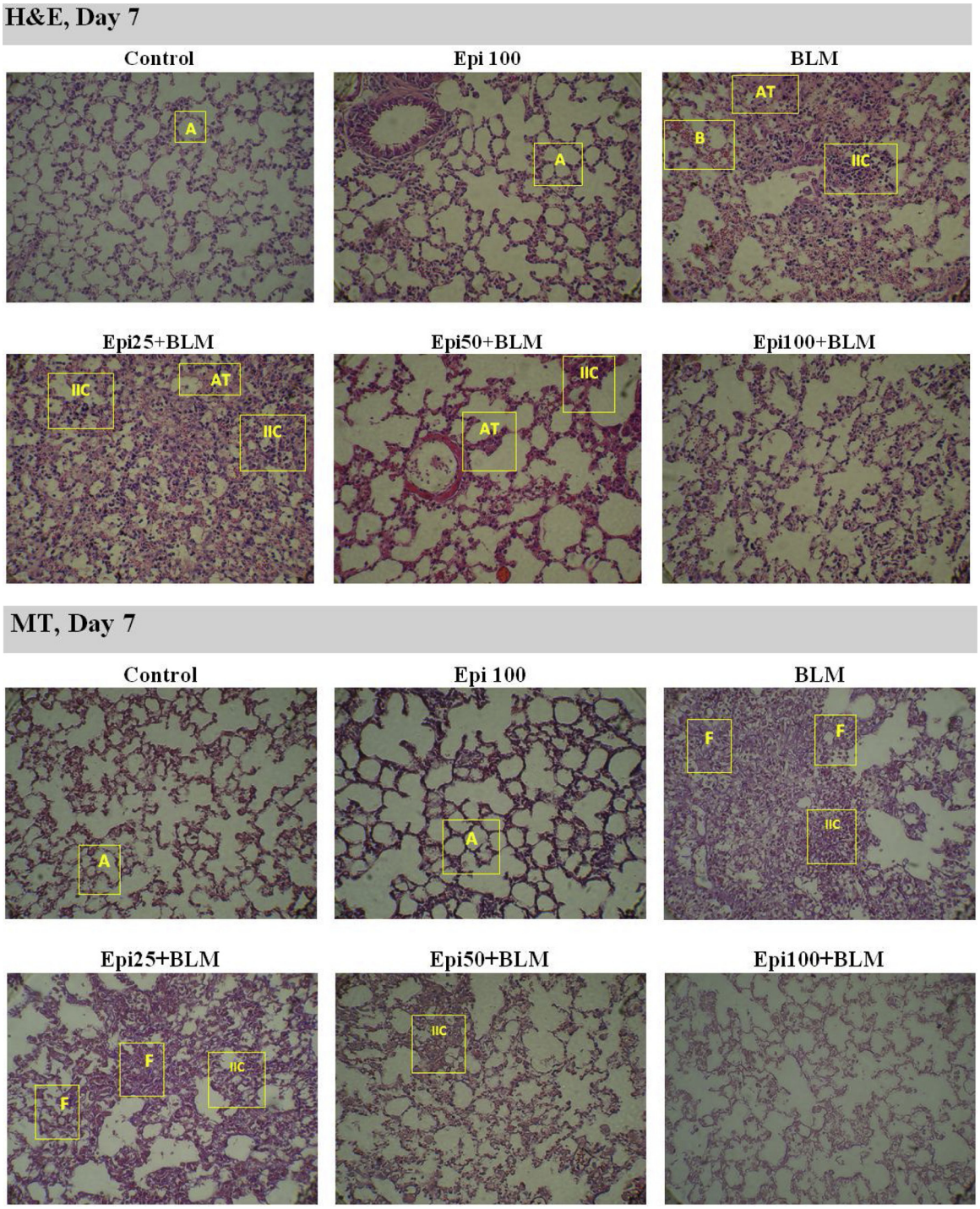

Fig. 7. Histopathological examination of lung tissue sections stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT) with original magnification x300 on day 7 after bleomycin (BLM). Anti-inflammatory and antifibrotic effects of epicatechin (Epi) on histopathological examination of lung tissue damage induced by BLM in mice are completely obvious. Alveoli in the control and Epi100 groups were normal and the structure of lung tissues had no lesion. In damaged alveoli in the BLM group, edema is associated with alveolitis and collagen accumulation. Collagen accumulations are shown with blue strings by MT staining. The Epi 25 + BLM group has no significant change in comparison with BLM group. In group Epi 50 + BLM, improvement was not complete, but lower than BLM group. In the Epi 100 + BLM group, the recovery is very favorable and almost similar to the control group. A: alveolus; AT: alveolar wall thickening; B; bleeding; IIC: infiltration of inflammatory cells; F: fibrosis.

confirmation, the data indicate that the level of TGF-β increases after BLM administration and their levels are well visible on days 7 and 14. TGF-β increases ROS overproduction and ROS activates TGF-β cytokine production. This can be a reason to keep oxidative stress high in the late phase of pulmonary injury on day 14. TGF-β is a pro-fibrotic agent that causes fibrosis by the proliferation of fibroblast and accumulation of excessive ECM. Furthermore, TGF-β can decrease GSH levels in the lung tissue [48–51]. BLM caused 25% mortality in one-week study and 50% in two-week study. Epi could reverse mortality percent of BLM treated animals. In a similar study, which pulmonary toxicity induced by BLM 0.1 U/100 µl/mouse, i.t., BLM induced 60% mortality after 15 days [27]. The increased body weight during administration of Epi 100 mg/

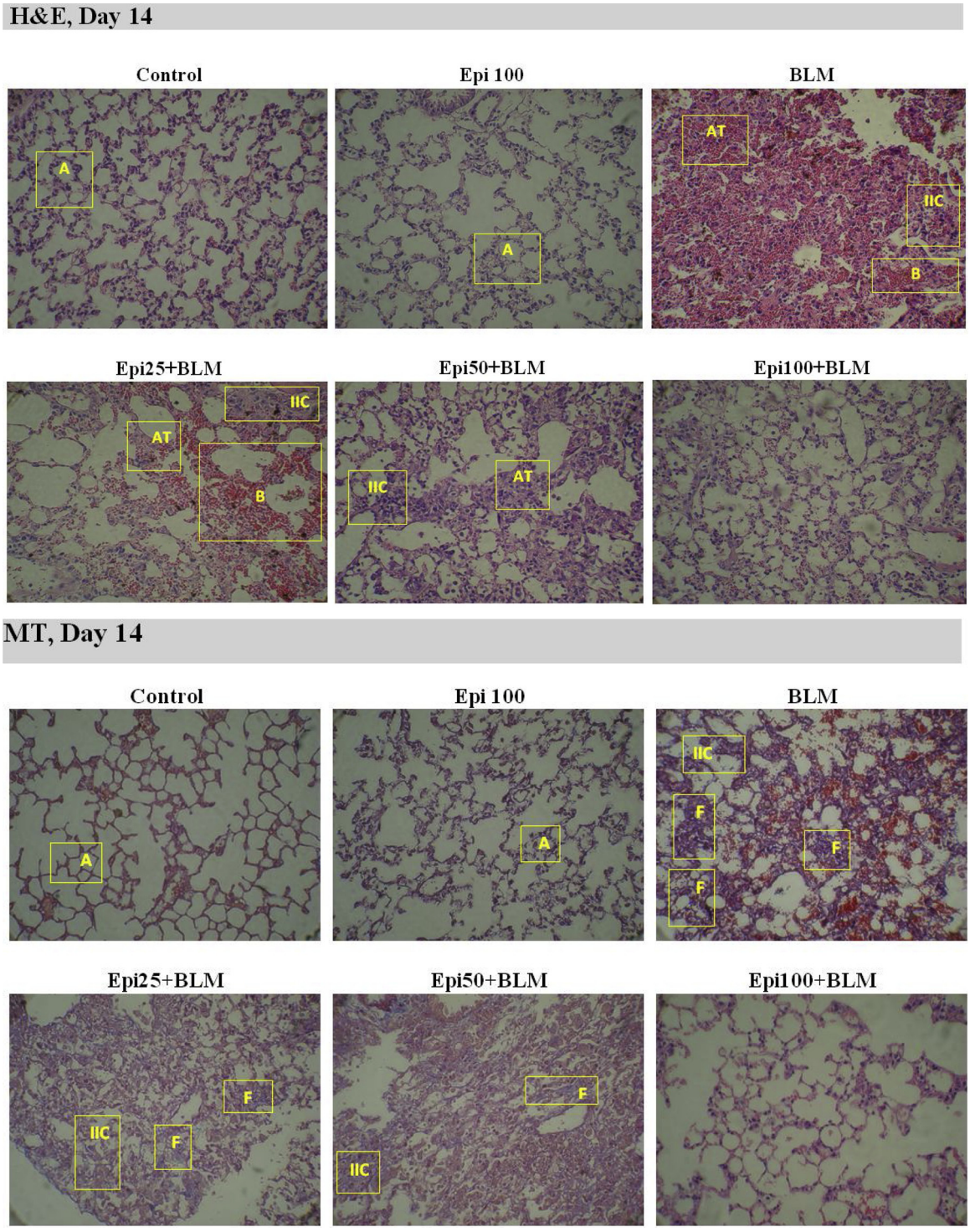

Fig. 8. Histopathological examination of lung tissue sections stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT) on day 14 after BLM. Photographs show anti-inflammatory and antifibrotic effects of epicatechin (Epi) on bleomycin (BLM) induced lung injury with original magnification ×300. Alveoli in the control and Epi100 groups are normal with intact alveolar space and the structure of lung tissue has no lesion. In the BLM group, the alveoli are destroyed and excessive collagen accumulated. Excessive collagen accumulation is shown with blue strings in MT staining. These pathological manifestations are more obvious and intensely indicate fibrotic changes on day 14. The Epi 25 + BLM group has no significant change in comparison with BLM group. In group Epi 50 + BLM, recovery was not complete, but lower than BLM group. In the Epi 100 + BLM group, the recovery is very favorable, so that the damage hardly seen and almost similar to the control group. A: alveolus; AT: alveolar wall thickening; B; bleeding; IIC: infiltration of inflammatory cells; F: fibrosis.

kg is similar to that of the control group. In BLM treated mice the body weight of animal decreases and this refers to the condition of the disease. In the present study, body weight returned to almost normal weight in Epi 100 mg/kg pretreated mice in the BLM model of lung injury. This suggests that Epi leads to recovery and weight gain by improving the damage induced by BLM. A significant increase in lung index on day 7 may be due to the role of both inflammation and profibrotic lesions. Pretreatment with Epi, especially with dose 100 mg/kg in the BLM lung injury model, has shown the best reversing effect according to increase the activity of GPX, SOD, CAT and GSH level. Epi

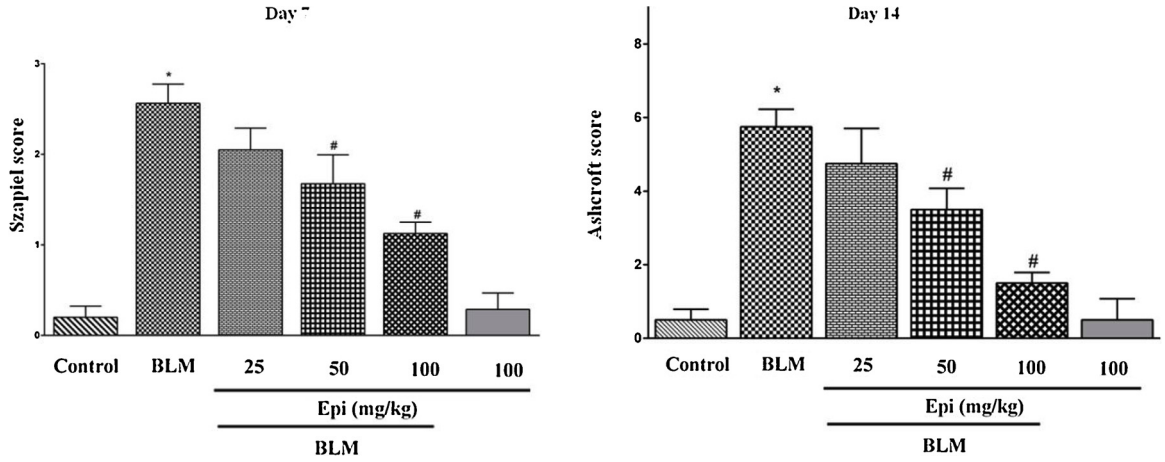

Fig. 9. Pretreatment with epicatechin (Epi) decreases alveolitis and inflammation (Szapiel score) on day 7 and inhibits fibrotic lesions (Ashcroft score) on day 14 in mice pulmonary damage induced by bleomycin (BLM). Scores in Szapiel score were 0–3 and in Ashcroft score were 0-8. n = 4–8 in each group. Data is taken from 3 slides in each mouse and 10 fields in each slide.
*P < 0.05 versus control group.
#P < 0.05 versus bleomycin group.

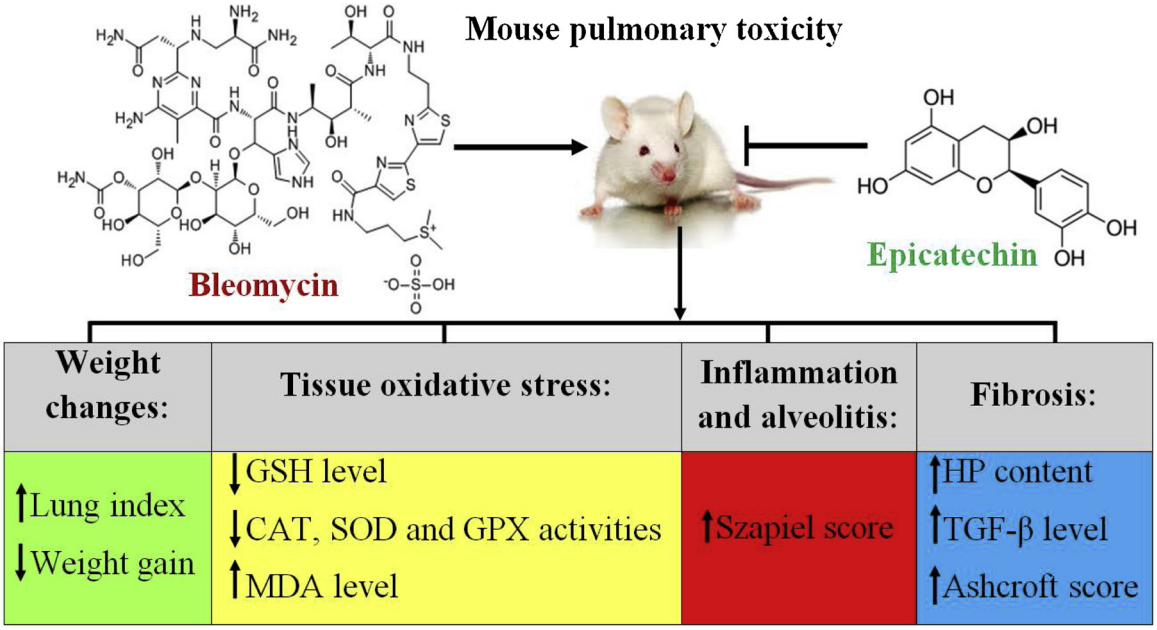

Fig. 10. The presented schematic shows the beneficial effects of epicatechin a natural flavonoid on bleomycin-induced pulmonary injury in mice.

reduced tissue levels of MDA, HP, TGF-β and lung index. The improvement of pathological manifestations in the lung tissue can be attributed the antioxidant activity of Epi in the lung. Pretreatment with Epi in dose of 50 mg/kg in the BLM model of lung fibrosis also provides recovery, but damage can still be seen and there is no complete recovery. There is no difference between Epi 25 mg/kg in BLM lung fibrosis with the BLM group and Epi with this dose cannot reverse the lung inflammatory and fibrotic damage. As previously reported, Epi 1 mg/kg by gavage for 14 days increases the expression of GPX, SOD and CAT enzymes in senile mice [52]. It can be concluded that enhanced activity of SOD, CAT and GPX in Epi pretreated mice of the lung injury may be due to the induction of expression of these enzymes by Epi in this model of lung injury. However, Epi dose in mentioned study (1 mg/kg) is low in comparison with the Epi doses (25, 50 and 100 mg/kg) in our study. It has been reported that pretreatment with Epi 50 mg/kg decreased the oxidative damage and showed hepatoprotective effects in the rat model of hepatitis [27]. In another study in doxorubicin model of brain toxicity in the rats, the used Epi dose was 10 mg/kg per day for 4 weeks [53]. It has been shown that the mice doses should be divided by 12.3 to convert animal doses to human equivalent doses [54]. Accordingly, effective Epi doses of this study (50 and 100 mg/kg) can be considered for the relevance of Epi doses in mice to humans. As a result, daily human Epi doses in the range of 4–8 mg/kg, which is equivalent to 280–560 mg for a person of 70 kg can be estimated for clinical application. However, careful pharmacokinetic and pharmacodynamic studies along with considerations of species differences and human variability should be made to estimate the right dose in humans. In this regard, a review article on the effects of Epi in the range of 25–447 mg on human cognition has reported that there is not enough evidence for the optimal Epi dose for positive cognitive effects [55].

Polyphenol compounds have poor bioavailability and short half-life, hence their prophylactic and therapeutic uses are limited [56]. Thus, there are ongoing efforts to enhance their bioavailability and consequently biological activity by using nanotechnology [56,57]. There is evidence that Epi absorption and excretion are dose-dependent in rats [58]. Thus, poor bioavailability of Epi can be compensated with high or

repeated doses of Epi. Overall, Epi repeated administration, nanotechnology techniques, Epi inhalation use and intravenous application of Epi are highlighted to overcome poor bioavailability of Epi. Epi and Epi containing foods have beneficial effects [56,59]. However, as a question in this study, can the inhibitory effects of Epi on BLM-induced pulmonary damage be extended to the reversing effects of Epi on anticancer activity of BLM? Can Epi be co-administered with BLM in clinical application for cancer patients? Hence, it is mentioned that green tea polyphenols induce selective toxicity in cancer cells and could be a valuable adjuvant in the treatment of cancer [60]. Furthermore, the beneficial effects of tea polyphenols in combination with anticancer agents have been widely accepted by cancer researchers [61]. In addition, Epi as a naturally known tea polyphenol compound can enhance the anti-proliferative effect of BLM on cancer cells without any toxicity to normal cells [62]. Nevertheless, Epi is believed to be able to alleviate the negative side effects of BLM and enhance its anticancer efficacy. Data from Epi 100 mg/kg without BLM treatment indicated that Epi has no harmful effect on the lungs and its effects are similar to the control group. Thus, Epi may be considered for inhalation or systemic use before using BLM or at the time of injury in cancer patients. In conclusion, our project showed that Epi could reverse the toxic effects of BLM through the attenuation of oxidative stress, inflammation and fibrosis in mice. Overall, based on the data of this study, Epi effects on BLM-induced pulmonary lesions are schematically shown in Fig. 10.

Epi as a restorative agent can improve and control lung damage induced by BLM and maybe increases the quality of life in cancer patients. However, further studies are needed to confirm the safety of Epi and co-treatment of systemic or inhaled Epi with BLM requires more safety and efficacy studies for clinical application in cancer patients.

Conflict of interest

The authors did not have any conflict of interest.

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References

[1] L. Nalysnyk, J. Cid-Ruzafa, P. Rotella, D. Esser, Incidence and prevalence of idiopathic pulmonary fibrosis: review of the literature, Eur. Respir. Rev. 21 (126) (2012) 355–361.
[2] J. Gauldie, M. Kolb, Animal models of pulmonary fibrosis: how far from effective reality? Am. J. Physiol. Lung Cell Mol. Physiol. 294 (2) (2008) L151.
[3] F. Chua, J. Gauldie, G.J. Laurent, Pulmonary fibrosis: searching for model answers, Am. J. Respir. Cell Mol. Biol. 33 (1) (2005) 9–13.
[4] G. Raghu, T. Freudenberger, S. Yang, J. Curtis, C. Spada, J. Hayes, J. Sillery, Cn. Pope, C. Pellegrini, High prevalence of abnormal acid gastro-oesophageal reflux in idiopathic pulmonary fibrosis, Eur. Respir. J. 27 (1) (2006) 136–142.
[5] T.E. King Jr, A. Pardo, M. Selman, Idiopathic pulmonary fibrosis, Lancet 378 (9807) (2011) 1949–1961.
[6] D.J. Lederer, F.J. Martinez, Idiopathic pulmonary fibrosis, N. Engl. J. Med. 378 (19) (2018) 1811–1823.
[7] D.K. Gidaris, K.N. Priftis, M.B. Anthracopoulos, Pulmonary Complications of Drug Therapy, Pulmonary Complications of Non-Pulmonary Pediatric Disorders, Springer, 2018, pp. 241–262.
[8] F.H. Green, Overview of pulmonary fibrosis, Chest 122 (6) (2002) 334S–339S.
[9] I. Adamson, Pulmonary toxicity of bleomycin, Environ. Health Perspect. 16 (1976) 119–125.
[10] H. Umezawa, M. Ishizuka, K. Maeda, T. Takeuchi, Studies on bleomycin, Cancer 20 (5) (1967) 891–895.
[11] R.H. Blum, S.K. Carter, K. Agre, A clinical review of bleomycin—a new antineoplastic agent, Cancer 31 (4) (1973) 903–914.
[12] A. Moeller, K. Ask, D. Warburton, J. Gauldie, M. Kolb, The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? Int. J. Biochem. Cell Biol. 40 (3) (2008) 362–382.
[13] H. Umezawa, K. Maeda, T. Takeuchi, Y. Okami, New antibiotics, bleomycin A and B, J. Antibiot. 19 (5) (1966) 200–209.
[14] G.L. Snider, B.R. Celli, R.H. Goldstein, J.J. O'Brien, E.C. Lucey, Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin: lung volumes, volume-pressure relations, carbon monoxide uptake, and arterial blood gas studies, Am. Rev. Respir. Dis. 117 (2) (1978) 289–297.
[15] N.I. Chaudhary, A. Schnapp, J.E. Park, Pharmacologic differentiation of inflammation and fibrosis in the rat bleomycin model, Am. J. Respir. Crit. Care Med. 173 (7) (2006) 769–776.
[16] S. Sleijfer, Bleomycin-induced pneumonitis, Chest 120 (2) (2001) 617–624.
[17] K.B. Pandey, S.I. Rizvi, Plant polyphenols as dietary antioxidants in human health and disease, Oxid. Med. Cell Longev. 2 (5) (2009) 270–278.
[18] J. Shay, H.A. Elbaz, I. Lee, S.P. Zielske, M.H. Malek, M. Hüttemann, Molecular mechanisms and therapeutic effects of (−)-epicatechin and other polyphenols in cancer, inflammation, diabetes, and neurodegeneration, Oxid. Med. Cell Longev. 2015 (2015) 181260.
[19] M. Ferrali, C. Signorini, B. Caciotti, L. Sugherini, L. Ciccoli, D. Giachetti, M. Comporti, Protection against oxidative damage of erythrocyte membrane by the flavonoid quercetin and its relation to iron chelating activity, FEBS Lett. 416 (2) (1997) 123–129.
[20] C. Rice-Evans, N. Miller, G. Paganga, Antioxidant properties of phenolic compounds, Trends Plant Sci. 2 (4) (1997) 152–159.
[21] M. Symonowicz, M. Kolanek, Flavonoids and their properties to form chelate complexes, Biotechnol. Food Sci. 76 (1) (2012) 35–41.
[22] H.-R. Kim, B.-K. Park, Y.-M. Oh, Y.-S. Lee, D.-S. Lee, H.-K. Kim, J.-Y. Kim, T.-S. Shim, S.-D. Lee, Green tea extract inhibits paraquat-induced pulmonary fibrosis by suppression of oxidative stress and endothelin-l expression, Lung 184 (5) (2006) 287–295.
[23] S.I. Rizvi, M.A. Zaid, R. Anis, N. Mishra, Protective role of tea catechins against oxidation-induced damage of type 2 diabetic erythrocytes, Clin. Exp. Pharmacol. Physiol. 32 (1–2) (2005) 70–75.
[24] K. Tanabe, Y. Tamura, M.A. Lanaspa, M. Miyazaki, N. Suzuki, W. Sato, Y. Maeshima, G.F. Schreiner, F.J. Villarreal, R.J. Johnson, Epicatechin limits renal injury by mitochondrial protection in cisplatin nephropathy, Am. J. Physiol. Ren. Physiol. 303 (9) (2012) F1264–F1274.
[25] L. Zhao, X. Wang, Q. Chang, J. Xu, Y. Huang, Q. Guo, S. Zhang, W. Wang, X. Chen, J. Wang, Neferine, a bisbenzylisoquinline alkaloid attenuates bleomycin-induced pulmonary fibrosis, Eur. J. Pharmacol. 627 (1–3) (2010) 304–312.
[26] M.A. Mouratis, V. Aidinis, Modeling pulmonary fibrosis with bleomycin, Curr. Opin. Pulm. Med. 17 (5) (2011) 355–361.
[27] T. Genovese, R.D. Paola, E. Mazzon, C. Muià, A.P. Caputi, S. Cuzzocrea, Melatonin limits lung injury in bleomycin treated mice, J. Pineal Res. 39 (2) (2005) 105–112.
[28] B. Shanmugam, K.R. Shanmugam, S. Ravi, G.V. Subbaiah, C. Ramakrishana, K. Mallikarjuna, K.S. Reddy, Exploratory studies of (-)-Epicatechin, a bioactive compound of Phyllanthus niruri, on the antioxidant enzymes and oxidative stress markers in D-galactosamine-induced hepatitis in rats: a study with reference to clinical prospective, Pharmacogn. Mag. 13 (Suppl. 1) (2017) S56–S62.
[29] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1–2) (1976) 248–254.
[30] L. Goth, A simple method for determination of serum catalase activity and revision of reference range, Clin. Chim. Acta 196 (2–3) (1991) 143–151.
[31] D.E. Paglia, W.N. Valentine, Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase, J. Lab. Clin. Med. 70 (1) (1967) 158–169.
[32] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1) (1959) 70–77.
[33] M. Uchiyama, M. Mihara, Determination of malonaldehyde precursor in tissues by thiobarbituric acid test, Anal. Biochem. 86 (1) (1978) 271–278.
[34] C. Edwards, W. O'Brien Jr, Modified assay for determination of hydroxyproline in a tissue hydrolyzate, Clin. Chim. Acta 104 (2) (1980) 161–167.
[35] S.V. Szapiel, N.A. Elson, J.D. Fulmer, G.W. Hunninghake, R.G. Crystal, Bleomycin-induced interstitial pulmonary disease in the nude, athymic mouse, Am. Rev. Respir. Dis. 120 (4) (1979) 893–899.
[36] T. Ashcroft, J.M. Simpson, V. Timbrell, Simple method of estimating severity of pulmonary fibrosis on a numerical scale, J. Clin. Pathol. 41 (4) (1988) 467–470.
[37] X.-Y. You, Q. Xue, Y. Fang, Q. Liu, C.F. Zhang, C. Zhao, M. Zhang, X.-H. Xu, Preventive effects of Ecliptae Herba extract and its component, ecliptasaponin A, on bleomycin-induced pulmonary fibrosis in mice, J. Ethnopharmacol. 175 (2015) 172–180.
[38] A.T. Society, Idiopathic pulmonary fibrosis: diagnosis and treatment: international consensus statement, Am. J. Respir. Crit. Care Med. 161 (2000) 646–664.
[39] G. Izbicki, M. Segel, T. Christensen, M. Conner, R. Breuer, Time course of bleomycin-induced lung fibrosis, Int. J. Exp. Pathol. 83 (3) (2002) 111–119.
[40] L. Safaeian, A. Jafarian-Dehkordi, M. Rabbani, H. Sadeghi, N. Afshar-Moghaddam, S. Sarahroodi, Comparison of bleomycin-induced pulmonary apoptosis between NMRI mice and C57BL/6 mice, Res. Pharm. Sci. 8 (1) (2013) 43.
[41] U. Galm, M.H. Hager, S.G. Van Lanen, J. Ju, J.S. Thorson, B. Shen, Antitumor antibiotics: bleomycin, enediynes, and mitomycin, Chem. Rev. 105 (2) (2005) 739–758.
[42] J. Hay, S. Shahzeidi, G. Laurent, Mechanisms of bleomycin-induced lung damage, Arch. Toxicol. 65 (2) (1991) 81–94.
[43] D.B. Chandler, J.C. Barton, D. Briggs III, T.W. Butler, J.I. Kennedy, W.E. Grizzle, J.D. Fulmer, Effect of iron deficiency on bleomycin-induced lung fibrosis in the hamster, Am. J. Respir. Crit. Care Med. 137 (1) (1988) 85–89.
[44] S.A. van Acker, G.P. van Balen, D.J. van den Berg, A. Bast, W.J. van der Vijgh, Influence of iron chelation on the antioxidant activity of flavonoids, Biochem. Pharmacol. 56 (8) (1998) 935–943.

[45] M. Mittal, M.R. Siddiqui, K. Tran, S.P. Reddy, A.B. Malik, Reactive oxygen species in inflammation and tissue injury, Antioxid. Redox Signal. 20 (7) (2014) 1126–1167.
[46] B.J. Day, Catalase and glutathione peroxidase mimics, Biochem. Pharmacol. 77 (3) (2009) 285–296.
[47] A.E. Filderman, L.A. Genovese, J.S. Lazo, Alterations in pulmonary protective enzymes following systemic bleomycin treatment in mice, Biochem. Pharmacol. 37 (6) (1988) 1111–1116.
[48] A. Santana, B. Saxena, N.A. Noble, L.I. Gold, B.C. Marshall, Increased expression of transforming growth factor beta isoforms (beta 1, beta 2, beta 3) in bleomycin-induced pulmonary fibrosis, Am. J. Respir. Cell Mol. Biol. 13 (1) (1995) 34–44.
[49] M. Peltoniemi, R. Kaarteenaho-Wiik, M. Säily, R. Sormunen, P. Pääkkö, A. Holmgren, Y. Soini, V.L. Kinnula, Expression of glutaredoxin is highly cell specific in human lung and is decreased by transforming growth factor-β in vitro and in interstitial lung diseases in vivo, Hum. Pathol. 35 (8) (2004) 1000–1007.
[50] K.N. Islam, Y. Kayanoki, H. Kaneto, K. Suzuki, M. Asahi, J. Fujii, N. Taniguchi, TGF-β1 triggers oxidative modifications and enhances apoptosis in HIT cells through accumulation of reactive oxygen species by suppression of catalase and glutathione peroxidase, Free Radic. Biol. Med. 22 (6) (1997) 1007–1017.
[51] R.-M. Liu, K.G. Pravia, Oxidative stress and glutathione in TGF-β-mediated fibrogenesis, Free Radic. Biol. Med. 48 (1) (2010) 1–15.
[52] A. Moreno-Ulloa, L. Nogueira, A. Rodriguez, J. Barboza, M.C. Hogan, G. Ceballos, F. Villarreal, I. Ramirez-Sanchez, Recovery of indicators of mitochondrial biogenesis, oxidative stress, and aging with (−)-epicatechin in senile mice, J. Gerontol. A. Biomed. Sci. Med. Sci. 70 (11) (2015) 1370–1378.
[53] R.H. Mohamed, R.A. Karam, M.G. Amer, Epicatechin attenuates doxorubicin-induced brain toxicity: critical role of TNF-α, iNOS and NF-κB, Brain Res. Bull. 86 (1–2) (2011) 22–28.
[54] A.B. Nair, S. Jacob, A simple practice guide for dose conversion between animals and human, J. Basic Clin. Pharm. 7 (2) (2016) 27.
[55] C. Haskell-Ramsay, J. Schmitt, L. Actis-Goretta, The impact of epicatechin on human cognition: The role of cerebral blood flow, Nutrients 10 (8) (2018) 986.
[56] M. Prakash, B. Basavaraj, K.C. Murthy, Biological functions of epicatechin: plant cell to human cell health, J. Funct. Foods 52 (2019) 14–24.
[57] T. Khushnud, S.A. Mousa, Potential role of naturally derived polyphenols and their nanotechnology delivery in cancer, Mol. Biotechnol. 55 (1) (2013) 78–86.
[58] S. Baba, N. Osakabe, M. Natsume, Y. Muto, T. Takizawa, J. Terao, Absorption and urinary excretion of (−)-epicatechin after administration of different levels of cocoa powder or (−)-epicatechin in rats, J. Agric. Food Chem. 49 (12) (2001) 6050–6056.
[59] I. Bernatova, Biological activities of (−)-epicatechin and (−)-epicatechin-containing foods: focus on cardiovascular and neuropsychological health, Biotechnol. Adv. 36 (3) (2018) 666–681.
[60] Z. Bedrood, M. Rameshrad, H. Hosseinzadeh, Toxicological effects of Camellia sinensis (green tea): a review, Phytother. Res. 32 (7) (2018) 1163–1180.
[61] J. Cao, J. Han, H. Xiao, J. Qiao, M. Han, Effect of tea polyphenol compounds on anticancer drugs in terms of anti-tumor activity, toxicology, and pharmacokinetics, Nutrients 8 (12) (2016) 762.
[62] S.J. Hosseinimehr, M. Rostamnejad, V. Ghaffari-rad, Epicatechin enhances antiproliferative effect of bleomycin in ovarian cancer cell, Res. Mol. Med. 1 (3) (2013) 25–28.