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Nigella sativa attenuates bleomycin-induced pulmonary fibrosis in rats by inhibition of inflammation, fibrosis, and inducible nitric oxide synthase

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ABSTRACT

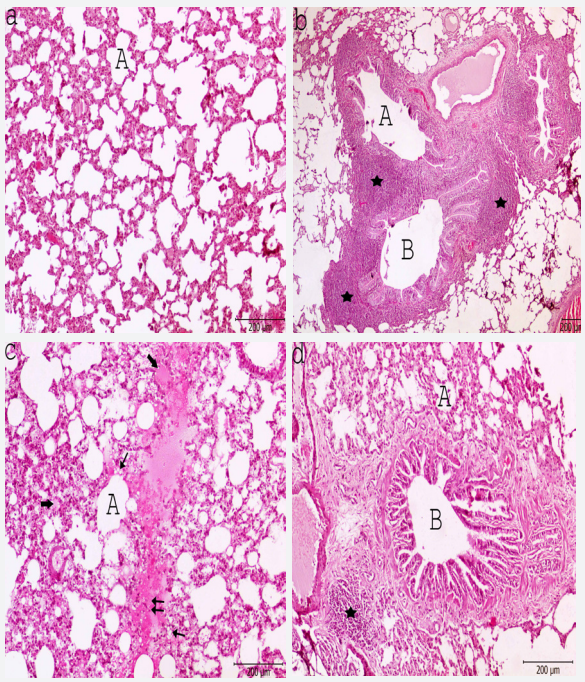
The present study investigated the anti-oxidant, anti-inflammatory and anti-fibrotic potential of *Nigella sativa (NS)* against bleomycin (BLC)-induced lung injury and fibrosis, an experimental animal model. A total of 18 male Sprague-Dawley rats were divided into three groups: Control, BLC, BLC+*NS*; each group consist of 6 animals. Pulmonary fibrosis was induced by a single intratracheal instillation of 2.5 mg/kg BLC. BLC+*NS* group rats were intragastric administered daily 400 mg/kg of *NS* from day 1 to 28. At the end of the study, lung tissues were removed for histopathological and immunohistochemical investigation. Lung tissues were stained with hematoxylin and eosin and Masson's Trichrome for histological evaluation. Moreover, the inducible nitric oxide synthase (iNOS) expression in the lung tissues was determined by immunohistochemical staining. BLC-induced histological changes including lung inflammation and lung fibrosis were significantly detected compared to the control group. *NS* treatment significantly ameliorated the BLC mediated histological changes and reduced the inflammatory cell infiltrate in lung tissues. *NS* significantly blocked collagen accumulations with parallel reduction in the fibrosis score. In addition, *NS* also markedly decreased the positive staining of iNOS in lung tissues. Our study provides evidence that *NS* significantly ameliorated BLM-induced pulmonary fibrosis in rats via the inhibition of inflammation score, fibrosis score, and iNOS expression. Therefore, *NS* may be a potential therapeutic reagent for the treatment of lung fibrosis.

1. Introduction

Idiopathic pulmonary fibrosis, in which alveolitis and pulmonary fibrosis pathogenesis is unrecognized, is a commonly observed and generally fatal interstitial lung disease of unknown etiology (ATS, 2000). Current treatments have very limited effect on the prognosis of this disease.

Bleomycin (BLC) is an antibiotic with anti-tumor effects obtained from *Streptomyces verticillus* (Adamson, 1976). Since BLC tends to concentrate in the lungs, kidneys, peritoneum and the lymph system following administration, it is used in the treatment of skin cancers, head and neck cancers, uterine cancer, cervical cancer, Hodgkin's disease, reticulosarcoma, lymphosarcoma, choriocarcinoma with embryonal cells and teratocarcinoma. BLC forms BLC-iron complexes that convert molecular oxygen into superoxide and hydroxyl radicals, which in turn cause DNA strand breaks and damage in DNA-RNA-protein synthesis (transcription and translation), leading to an anti-neoplastic effect (Claussen and Long, 1999; Chaudhary et al., 2006).

The lung fibrosis model, developed by using BLC, is an important model for understanding the stages of the disease as well the relation between lung structure and function (Moeller et al., 2008; Mouratis and Aidinis, 2011). It has been reported that histological hallmarks, such as intra-alveolar buds, mural incorporation of collagen and obliteration of the alveolar space, are present in BLC treated animals similar to idiopathic pulmonary fibrosis patients (Usuki, 1995).



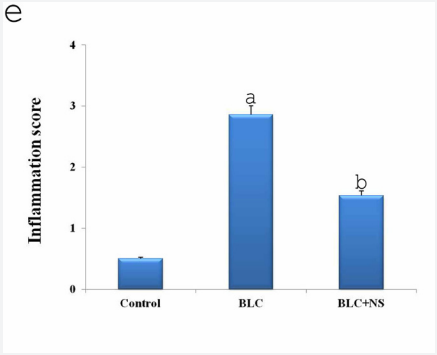


Fig. 1. Representative lung tissue photographs of H&E staining. **a:** Control group animals presented normal lung tissue. **b,c:** The tissue specimens were performed for the presence of severe inflammatory cell infiltration, wider and thickened interalveolar septa, alveolar edema, alveolar exudate, diffuse hemorrhagic area, and collapsed alveolar spaces with inflammatory exudates in BLC-treated group animals. **d:** Less histopathological parameters were seen in the *NS* treatment group. **e:** Inflammation score was significantly decreased in the BLC+NS group when compared to BLC group. **A:** Alveol; **B:** Bronchiole; Asterisks: Inflammatory cell infiltration; thick arrow: Alveolar exudate; thin arrow: Alveolar edema; double arrows: Diffuse hemorrhagic area. (H&E, scale bar, 200 μm). aP<0.001 compared to control group, bP<0.01 compared to BLC group.

Nigella sativa (NS) is a plant species belonging to the Ranunculaceae family. The part of the plant used as food is the seed within its capsule (Salem, 2005). The plant has been used since ancient times by the people of the Middle East and Far East as a traditional remedy for a broad range of diseases including asthma, bronchitis, headache, dysentery, obesity, back pain, hypertension and gastrointestinal problems (Salem et al., 2010; Ahmad et al., 2013). Studies have shown that black cumin from *NS* can target and kill various cancer cells, induce the formation of antibodies against tumors, and cause an increase in the number and activity of macrophage cells (Swamy and Tan, 2000; Bourgou et al., 2012). The literature describes black cumin oil as having antioxidant, antibacterial, antifungal, antidiabetic, immunomodulatory, anti-inflammatory, analgesic, antiviral and antihyperlipidemic effects (Ahmad et al., 2013; Alemi et al., 2013; Sultan et al., 2014).

However, the effects of *NS* on pulmonary fibrosis have not yet been studied in detail. Our study aimed to investigate the protective effects of *NS* on pulmonary fibrosis induced by intratracheal instillation of BLC by using histopathological and immunohistochemical evaluation in damaged lung tissue in rats.

2. Material and methods

Animals

Eighteen Sprague-Dawley rats, weighing 200-220 grams and averaging 8 weeks old were used in the present study. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. The study was approved by Namik Kemal University, Local Animal Ethics Committee, and ethical rules were observed during the study (Permission number: 2015/06-10, 07.05.2015).

Experimental model of pulmonary fibrosis

An animal model of BLC-induced pulmonary fibrosis as reported earlier was used in this study (Ermis et al., 2013; Kilic et al., 2014). The rats were anesthetized with an intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). The skin and subcutaneous tissue overlying the proximal portion of the trachea were exposed by blunt dissection. A single intratracheal instillation of 2.5 mg/kg of BLC in sterile 0.9% NaCl was administered to the rats to develop the model for pulmonary fibrosis.

Experimental design

A total of 18 Sprague-Dawley rats were divided into three groups: control, BLC, BLC+*NS*; each group consisted of 6 animals. Control group animals received intratracheal injection of physiological saline alone. BLC group animals were subjected to a single intratracheal instillation of BLC as previously mentioned. BLC+*NS* group animals were considered as a preventive model, which received the same amount of BLC as the BLC group animals and were then treated with *NS* (400 mg/kg intragastric) for 28 days. Twenty-eight days after the BLC treatment, the experiment was terminated and animals were sacrificed by cervical dislocation and lung tissues were removed for histopathological and immunohistochemical investigation. The dose of *NS* was selected by previous studies (Akhtar et al., 2013).

Histological evaluation

The lung specimens were individually immersed in Bouin's solution, dehydrated in alcohol and embedded in paraffin. Sections of 5 µm were obtained, deparaffinized and stained with hematoxylin and eosin (H&E) and Masson's trichrome using standard procedures. The lung tissues was examined and evaluated in random order under blindfold conditions with standard light microscopy (Olympus CX41 microscope (Olympus, Japan)) by a histologist. The H&E staining method was used to investigate the degree of lung tissue

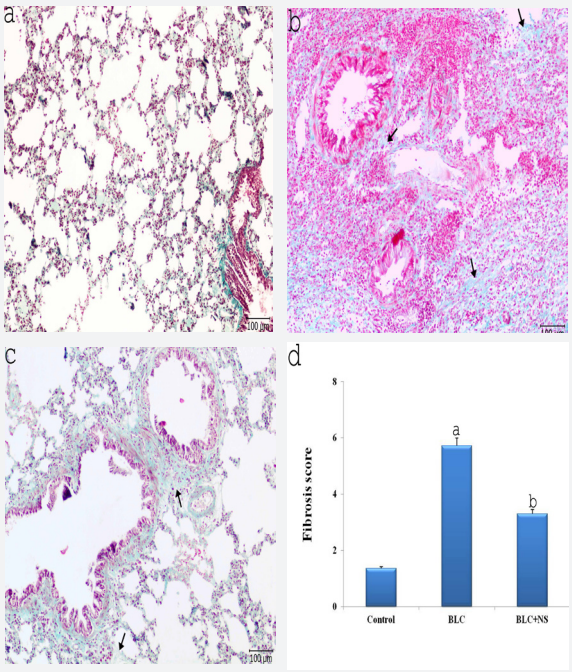


Fig. 2. Representative lung tissue photographs of Masson's trichrome staining. **a:** Control group animals showing normal collagen fibers distribution. **b:** BLC group animals showing extensive collagen deposition are recognized as green in the lung interstitium and around bronchioles. **c:** *NS* treatment significantly decreased collagen deposition in the lung. **d:** Fibrosis score was significantly decreased in the BLC+NS group when compared to BLC group. Arrow: Collagen fibers. (Masson's trichrome, scale bar, 100 μm). aP<0.001 compared to control group, bP<0.01 compared to BLC group

inflammation. A score ranging from 0 to 4 was assigned according to severity (Chen et al., 2010), and the scores were averaged per group. The Masson's trichrome staining method was used to investigate fibrotic degree. Successively increasing microscopic magnification fields were used to determine the severity of fibrosis using the semiquantitative grading system described by Hubner et al. (2008). The grade of pulmonary fibrosis was blindly scored by examining 10 randomly selected regions per sample at 200X magnification. A score ranging from 0 (normal lung) to 8 (total fibrosis) was assigned, and the scores were averaged per group.

Immunohistochemical evaluation

Immunohistochemical reactions were performed according to the avidin biotin complex technique described by Hsu et al. (1981). The sections were incubated with specific polyclonal anti-inducible nitric oxide synthase (iNOS) antibody (Cat. # RB-1605-P, Neomarkers, USA).

The positive staining of iNOS cell numbers were scored in a semi-quantitative manner to determine the differences between the control and experimental groups in the distribution patterns of intensity of immunolabeling of lung tissue. The numbers of the positive staining were recorded as absence (-), a few (±), few (+), medium (++), high (+++), and very high (++++). This analysis was performed in at least randomly selected eight microscopic high-power fields from each lung lobe section, in two sections from each animal at X400 magnification. The final score determined in each category for each individual animal was the average of the scores from the sections of the lungs examined.

Statistical analysis

All statistical analyses were carried out using SPSS statistical software (S0064 Minitab Release 13, License number: WCP1331.00197). All data were presented in mean±SD. Differences in the measured parameters among the three groups were analyzed with a nonparametric test (Kruskal-Wallis). Dual comparisons between groups exhibiting significant values were evaluated with a Mann-Whitney U-test. These differences were considered significant when probability was less than 0.05.

Table 1. Semiquantitative comparison of the number of iNOS positive cells in lung tissues for each group. Control, BLC and BLC+NS group (n: 6 for each gruop)

	Control	BLC	BLC+NS
iNOS	±	++++	++

The numbers of the positive staining were recorded as absence (-), a few (±), few (+), medium (++), high (+++), and very high (++++).
iNOS: Inducible nitric oxide synthase; **BLC:** Against bleomycin; ***NS:*** *Nigella sativa*

3. Results

Histopathological findings

The H&E staining method was used to investigate the degree of lung tissue inflammation. The lungs of rats in the control group presented normal lung architecture and there were no lesions (Fig. 1a). The lung tissue sections of BLC-treated animals showed markedly histopathological abnormalities, alveolar edema and exudate, extensive alveolar injury with intra alveolar septal thickening, large fibrous areas, diffuse hemorrhagic area, collapsed alveolar spaces, and including severe inflammatory cell infiltration. Inflammatory cells were also apparent interstitium, around small airways and mucosal epithelium (Fig. 1b). However, *NS* treated rats significant less all these changes. The interstitium of the lungs appeared thinner and the number of inflammatory cells apparently reduced (Fig. 1c). Moreover, inflammation score were decreased significantly in *NS* treatment rats compared to BLC-treated rats (Fig. 1d).

The Masson's trichrome staining method was used to investigate fibrotic extent. Masson's trichrome staining showed that the BLC-treated animals had an abnormal collagen deposition and distorted lung morphologies compared with the control animals (Fig. 2b). Collagen deposition was suppressed in the lungs of the BLM-treated rats with *NS* treatment (Fig. 2c). No such abnormalities were apparent in control group rats (Fig. 2a). Moreover, fibrosis score were decreased significantly in *NS* treatment rats compared to BLC-treated rats (Fig. 2d).

Immunohistochemical findings

In control rats, a few iNOS positive cells were observed (Fig. 3a). BLC induced lung fibrosis led to statistically significant increase in the number of iNOS positive cells in lung tissues. Many cells in the wall of alveoli, alveolar sacs and alveolar ducts were strongly positive for iNOS (Fig. 3b). Treatment with *NS* markedly reduced the number of iNOS positive cells (Fig. 3c, Table 1).

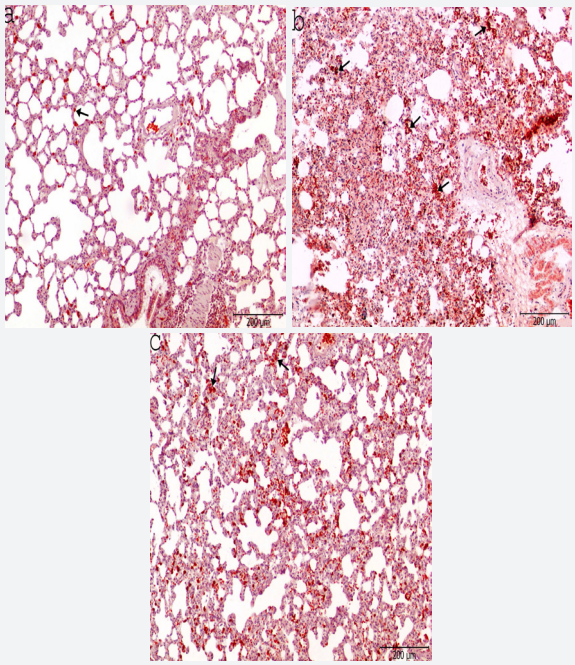


Fig. 3. Immunohistochemical expression of iNOS in lung tissue. **a:** A few iNOS positive cells in control group. **b:** Number of iNOS positive cells are significantly increased after BLC treatment **c**: Treatment of infliximab markedly reduced the number of iNOS positive cells. Arrow: iNOS positive cells (Immunoperoxidase, haematoxylin counterstain, scale bar, 200 μm).

4. Discussion

The lung fibrosis model developed using BLC is the most important model for understanding the stages of the disease, as well the relation between lung structure and function. Being an antineoplastic agent, BLC's mechanism of action involves the formation of BLC-iron complexes that convert molecular oxygen into superoxide and hydroxyl radicals, which in turn cause DNA strand breaks. In vitro studies have shown that antioxidant treatments inhibit BLC-induced DNA and cell damage. Reactive oxygen radicals and proteases are generally released by inflammatory cells, causing damage in the lung tissue; the repair process of this damage will in turn lead to excessive fibrosis (Erden et al., 2008).

The pathophysiology of BLC-induced lung fibrosis consists of two phases. The first inflammatory phase involves the accumulation of inflammatory cells such as neutrophils, lymphocytes and macrophages in the interstitial area, while the second phase involves the development of late fibrosis. Reactive oxygen radicals and proteases are generally released by these inflammatory cells, causing damage to the lung tissue, and triggering a repair process which results in excessive fibrosis (Hagiwara et al., 2000). For this reason, stimulating the antioxidant system in order to treat the lung fibrosis caused by BLC would be a logical course to follow (Iraz et al., 2006).

Previous studies have demonstrated that various antioxidant substances can alleviate BLC-induced lung damage (Li et al., 2012; Larki et al., 2013; Verma et al., 2013; Karimfar et al., 2015). In our study, we have employed *NS*, whose antioxidant effects were well known. In a previous study, Kilic et al. (2015) developed an experimental lung fibrosis model with BLC, in which they identified severe alveolar damage, thickening of alveolar walls, inflammation and fibrosis as a result of BLC application. They also demonstrated that this damage was reduced with the administration of antioxidant substances (Kilic et al., 2015). In another study, Gao et al. (2013) induced lung damage with BLC, and then applied both H&E and Masson trichrome staining to visualize the extent and nature of this damage. The H&E staining demonstrated disruption of the normal lung tissue structure, thickening of the intra-alveolar septal area, and a high level of inflammation, while the Masson trichrome staining revealed high levels of collagen accumulation in the lung tissue. Gao et al. (2013) also observed that baicalein treatment reduced and reversed these degenerative changes. In line with the literature, we also observed degenerative changes in the lung tissue during our study. These BLC-caused damages were reduced through the application of the *NS* treatment.

Previous studies have demonstrated that an overproduction of nitric oxide (NO), resulting from the expression of iNOS, appears in pulmonary fibrosis in both animal models and humans (Barnes and Hansel, 2004; Kalayarasan et al., 2008). BLC has been shown to be a stimulator of iNOS expression (Di Paola et al., 2011; Galuppo et al., 2011). Increased pulmonary expression of iNOS and increased NO production are observed in pulmonary fibrosis (PF) patients; however, no changes in the levels of other NOSs have been detected (Lakari et al., 2002). In addition, pharmacological inhibition of NO has been proposed as a potential therapeutic strategy for PF (Hobbs et al., 1999). These findings support the notion that NO over-produced by iNOS formation may have deleterious effects in the pathogenesis of PF. In the present study, we found that *NS* treatment reduced BLC-induced increased of iNOS positive cells.

In conclusion, we determined that the lung fibrosis caused by BLC in rats can be alleviated through the application of *NS*. We believe that the protective effect of *NS* is associated with its strong anti-oxidant, anti-inflammatory and anti-fibrotic effect, as well as its ability to prevent the formation or cause the elimination of free oxygen radicals. However, more comprehensive studies are necessary to support and substantiate the use of *NS* as a potential protective agent against PF.

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