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ORIGINAL ARTICLE

Amitriptyline attenuates bleomycin-induced pulmonary fibrosis: modulation of the expression of NF-κβ, iNOS, and Nrf2

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Abstract

Amitriptyline is a tricyclic antidepressant that was suggested to have antifibrotic potential. The current study aimed to investigate the modulatory effects of amitriptyline on bleomycin-induced pulmonary fibrosis in rats. Rats were randomly assigned into 4 groups: normal control, bleomycin control, amitriptyline+bleomycin, and amitriptyline only treated group. Lung injury was evaluated through the histological examination and immunohistochemical detection of α-smooth muscle actin (α-SMA) in lung tissue, in addition to the biochemical assessment of pulmonary contents of hydroxyproline and transforming growth factor beta-1 (TGF-β1). In addition, the following parameters were investigated for studying the possible mechanisms of amitriptyline antifibrotic effect: inducible nitric oxide synthase (iNOS), nuclear factor-κβ (NF-κβ), tumor necrosis factor-alpha (TNF-α), serpine-1, p53, nuclear factor erythroid 2-related factor 2 (Nrf2), lipid peroxides, and reduced glutathione (GSH). Amitriptyline exhibited potent antifibrotic effect that was reflected upon the histopathological examination and through its ability to suppress all the fibrotic parameters. Amitriptyline successfully suppressed the expression of NF-κβ, Nrf2, iNOS, and p53 in lung tissues besides the inhibition of other oxidative stress and inflammatory mediators. Amitriptyline could be a promising treatment to pulmonary fibrosis. Amitriptyline not only prevents the depression and its drawbacks in patients suffering from pulmonary fibrosis but also it can suppress fibrosis through variable mechanisms mainly via inhibition of NF-κβ/TNF-α/TGF-β pathway in addition to inhibition of Nrf2 and iNOS expression.

Keywords Amitriptyline · Pulmonary fibrosis · Nuclear factor-κβ · Nrf2 · iNOS

Introduction

Pulmonary fibrosis occurs following a cascade of events. Lung epithelial cells are first damaged and lose their protective function, leading to their transition into mesenchymal cells (epithelial–mesenchymal transition; EMT). Then, these mesenchymal cells or fibroblasts transdifferentiate into highly contractile, synthetic alpha-smooth muscle actin (α-SMA)-positive myofibroblasts, which are considered the key effector cells in pulmonary fibrosis (Lekkerkerker et al. 2012).

As a major fibrogenic cytokine, transforming growth factor beta-1 (TGF-β1) was demonstrated to induce the change from fibroblasts to myofibroblasts producing EMT in alveolar epithelial cells and hence playing a key role in pulmonary fibrosis (Tian et al. 2018).

There are many factors that play a major role in the pathogenesis of pulmonary fibrosis. Oxidative stress is considered one of the most important inducers for pulmonary fibrosis (Wuyts et al. 2013). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a significant transcription factor for regulating oxidative stress by activating downstream antioxidant proteins including heme oxygenase (HO-1) and NAD(P)H. It was demonstrated that Nrf2 blocked EMT progression in the bleomycin model of pulmonary fibrosis (Zhang et al. 2018).

Highlights
- Amitriptyline inhibited the experimentally induced pulmonary fibrosis in rats.
- It suppresses fibrosis through variable mechanisms.
- It can suppress NF-κβ, Nrf2, iNOS, and p53 expression in lungs.
- It inhibits oxidative stress and inflammatory mediators.
- It has no effect on serpine-1 expression.

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Inflammation leads to transformation of several cell types into myofibroblasts and eventually extracellular matrix deposition. When encountering the invaders, alveolar epithelium can secrete tumor necrosis factor-alpha (TNF-α), the mediator of inflammatory signal pathway, which can amplify the inflammation response by the activation of nuclear factor- beta (NF-κβ) transcription factor (Li et al. 2017a). The inflammatory response is responsible for the recruitments of leukocytes and the aggravation of further oxidative stress injury (Li et al. 2017b).

Plasminogen activator inhibitor-1 (PAI-1), also known as serpine-1, is a primary inhibitor of plasminogen activators, which converts plasminogen into plasmin playing a major role in fibrinolysis. Besides suppression of fibrinolysis, PAI-1 has many other functions, including modulation of cell adhesion, migration, and proliferation. It was demonstrated that PAI-1 plays a critical role in the development of lung fibrosis. In addition, it was reported that PAI-1 induces p53, activating p53-p21-Rb cell cycle repression pathway (Jiang et al. 2017).

Previous studies proved the increased incidence of depressive symptoms in patients with chronic respiratory diseases like pulmonary fibrosis. In addition, these studies have revealed that psychological factors affect the health-related quality of life in patients with pulmonary fibrosis (Matsuda et al. 2017). Therefore, it is suggested that the use of antidepressants can help in treatment of patients with pulmonary fibrosis.

A previous study has reported that tricyclic antidepressants (TCAs) like amitriptyline has a full range of actions that is not attributable solely to their antidepressant actions. They were reported to have antifibrotic effects and could protect against liver fibrosis through variable mechanisms (Chen et al. 2017). Amitriptyline has potent anti-inflammatory effects via alteration of cytokine activity, inhibiting the release of proinflamatory cytokines as interleukin-1, nuclear factor–kappa-β, and tumor necrosis factor-α, besides increasing the release of immunosuppressive and anti-inflammatory interleukin-10 (Qiu et al. 2017). In addition, amitriptyline exhibits antioxidant properties through inhibition of natural killer cell activity as well as nitric oxide production (Achar et al. 2009). Moreover, TCAs can inhibit fibrosis via increasing the accumulation of ceramide which is considered a promising therapeutic strategy to reverse liver fibrosis (Chen et al. 2017). Therefore, the current study aimed to investigate the modulatory effects of amitriptyline against bleomycin-induced pulmonary fibrosis in rats and studying the involved mechanisms of its effects.

Materials and methods

Animals

Male Wistar albino rats (150–200 g) were used in the present study. They were purchased from the Egyptian Company for Production of Vaccines, Sera and Drugs (EGYVAC; Cairo, Egypt), and allowed free access to water and standard pellet chow. Rats were kept under constant conditions (temperature 25 ± 3 °C, and humidity 50%) with 12/12 h light/dark cycles and were housed in plastic cages in the animal house at October University for Modern Science and Arts (MSA University).

Drugs and chemicals

Amitriptyline was obtained from Future Pharmaceutical Industries (Egypt), whereas bleomycin hydrochloride was obtained from Nippon Kayaku (Tokyo, Japan). All other chemicals used were of analytical grade.

Induction of pulmonary fibrosis

A single dose of bleomycin HCl (5 mg/kg) dissolved in 0.9% NaCl solution was instilled into the trachea to induce pulmonary fibrosis (Zaafan et al. 2016). Ketamine (80 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.) were used for anesthesia (Verma et al. 2013).

Experimental design

Rats were randomly allocated into 4 groups ($n = 6$). The first group of rats received a single intratracheal dose of sterile saline and served as normal control group. A bleomycin control group received bleomycin HCl (5 mg/kg; i.t.) for induction of pulmonary fibrosis. Another group was treated with amitriptyline (10 mg/kg; p.o.) for 21 days after bleomycin injection. Finally, the last group was treated with amitriptyline after only saline intratracheal instillation.

At the end of the experiment, rats were sacrificed by cervical dislocation under ketamine anesthesia, and then both lungs were rapidly dissected out and washed with ice-cold saline. In these animals, the right lungs were used for biochemical assessment of hydroxyproline, TGF-β1, TNF-α, NF-kβ, and serpine-1 as well as Nrf2, lipid peroxides, and reduced glutathione (GSH). The left lungs were used for histopathological examination and immunohistochemical detection of inducible nitric oxide synthase (iNOS), α-SMA, and p53.

Biochemical investigations

Hydroxyproline content was measured as an indication of collagen deposition using rat-specific immunoassay kit according to the method described by Yao et al. (2011). Pulmonary TNF-α and TGF-β1 were determined by ELISA technique using standard kits (MyBioSource, Inc., USA).

Lipid peroxidation in lung tissues was estimated by the determination of thiobarbituric acid reactive substance content that was evaluated as malondialdehyde (MDA) in lung

homogenate using a standard kit purchased from Biodiagnostic (Egypt). Pulmonary GSH content was determined using a commercial kit based on Ellman's reaction (Biodiagnostic, Egypt). Pulmonary serpine-1 and NF-κβ contents were determined by ELISA technique using standard kits (Elabscience Biotechnology, Inc. and Cloud Clone Corp., respectively). Cayman's nuclear extraction kit was used for preparation of the nuclear extract for determination of nuclear fraction of Nrf2 using standard kit (MyBioSource, Inc.).

Histopathologic assessment of lung tissue damage

The lung tissues of rats in different groups were fixed in 10% formol saline for 24 h. Lung tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin and eosin stain for routine examination as well as Masson stain for detection of the fibrous tissue and collagen through the light electric microscope according to the method previously described (Banchroft et al. 1996).

Immunohistochemical expression of iNOS, α-SMA, and p53

Lung tissue sections of 3-μm thickness embedded in paraffin were used for iNOS, α-SMA, and p53 expression detection through the immunostaining with primary antibody polyclonal immunoglobulin-G of rat iNOS, α-SMA, and p53 according the method previously described by Liu et al. (2018). Finally, grading of degree of positive immunohistochemical reactions from 1 to 5 was performed.

Statistical analysis

Data in the current study were presented as mean ± SEM. Comparisons between means of different groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test (Tukey 1951). The level of significance was taken as $p < 0.05$. GraphPad Prism software package, version 5 (GraphPad Software, Inc., USA), was used to carry out all statistical tests.

Results

Bleomycin intratracheal instillation produced marked pulmonary fibrosis that was detected through the significant increase in pulmonary contents of hydroxyproline and TGF-β1 as compared to the normal control rats (Fig. 4). In addition, bleomycin produced many histological alterations as the desquamation of the bronchiolar lining epithelium with marked inflammatory cell infiltration and fibrosis in collapsed air alveoli (Fig. 1). The Masson trichrome staining showed obvious increase in collagen deposition and peribronchiolar fibrosis in lung tissue as demonstrated by the blue color (Fig. 2, Table 1) in the bleomycin control group.

Amitriptyline treatment produced marked antifibrotic effects that were revealed through significant suppression of the elevated hydroxyproline and TGF-β1 contents (Fig. 4). Moreover, amitriptyline treatment produced almost normal histological structure with marked decrease in inflammatory cell infiltration and fibrosis (Fig. 1) and obvious suppression of collagen deposition and fibroblastic cell proliferation in peribronchiolar tissue as compared to the bleomycin control group (Fig. 2, Table 1).

A significant increase in the pro-inflammatory and inflammatory mediators was detected in the bleomycin control rats like NF-κβ, TNF-α, and iNOS, while amitriptyline successfully suppressed these mediators in the amitriptyline-treated rats (Figs. 3, 4). Moreover, amitriptyline treatment decreased significantly the oxidative stress produced upon bleomycin intratracheal instillation that was reflected through the increased pulmonary contents of Nrf2 and GSH, concurrently with marked suppression of the lipid peroxide contents in lung tissue as compared to the bleomycin control group (Fig. 5).

Moreover, immunohistochemical staining of lung sections indicated a decreased amount of α-SMA and p53 expression in the lung tissue of rats in the amitriptyline-treated group compared with the bleomycin control group (Fig. 3).

On the other hand, pulmonary contents of serpine-1 showed no significant change from the normal rats in the bleomycin control group and in the amitriptyline-treated groups (Fig. 5).

Discussion

Despite the significant advances in understanding pulmonary fibrogenesis, it remains a severely debilitating disease with a high mortality rate (Liu et al. 2017). A previous study showed the beneficial effects of tricyclic antidepressants (TCAs) in liver fibrosis through promotion of ceramide accumulation and regulation of collagen production in human hepatic stellate cells (Chen et al. 2017). In addition, another study has suggested that depression is a significant determinant of the health-related quality of life or health status in patients with pulmonary fibrosis and reported that screening and management of depression should be considered clinically important in pulmonary fibrosis patients, even if their pulmonary function impairment is mild to moderate (Matsuda et al. 2017). Therefore, the current study aimed to investigate the beneficial effects of amitriptyline as a tricyclic antidepressant on pulmonary fibrosis.

Results of the current study showed that bleomycin produced marked pulmonary fibrosis demonstrated through the histological examination that detected the obvious collagen deposition and peribronchiolar fibrosis in lung tissue as well

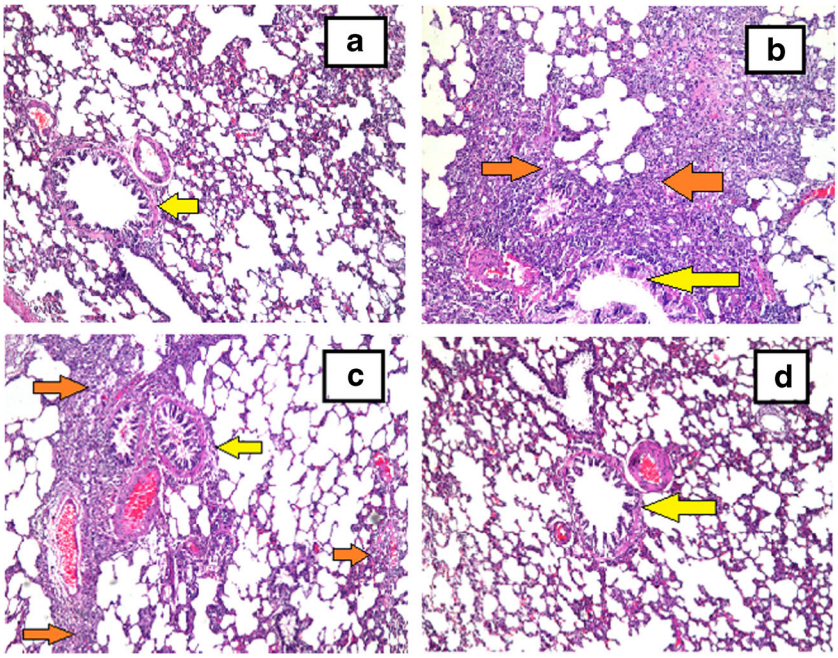

Fig. 1 Effect of amitriptyline treatment on the histological structure of the lung tissue in rats with bleomycin induced pulmonary fibrosis. Hematoxylin and eosin staining (H&E × 16). **A**, **D** The normal histological structure of lung tissue of a rat in normal control group in which the spongy structure of the lung appeared with thin inter-alveolar septa and normal clear alveoli. Bleomycin administration produced desquamation of bronchiolar lining epithelium with fibrosis and inflammatory cell infiltration in collapsed air alveoli. **B** On the other hand, treatment with amitriptyline produced almost normal histological structure with marked decrease in inflammatory cell infiltration and focal fibrosis. **C** The yellow arrows refer to the lining epithelium of the bronchioles, while the red arrows refer to the fibrosis and the collapsed air alveoli

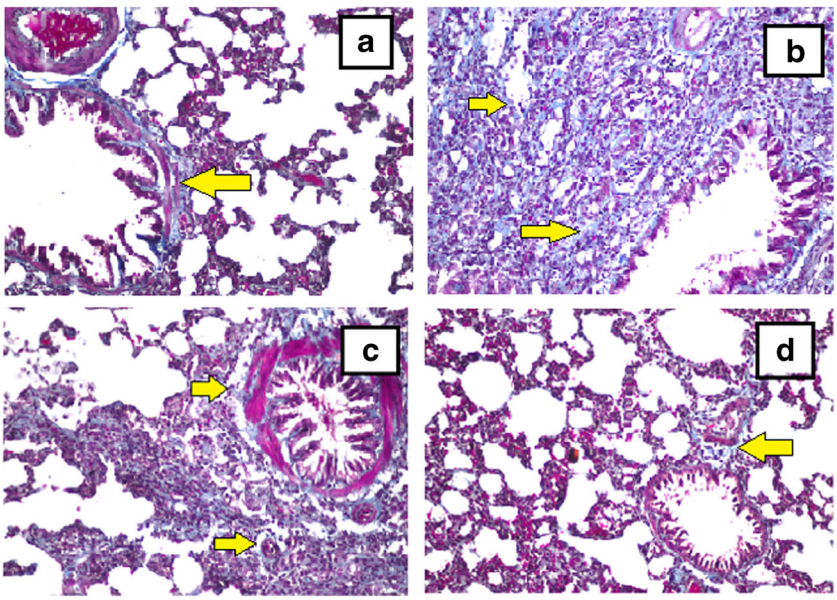

Fig. 2 Effect of amitriptyline treatment on the histological structure of the lung tissue in rats with bleomycin-induced pulmonary fibrosis. Masson trichrome staining (× 16). Lung tissue from normal rats and rats treated with amitriptyline alone showed normal histological structure with typical open alveoli, interalveolar spaces, and bronchioles with lack of inflammatory cell infiltration and fibrosis. **A**, **D** Bleomycin injection produced obvious high increase in collagen deposition in lung tissue and peribronchiolar fibrosis as demonstrated by the blue color. **B** Meanwhile, lungs of the rats treated with amitriptyline after bleomycin injection showed marked decrease in collagen and fibroblastic cell proliferation in peribronchiolar tissue as compared with bleomycin control group. **C** The yellow arrows refer to the collagen deposition presented in blue stain. The normal rats showed minor blue color around the bronchioles, while the bleomycin control rats showed obvious diffused blue color throughout all the lung tissue. The amitriptyline-treated rats showed minor focal areas of the blue stain

Table 1 Effect of amitriptyline on grades of bleomycin-induced pulmonary fibrosis, collagen deposition, and inflammatory cell infiltration in histological examination of lung tissues

Groups Histological alterations	Normal control	Bleomycin control	Amitriptyline+bleomycin	Amitriptyline
Inflammatory cell infiltration (H&E)	–	+++	+	–
Focal fibrosis (H&E)	–	+++	+	–
Collagen deposition (Masson Trichrome)	–	+++	+	–

as the significant increase in pulmonary contents of hydroxyproline and TGF-β1 as compared to the normal control rats. These results are in harmony with those of previous studies (Tian et al. 2018; Liu et al. 2018). Amitriptyline treatment successfully produced potent antifibrotic effect that was demonstrated in the present study via the obvious suppression of collagen deposition and fibroblastic cell proliferation in peribronchiolar tissue upon the histological examination in addition to the significant suppression of the elevated pulmonary contents of hydroxyproline and TGF-β1. Previous

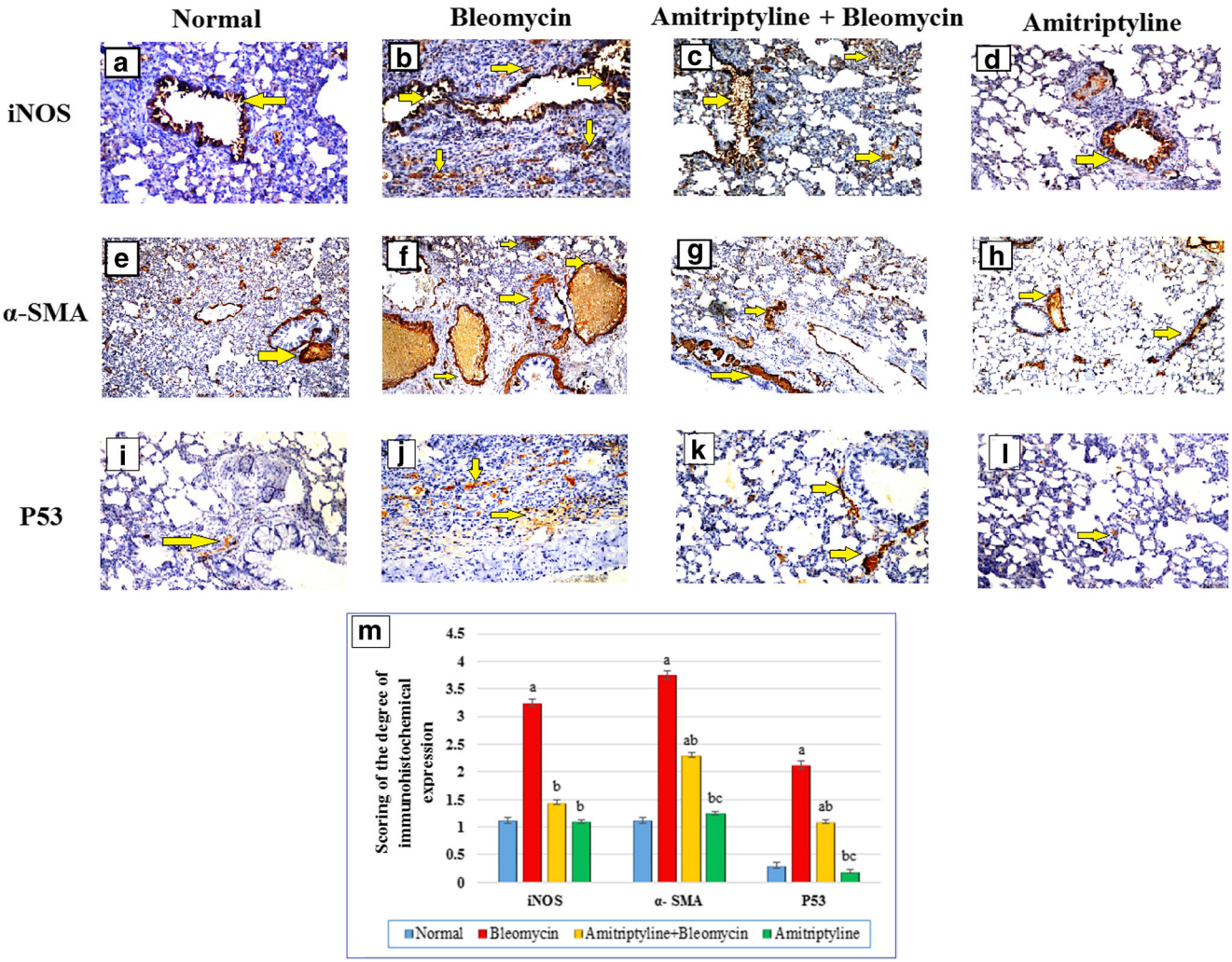

Fig. 3 Effect of amitriptyline on the immunohistochemical expression of iNOS, α-SMA, and p53 in lung tissue. Lung sections from normal control rats (A, E, and I) and rats treated with only amitriptyline (D, H, and L) showed a small degree of immunostaining for iNOS and α-SMA in the peribronchiolar tissues and alveoli with minor degree of immunostaining for p53. On the other hand, intratracheal instillation of bleomycin produced marked increase in the immunohistochemical expression of iNOS, α-SMA, and p53 in lung tissues (B, F, and J). While, sections from rats with bleomycin-induced fibrosis and treated with amitriptyline showed moderate immunohistochemical expression of iNOS and α-SMA as well as a small degree of immunostaining for p53 (C, G, and K). The yellow arrows refer to the brown color representing the positive immunostaining. Comparative quantification of the immunohistochemical expression for iNOS, α-SMA, and p53 in lung tissues of rats from all groups is illustrated in M

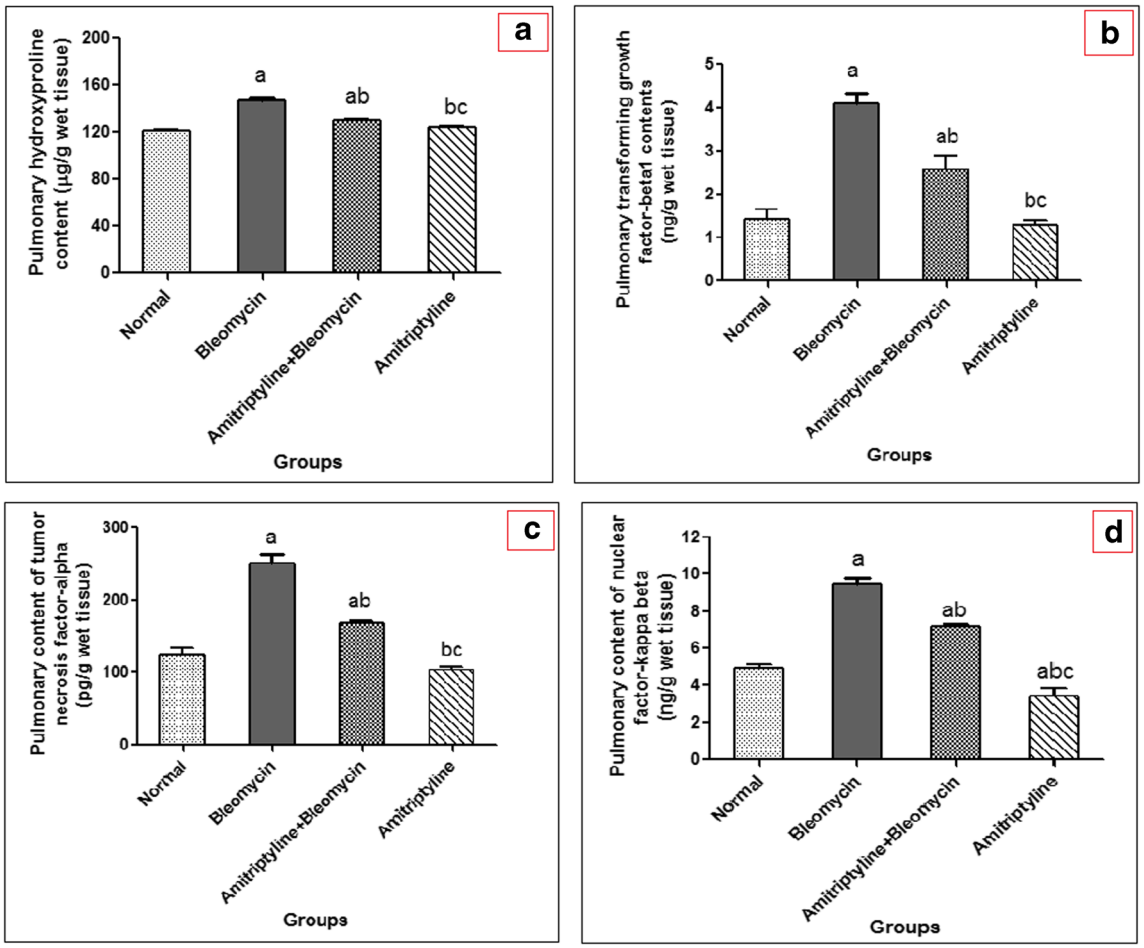

Fig. 4 Effect of amitriptyline treatment on the pulmonary contents of hydroxyproline, transforming growth factor-beta1 (TGF-β1), tumor necrosis factor-alpha (TNF-α), and nuclear factor-κ beta (NF-κβ). Each value represents mean ± SEM ($n = 6$). a, significantly different from normal control group. b, significantly different from bleomycin control group. c, significantly different from amitriptyline+bleomycin group; at $p < 0.05$

studies reported the possible antifibrotic effects of amitriptyline against hepatic and renal fibrosis (Chen et al. 2017; Achar et al. 2009).

Different mechanisms are suggested to be involved in the pathogenesis of pulmonary fibrosis. The inflammatory response following injury is crucial to the process of pulmonary fibrosis. TNF-α has been proved to be associated with the pathogenesis of pulmonary fibrosis (Luzina et al. 2015). Results of the present study have shown that pulmonary TNF-α content was decreased significantly in amitriptyline-treated rats as compared to the bleomycin control rats. Sufficient evidence demonstrated that bleomycin triggers NF-κβ signaling stimulation, which in turn induces inflammation and pulmonary fibrogenesis (Li et al. 2017b). The results of the present study provide evidence that amitriptyline interferes with NF-κβ signaling. These results suggest that the anti-inflammatory effect of amitriptyline can aid in alleviating fibrosis and contribute to its protective effect against pulmonary fibrosis.

The increased oxidative stress and stimulation of reactive oxygen species (ROS) production are also demonstrated to be involved in the pathogenesis of pulmonary fibrosis (Liu et al. 2018; Cheresh et al. 2013). Results of the current study showed that bleomycin produced remarkable elevation of MDA with a parallel decline in GSH in lung tissue. On the other hand, amitriptyline treatment was successfully able to suppress the elevated MDA and increase GSH in lung tissue when compared to the bleomycin control rats.

Establishment of Nrf2-mediated antioxidative protection signifies a new therapeutic strategy to stop the progression in fibrotic injury. Accumulation of Nrf2 in the nucleus stimulates the expression of antioxidation defense gene HO-1 by leveraging its association with certain antioxidant response factor (Kikuchi et al. 2010). Several studies underscore the importance of Nrf2 in regulating pulmonary fibrosis. Nrf2 knockout mice were reported to be more sensitive to bleomycin and paraquat-induced pulmonary fibrosis as compared to wild-type animals (He et al. 2012). Decreased Nrf2 expression was associated with increased α-SMA and type 1 collagen expression, while Nrf2 activation increased antioxidant defenses and myofibroblastic dedifferentiation in IPF fibroblasts (Artaud-Macari et al. 2013).

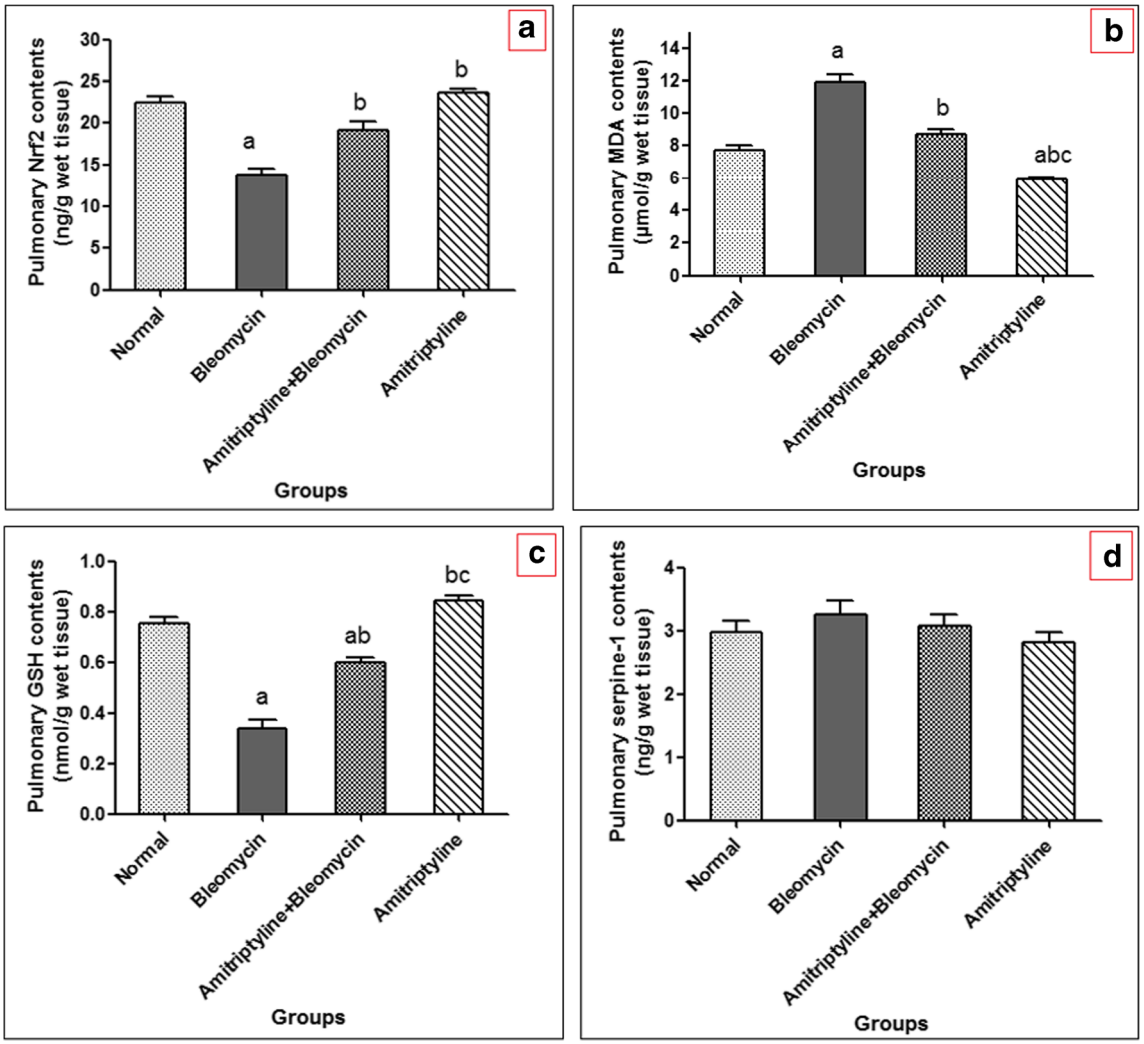

Fig. 5 Effect of amitriptyline treatment on pulmonary contents of nuclear factor-erythroid 2-related factor 2 (Nrf2), malondialdehyde (MDA), reduced glutathione (GSH), and serpine-1. Each value represents mean ± SEM ($n = 6$). a, significantly different from normal control group. b, significantly different from bleomycin control group. c, significantly different from amitriptyline+bleomycin group; at $p < 0.05$

The present study revealed that amitriptyline boosts Nrf2 accumulation in nuclei during fibrotic lung injury. These results suggest that amitriptyline can protect against pulmonary fibrosis through the suppression of the oxidative stress injury.

Reactive nitrogen species and in particular NO have been demonstrated to play a major role in the pathogenesis of pulmonary fibrosis. Bleomycin generated RNS in lung tissue resulting in DNA injury, lipid peroxidation, and an increase in collagen synthesis and deposition (Guo et al. 2016). Results of the present study showed a significant increase in iNOS expression in lung tissue in bleomycin control rats. These results are in harmony with those of a previous study (Zaafan et al. 2016). In addition, the current study demonstrated that amitriptyline treatment was able to decrease iNOS expression significantly in lung tissue as compared to bleomycin control group.

Epithelial shifts triggered by bleomycin in rat lung tissue were assessed by the expression of α-SMA in lungs. Bleomycin produced a significant increased expression in α-SMA. This shift was attenuated by amitriptyline, which suggests that it can inhibit EMT-like changes in injured lungs.

Results of the present study also revealed increased expression of p53 in the bleomycin control rats. These results are in agreement with those of a previous study that reported that increased expression of p53 can be considered as one of the players in pulmonary fibrosis pathogenesis through the induction of cellular senescence (Marudamuthu et al. 2015). On the other hand, rats treated with amitriptyline showed marked decrease in p53 expression that demonstrates that the antifibrotic potential of amitriptyline can be attributed also to its ability to suppress p53 expression.

Serpine-1 or plasminogen activator inhibitor was reported to further mediate the incidence of pulmonary fibrosis (Marudamuthu et al. 2015). A previous study showed that elevation of serpine-1 contributes importantly to alveolar type-2 cell senescence in fibrotic lung diseases (Jiang et al.

2017). On the contrary, the current study showed non-significant change in pulmonary serpine-1 upon bleomycin treatment. While this previous study (Jiang et al. 2017) reported that the increase in serpine-1 is the inducer to p53 expression, the current study showed marked increase in p53 expression without any significant change in serpine-1 expression.

In conclusion, amitriptyline can be a promising treatment in pulmonary fibrosis. Amitriptyline not only prevents the depression and its drawbacks in pulmonary fibrosis patients but also it can suppress fibrosis through variable mechanisms mainly the inhibition of NF-κβ/TNF-α pathway in addition to the inhibition of iNOS and Nrf2 expression.

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Author contribution MA and AR conceived and designed the research. MA and AM conducted experiments. AM and AR analyzed data. MA wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

The study was done with compliance to the ethics standards and approval from the ethics committee of the October University for Modern Sciences and Arts, Egypt.

Conflict of interest The authors declare that they have no conflict of interest.

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