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Original Article

N-Acetylcysteine attenuated pulmonary fibrosis induced by bleomycin *via* immunomodulation responses

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Abstract

Background and purpose: Pulmonary fibrosis (PF) is a chronic and life-threatening interstitial lung disease. N-acetyl cysteine (NAC) is an antioxidant pharmaceutically available to reduce endothelial dysfunction, inflammation, and fibrosis, however, the therapeutic effect of NAC on PF has not been clearly identified. This research aimed to investigate the possible therapeutic impact of NAC on PF induced by bleomycin in the rat model.
Experimental approach: Rats received intraperitoneal injections of NAC at 150, 300, and 600 mg/kg for 28 days before bleomycin, while the positive and negative control groups were treated with bleomycin alone and normal saline, respectively. Then, rats' lung tissues were isolated and leukocyte infiltration and also collagen deposition were evaluated using hematoxylin and eosin and Mallory trichrome stainings, respectively. In addition, the levels of IL-17, and TGF-β cytokines in bronchoalveolar lavage fluid and hydroxyproline in homogenized lung tissues were assayed using the ELISA method.
Findings/Results: Histological findings indicated that NAC decreased leukocyte infiltration, collagen deposition, and fibrosis score in the bleomycin-induced PF tissue. Moreover, NAC significantly reduced TGF-β and hydroxyproline levels at 300-600 mg/kg, as well as IL-17 cytokine at 600 mg/kg.
Conclusion and implications: NAC showed a potential anti-fibrotic effect by reducing hydroxyproline and TGF-β as well as an anti-inflammatory effect by decreasing IL-17 cytokine. So, it may be administered as a prophylactic or therapeutic candidate agent to attenuate PF *via* immunomodulatory effects. Although, future studies are suggested.

Keywords: Bleomycin; Collagen; Cytokines; N-acetylcysteine; Pulmonary fibrosis.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a progressive, irreversible, and life-threatening illness known as a disease, that occurs due to parenchymal lung injury because of inflammatory agents and fibrosis (1-3). It is linked to high mortality and has found resistance against therapies. PF cases have a survival rate of 2-3 years (3). IPF is commonly seen in adults aged between 50 and 70 years, especially in cases with a history of cigarette smoking, with men more vulnerable compared to women (1,4). PF is directly or indirectly affected by different parameters, like reactive oxygen species (ROS), inflammatory immune cells, growth factors, and cytokines (5,6). In addition, herpes viruses and bacteria, and chemical compounds such as asbestos, silica, bleomycin, and methotrexate are other factors related to the damage in this disease (7).

Bleomycin as a strong antibiotic has a therapeutic impact on testicular, head, and neck cancers, soft tissue sarcomas, and lymphogranuloma (8).

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Bleomycin damages alveolar lung cells by an increase in accumulating inflammatory cells and collagen tissues in airbags, as well as stimulating fibroblast proliferation *via* immunopathological reactions (9). However, bleomycin is associated with producing transforming growth factor beta (TGF-β) protein in fibroblast proliferation and PF development (10). At the present, N-acetyl cysteine (NAC) as an antioxidant, as well as vitamin E and bilirubin are given to remove free radicals and prevent inflammatory processes in PF (11-16).

NAC is an antioxidant and a precursor to glutathione, which acts as a direct ROS scavenger in cells. It can regulate cell apoptosis, angiogenesis, growth, arrest, redox reaction, and inflammatory responses (17).

Among the compounds in NAC, thiol groups may be compatible with the proposed prophylactic mechanisms and inhibitors of PF induced by bleomycin, including the antioxidant-oxidative imbalance occurring in the pulmonary tissue of IPF cases. NAC effectively increases the non-protein thiol levels in the homogenized lung fluid which is capable to scavenge ROS and inhibit lipid peroxidation (17).

Therefore, the present study investigated the immunomodulatory impact of NAC in PF induced by bleomycin through the assessment of cytokines, hydroxyproline, and leukocyte infiltration in the lung tissue.

MATERIALS AND METHODS

Animal treatment protocol

Male Wistar rats (weighing approximately 200 g) were prepared and maintained from the laboratory of the animal production center, Babol University of Medical Sciences. The rats were divided into five groups (n = 5). NAC was given intraperitoneally as a pretreatment at 150, 300, and 600 mg/kg for 28 consecutive days. After the last administration of NAC doses, the rats were exposed to intratracheal instillation of bleomycin 5 IU/Kg. The animal study standard protocol was performed regarding Ethical no. IR.MUBABOL.HRI.REC.1395.10 given from Babol University of Medical Sciences.

Chemicals, reagents, and drugs

NAC from Akorn Company (USA), bleomycin from BLEO-CELL, STADAPHARM GmBH (exCell Pharm, Germany), hematoxylin and eosin (H&E), Mallory trichrome stain and phosphate-buffered saline (PBS) from Armin Teb (Iran), TGF β and interleukin (IL)-17A ELISA kits from Invitrogen (USA) as well as hydroxyproline ELISA kit from MyBioSource (USA) were obtained.

Collection of bronchoalveolar lavage fluid

Intratracheal bleomycin injection was used in anesthetized rats. After exposure, an oblique incision was done in the trachea and a scalp vein set was placed in it. Cold PBS (0.9%, PH = 7.4) was injected into the lungs and bronchoalveolar fluid following lavage was drawn with a syringe. The samples were centrifuged and the supernatant was kept at -70 °C for cytokines measurement.

Measurement of hydroxyproline

Lung collagen was assessed through the estimation of the hydroxyproline level in the lung tissue samples using rat-specific hydroxyproline ELISA ki. In brief, the lung tissues were isolated and minced into small pieces. The tissues (10 mg) were homogenized in 100 µL cold PBS and then the supernatants were collected after centrifuging at 1500 *g* for 15 min. Fifty µL of supernatant was transferred into a 96-well plate, then 100 µL of horseradish peroxidase-conjugate reagent was added and incubated for 60 min at 37 °C. The plate was washed four times and then 50 µL of chromogen solution A and 50 µL of chromogen solution B were added to each well. The plate was kept for 15 min at 37 °C. Finally, after adding 50 µL stop solution to the wells and the color changed from blue to yellow. Absorbance was determined at 450 nm. Results were presented as µg of hydroxyproline per mL.

Histological assessment

To examine histopathological changes, the right lungs of the five groups were collected after bleomycin treatment followed by instilling with neutral buffered formalin (10%) and immersing in the fixator through 16-18 h at

room temperature, embedding in paraffin, and sectioning at 5 μm thicknesses. Following the removal of paraffin and rehydration, H&E staining of the lungs was done for evaluating lung leukocyte infiltration and Mallory trichrome stain for identifying the areas and amount of collagen deposition. Semi-quantitative grading system, called the Ashcroft score was applied to measure the amount and intensity of PF (18). Ten areas were assessed for each lung section for assigning grades to the PF as follows; normal lung: grade 0; minimal fibrous thickening: grade 1; moderate thickening: grades 2 or 3; increased fibrosis with defined damage affecting lung architecture: grades 4 or 5; serious structure deformity: grades 6 or 7, and total fibrous obliteration: grade 8. The average score obtained from all fields was considered the fibrosis score of the studied lung section.

Cytokine measurements

The cytokines level of TGF-β using TGF β rat ELISA kit and IL-17A using rat IL-17A ELISA kit in bronchoalveolar lavage fluid (BALF) were determined. The samples were centrifuged and supernatants were examined using ELISA kits considering the producer's guidelines. Briefly, regarding the ELISA kit protocol, the samples were diluted and added to the wells. Then, the conjugated antibody was added. In the end, the stopping solution was added and the absorbance of each well was read with an ELISA reader at a wavelength of 450 nm.

Statistics analysis

The results are presented as means ± SD. One-way ANOVA was used for data analysis. The differences were considered statistically significant at $P < 0.05$.

RESULTS

NAC reduced bleomycin-induced histo-pathological and fibrotic changes in the lung

The results showed lung tissue has a normal architecture, no bleeding sites, and symptoms of edema and inflammation around the vessels and bronchioles in the control group compared to the bleomycin-treated group, and also fibrosis was not observed (Fig. 1 A and B). Conversely, the lung sections of rats exposed to bleomycin (positive control), showed a marked increase of inflammatory cells and amount of collagenous extracellular matrix and large fibrous areas in comparison to the negative control (Fig. 1 C and D).

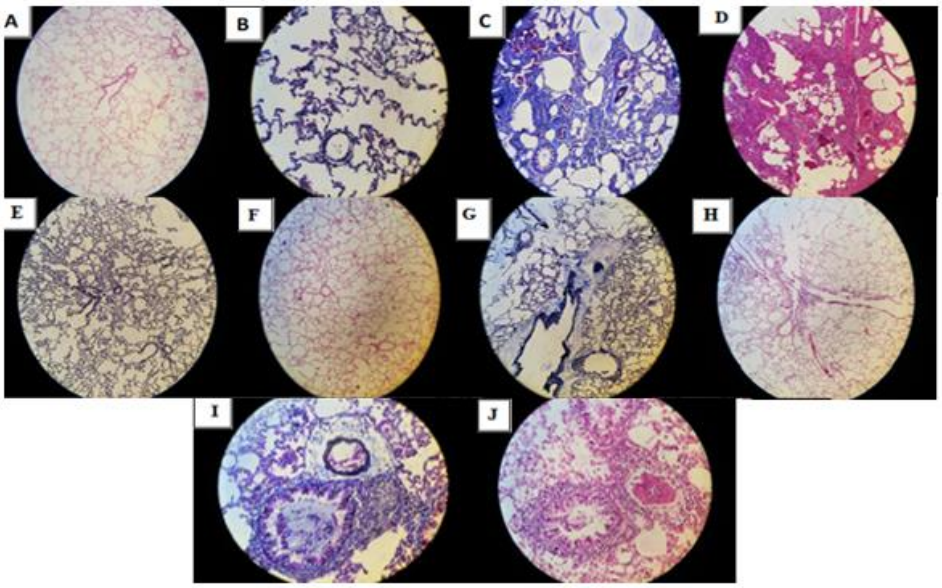

Fig. 1. Representative photomicrographs of lung histology. The animals were treated with (A and B) saline, (C and D) bleomycin, (E and F) bleomycin + NAC 600 mg/kg, (G and H) bleomycin + NAC 300 mg/kg, and (I and J) bleomycin + NAC 150 mg/kg after bleomycin or saline instillation. Sections were stained with (A, C, E, G, I) Mallory's trichrome and (B, D, F, H, J) hematoxylin and eosin (H&E). The semi-quantitative score of lung extracellular matrix content was calculated; n = 5. NAC, N-acetyl cysteine.

The investigation of the lung sections in NAC-treated rats (600 mg/kg) after bleomycin treatment didn't show bleeding sites and signs of congested edema compared to the bleomycin-treated group. NAC (600 mg/kg) attenuated the morphological changes induced by bleomycin and significantly decreased the extent of lung fibrosis areas (Fig. 1 E and F). The dose of 300 mg/kg NAC decrease the number of inflammatory cells, the level of alveolar wall thickness, and the fibrosis areas compared to the positive control and NAC 600 mg/kg groups, (Fig 1. G and H). Nevertheless, the group treated with NAC at 150 mg/kg showed moderate to severe tissue changes with a significant increase in the extent of fibrosis areas compared to the other groups (Fig.1 I and J).

NAC attenuated inflammatory and fibrogenic cytokines

Our findings indicated that NAC at both 300 and 600 mg/kg and only at 600 mg/kg significantly decreased the amount of TGF-β and IL-17A, respectively, compared to the positive control group. These cytokines were raised in the bleomycin group unlike the NAC-treated groups (Fig. 2A and B). Moreover, results showed that these cytokines do have not a significant reduction after treatment with 150 mg/kg of NAC. Our findings showed that NAC could modulate the immune response by decreasing inflammatory and fibrogenic cytokines in PF induced by bleomycin.

NAC attenuated the amount of hydroxyproline

In this study, the level of lung tissue hydroxyproline as an indicator for measuring collagen levels in connective tissues was evaluated using a hydroxyproline kit assay. According to the results, a significant change was observed in the NAC-treated groups compared to the positive group.

Our findings indicated that the BALF levels of hydroxyproline (collagen content) at 600 and 300 mg/kg of NAC significantly decreased in the PF induced by bleomycin (Fig. 3).

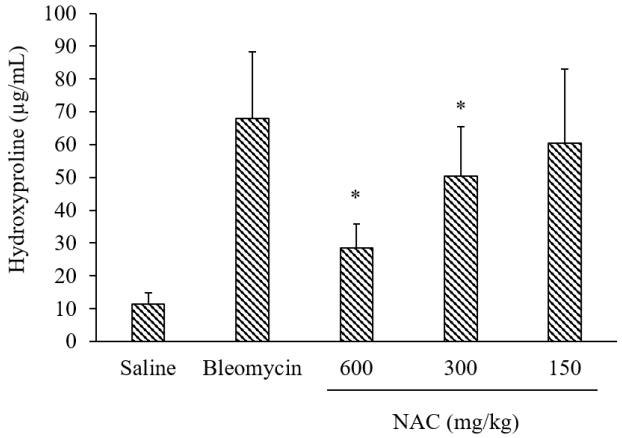

Fig. 3. Lung collagen content measured by hydroxyproline assay after bleomycin + NAC treatment, Data represents mean ± SD; n = 5. *P < 0.05 Indicates significant differences compared to the positive control group (bleomycin-treated group). NAC, N-acetyl cysteine.

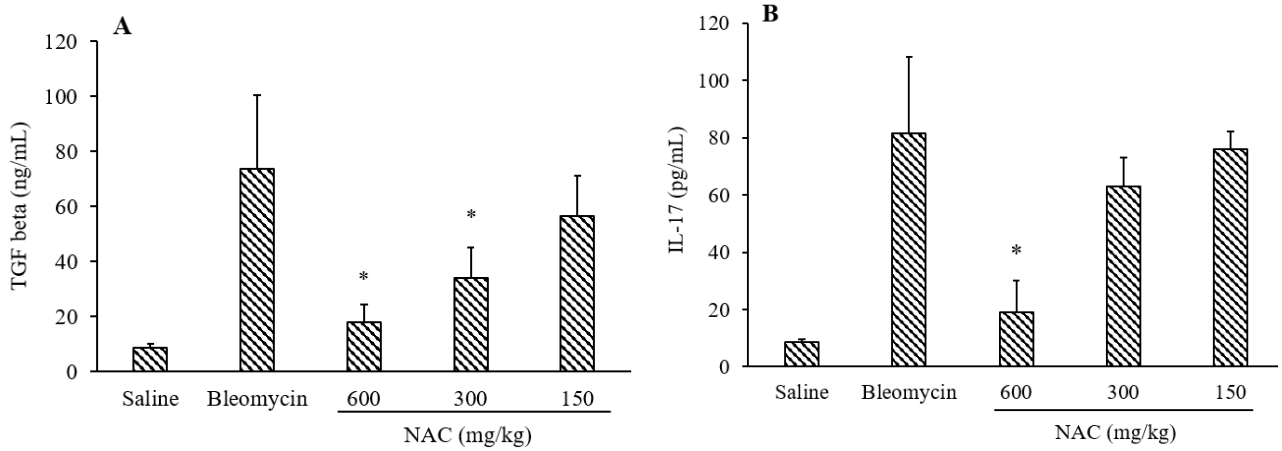

Fig. 2. Effect of NAC on inflammatory and fibrotic cytokines. (A) TGF-β (B) IL17-A levels in bronchoalveolar lavage fluid harvested after bleomycin + NAC treatment. Data represent mean ± SD; n = 5. *P < 0.05 Indicates significant differences compared to the positive control group (bleomycin-treated group). NAC, N-acetyl cysteine; TGF, transforming growth factor beta; IL, interleukin.

DISCUSSION

The bleomycin-induced fibrosis is generally applied for investigating the mechanisms of lung fibrosis. Finding chemical drugs and herbal remedies with immunomodulatory and anti-tumor effects to control inflammatory diseases can be useful for controlling inflammatory disease (19-21).

In this study, we showed that NAC administration reduced inflammation and suppressed lung fibrosis. We found that the group treated with bleomycin indicated the highest levels of IL-17, as an inflammatory cytokine, TGF-β, as a fibrogenic cytokine, lung tissue hydroxyproline, as a collagen index as well as lung histopathological changes compared to the healthy group (negative control).

Previous studies indicated that NAC is an antioxidant and a free radical scavenger as well as a glutathione precursor in the cellular environment. In addition, thiol compounds in NAC have preventive and inhibitory effects on the PF caused by bleomycin (22,23).

On the other hand, NAC is able to increase the number of thiols in the lung fluid that collect ROS, chelate iron ions, and inhibit pro-oxidative lipids (22,24). Also, antioxidants such as NAC in the intracellular fluids reduce BALF inflammatory and fibrogenic cytokines concentration (25-27). Our findings showed that the TGF-β and IL-17A decreased after NAC treatment in the PF induced by bleomycin. Moreover, according to a similar previous study, NAC reduced TGF-β cytokine (26).

Other studies showed that NAC reduces the inflammatory response by inhibiting of arachidonic acid and eicosanoid production (27-29). Several studies have demonstrated a direct correlation between the progression of PF and IL-17 as well as TGF-β cytokines (30-35).

In this study, we think that decreasing IL-17 as inflammatory and TGF-β as a fibrogenic cytokine may be associated with antioxidant properties of NAC by increasing the glutathione level and other effects on the inflammatory and fibrogenic nuclear factors such as NF-κB, signal transducer and activator of transcription 3 (STAT3), and Smad3, however further studies are needed for determining whether an increase or a reduction in cytokine synthesis is associated with the direct effect of NAC.

The previous study showed that IL-17A was associated with the progression of PF in TGF-β-dependent conditions (31).

IL-17A is a cytokine secreted from TH17 cells playing an important role in chronic inflammation. An earlier study reported that IL-17A enhances collagen synthesis as well as the transfer of mesenchymal-epithelial cells into alveolar epithelial cells through TGF-β-dependent signaling pathways in fibrous pulmonary tissues (31). Also, it has been shown that the neutralization of IL-17A with monoclonal antibody reduced acute bleomycin-induced inflammation and pulmonary fibrosis.

In this study, our results showed that groups of rats with bleomycin-associated lung fibrosis which received NAC at 600 mg/kg exhibited a significant decrease in IL-17A inflammatory cytokine levels in the lung fluid (BALF). Therefore, our findings suggest that NAC by reducing the IL-17 cytokine, may be able to decrease the transfer of mesenchymal-epithelial cells into alveolar epithelial cells and reduce the differentiation and proliferation of fibroblasts into myoblast and ultimately reduce collagen synthesis and aggregation in a TGF-β-dependent manner, however, more studies are needed. NAC is likely to modulate immune responses to TH1 cellular immunity, which is associated with a decrease in the acute effects of bleomycin-induced PF. However, the effect of NAC on the activity of Th1 deserves further study.

In addition, another study indicated that neutrophilia, IL-1β, and IL-17A production in the bleomycin-induced PF was dependent on TGF-β. Their findings showed the cooperation of IL17A and TGF-β in the development of PF (33).

In line with previous investigations, both IL-17A and TGF-β levels were increased in the fibrosis-positive control model, while both factors were significantly decreased after pretreatment with NAC. Our findings indicated that both of these cytokines were associated

with the development of PF and NAC was able to reduce both fibrosis-inducing agents including IL-17A as inflammatory and TGF-β as fibrogenic cytokines that work together to cause injury. Therefore, the identification of molecules and other nuclear factors involved in this mechanism is suggested.

Additionally, Murray *et al*. reported that serum amyloid P is an inhibitor of bleomycin-induced PF through the inhibition of pulmonary fibrocytes and profibrotic macrophage (M2) differentiation. Also, they showed that serum amyloid P inhibits TGF-β-induced pathological effects including apoptosis, airway inflammation, and pulmonary collagen accumulation (35).

Our findings indicated that groups of rats that received NAC at 300 and 600 mg/kg showed significantly decreased TGF-β cytokine levels compared to the positive control group. Possibly the NAC could inhibit the activity of pulmonary M2 macrophages which are the major producers of TGF-β fibrogenic cytokine as activator and proliferator of fibroblasts. However, this hypothesis should be examined in a future study.

Previous studies demonstrated that TGF-β has an important role in controlling lung immunopathological injuries caused by neutrophil and M2 macrophage over-activation (32,34). Further studies are required to determine the NAC effect on neutrophil and M2 macrophage activity in lung fibrosis.

In addition, Liu *et al*. indicated that antiflammin-1 could prevent bleomycin-related inflammation and fibrosis in the lungs by reducing tumor necrosis factor (TNF)-α and IL-1β as well as decreasing inflammatory cells in BALF and reduction of hydroxyproline content in lung homogenates. Furthermore, they revealed that antiflammin-1 significantly prevented the TGF-β-related NIH3T3 cells proliferation (32). Our results showed that the use of NAC reduced the accumulation of inflammatory cells in lung tissue samples in histological studies. However, the effect of this antioxidant on the production of pro-inflammatory cytokines and nuclear factors, like TNF α, IL-1β, IL-6, NF-κB, STAT3, and Smad3 is suggested in the bleomycin-based fibrosis model.

On the other hand, the measurement of hydroxyproline as a collagen accumulation index showed that the higher NAC concentration caused a further decrease in hydroxyproline that indicates the efficacy of NAC in a dose-dependent manner in the inhibition of extracellular matrix-producing myofibroblasts, including type 1 collagen. Also, PF is a result of collagen deposition and cell proliferation in interstitial tissues (22,33). In our study, NAC decreased the thickness of alveolar walls and restricted the progression of fibrosis in bleomycin-treated rats. Our findings showed that hydroxyproline level as a collagen accumulation index at the highest dose of NAC (600 mg/kg) was similar to the animals in the negative control group. Further research is suggested to identify the mechanisms of NAC on pulmonary fibrocytes as well as its effect on the differentiation of profibrotic macrophages (M2). Based on our findings, NAC at 300 and 600 mg/kg was able to significantly delay the inflammation and fibrosis caused to bleomycin with a reduction of hydroxyproline content along with inflammatory and fibrogenic cytokines.

CONCLUSION

Based on these findings, NAC could decrease leukocyte infiltration, collagen deposition, and also fibrosis score in the bleomycin-induced PF tissue and decrease the level of IL-17 as an inflammatory as well as TGF-β as a fibrogenic cytokine in the bronchoalveolar fluid. Such effects of NAC can ultimately inhibit the progression of PF. Future clinical investigations are suggested for determining the preventive or therapeutic effect of NAC on pulmonary fibrosis and other inflammatory disorders.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

A. Azadmehr and AA. Moghadamnia designed and supervised the study, drafted the manuscript, and gave the final approval of the manuscript to be submitted for publication; F. Feizi advised and analyzed the histological experiment; Z Maghsadi and N. Hamidi conducted the experiments and analyzed the data. The finalized article was approved by all authors.

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