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Preventive and early therapeutic effects of β-Glucan on the bleomycin-induced lung fibrosis in rats

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Abstract. – OBJECTIVE: The β-glucans are long-chain polymers of glucose, which comprise the fungal cell wall, stimulate cells of the innate immune system, enhance disturbed epithelization, and have antioxidant effects. Oxidative stress has been implicated in the pathogenesis of bleomycin-induced lung fibrosis and various antioxidant agents have been studied for prevention and treatment of the disease. In this experimental animal study, we assessed effects of β-glucan, extracted from barley, on the bleomycin-induced lung fibrosis, and evaluated differences of starting before and after bleomycin instillation.

MATERIALS AND METHODS: Male Spraque-Dawley rats were given a single dose of bleomycin in pulmonary fibrosis groups. First dose of β-glucan and NAC was given three days before the bleomycin injection, and at one of the other group β-glucan was started 12 hours after bleomycin and continued until 14th day. Fibrotic changes in lung were estimated by using Aschoft's criteria and measuring lung hydroxyproline content.

RESULTS: Bleomycin induced severe pulmonary fibrosis with marked increase in hydroxyproline content of lung tissue and typical lung fibrosis, which was prevented by β-glucan. Hydroxyproline level was significantly higher in bleomycin treated rats than the other groups, and its level was decreased in the therapeutic groups, especially in the β-glucan post-bleomycin group fibrosis score, hydroxyproline and MDA levels returned to the control levels. On the other hand, reduced glutathione level elevated in the same group.

CONCLUSIONS: The data suggest that β-glucans have protective and early therapeutic effects against bleomycin-induced lung fibrosis in rats.

Key Words:
β-glucan, Bleomycin, Lung fibrosis, Hydroxyproline, Glutathione, Therapeutic.

Introduction

Idiopathic pulmonary fibrosis (IPF) is defined as a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause, occurring primarily in older adults. It is limited to the lungs with histopathologic pattern of usual interstitial pneumonia. It starts usually with non-productive cough and exertional dyspnea. Severe resting dyspnea and sight of right heart failure develops with involvement of larger areas of the lung[1]. The majority of patients get worse rapidly. In a recent analysis based on health care data of a large health plan in the United States estimates the prevalence 14.0 and 42.7 per 100,000 persons depending on the used case definition system[2]. Based on the evidence published to date, no pharmacological therapy has been proven for IPF[1]. Recent studies have suggested some promising agents for IPF include antioxidants[3-5], interferon gamma[6], erdosteine[7], N-acetylcysteine (NAC)[8,9], aminoguanidine[10], Ginkgo biloba[11], bosentan[12], prostacyclin[13], blockers of tumor necrosis factor alpha and transforming growth factor beta[14]. Despite, the large number of antifibrotic drugs being described in experimental animal studies, the translation of a serious alternative treatment protocol into clinical practice has not been accomplished yet. Practically, treatment protocols are focused on complications such as heart failure, infections, etc., and supportive care. Therefore, recently researchers have concentrated on the discovering an effective agent in the treatment of IPF.

Bleomycin is a chemotherapeutic antibiotic, produced by the bacterium "*Streptomyces verticillus*"[15,16]. Its use in animal models for creating pulmonary fibrosis is based on the fact that fibro-

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sis is one of the major adverse drug effects of bleomycin in human cancer therapy. Bleomycin plays an important role in the treatment of lymphoma, squamous cell carcinomas, germ cell tumors and malignant pleural effusion. It is believed that bleomycin acts by causing single and double-strand DNA breaks in tumor cells and, thereby, interrupting the cell cycle. This happens by chelation of metal ions, and reaction of the formed pseudo enzyme with oxygen, which leads to production of DNA-cleaving superoxide and hydroxide free radicals[17]. An over production of reactive oxygen species can lead to an inflammatory response causing pulmonary toxicity, activation of fibroblasts and subsequent fibrosis[18,19]. It causes inflammatory and fibrotic reactions within a short period of time, even more so after intratracheal instillation. The initial elevation of pro-inflammatory cytokines (interleukin-1, tumor necrosis factor-α, interleukin-6, interferon-γ) is followed by increased expression of pro-fibrotic markers (transforming growth factor-β1, fibronectin, procollagen-1). The "switch" between inflammation and fibrosis appears to occur around 9th day after bleomycin[18]. Therefore, immune system stimulation might be logical in the treatment of bleomycin-induced pulmonary fibrosis.

β-Glucans are cell-wall polysaccharide present in yeast, fungi, and cereal plants. Differences in the linkage arrangement compared with the cereal glucans have been reported[20-22]. The β-(1 → 3) linkages always occur in isolation and interrupt the regular cellulose-like structure in a random fashion. This irregularity makes (1 → 3)(1 → 4)-β-glucans water soluble. In the brewing process barley β-glucan tends to form gels[23] and they are accepted to be among the most powerful immune response modifiers. Since the beneficial effects of β-glucans on the immune system are devoid of toxic and adverse effects, studies have focused on β-glucan molecules. Several studies have reported that β-glucans inhibit tumor development, protect from ototoxicity and lipopolysaccharide induced mortality[24], restore hematopoiesis, promote wound healing, and activates macrophages[25,26]. β-glucan molecules act on macrophages, B lymphocytes, suppressor T cells, and natural killer cells. It enhances neutrophil mobilization and migration by binding to the receptors located on monocytes[27]. It has also been found to be an effective antioxidant and free-radical scavenger[28,29]. It has been reported to be a critical inducer of mucosal immunity in the digestive tract[30].

The present study aimed to investigate protective and early therapeutic effects of β-glucan on lung fibrosis induced by bleomycin exposure by using Ashcroft criteria which provide semi-quantitative assessment of lung fibrosis, and by the measurement of hyroxyproline content and oxidative stress markers in damaged lung tissue in rats.

Materials and Methods

Animals

The experiments were carried out on a total of 44 male 12-week-old Sprague-Dawley rats, weighing between 210-260 g. All rats were maintained under the same conditions before the surgical procedure. The animals were housed at 22°C ± 2°C and 40% to 45% relative humidity in wire bottom colony cages (4 rats/cage) with a 12-hour light/dark cycle (7:00 am-7:00 pm) and fed standard rat chow and tap water *ad libitum*. The experiments were in compliance with the Principles of Laboratory Animal Care formulated by the National Institutes of Health[31]. The study protocol was approved by the Committee for the Ethical Care and Use of Laboratory Animals of Inonu University, Faculty of Medicine.

Experimental Protocols

Animal Model of Bleomycin-Induced Pulmonary Fibrosis

All rats were anesthetized with 80 mg/kg ketamine (Ketalar, Pfizer, Istanbul, Turkey) and 5 mg/kg xylazine HCl (Rompun, Bayer, Germany). Following anesthesia, a midline incision on the neck and the trachea was exposed by blunt dissection. A needle of insulin injector was inserted into the trachea under direct visualization. Bleomycin hydrochloride (Nippon Kayaku, Tokyo, Japan) was dissolved in 250 μl of phosphate-buffered saline solution and instilled into the animal's lungs at a dose of 7.5 mg/kg. The rats were killed 14 days after bleomycin injection. Sterile saline solution and β-glucan were administered to the control groups. The animals were shaken to facilitate distribution of the bleomycin and saline. Pulmonary fibrosis was assessed based on the measurement of lung hydroxyproline content as well as evaluation of lung histopathology.

Experimental Groups

The animals were divided randomly into the following five groups: Control (n = 7), Bleomycin (n=10), N-acetylcysteine+Bleomycin (n=9), β-Glucan+Bleomycin (n = 9), and Bleomycin+β-Glucan (n = 9). The control group rats did not take any treatment except equal volume of vehicle of bleomycin. The rats received 1 mg/kg β-glucan (Sigma, St. Louis, MO, USA), supplied as a dry powder, by intragastric gavage for 3 days before the bleomycin instillation in β-Glucan+Bleomycin (pre-Bleomycin) group, but 12 hours after the bleomycin instillation in the Bleomycin+β-Glucan (post-Bleomycin) group. N-acetylcysteine was administrated by intraperitoneal injection at a dose of 50 mg/kg; it started 3 days before the bleomycin instillation in N-acetylcysteine+Bleomycin group. N-acetylcysteine and β-Glucan applied daily until the end of the study. Fourteen days after the bleomycin instillation, lung tissue samples were collected under anesthesia.

Histopathological Examination

The right lung samples were fixed in 10% neutral formalin solution for 24 h. After routine tissue processing, paraffin-embedded tissues sectioned at 5 μm and stained with hematoxylin and eosine (H&E) were examined for pulmonary fibrosis under light microscope. The entire lung section composed of 30-35 microscopic fields was examined at a magnification of ×100. The lung fibrosis was scored according to the Ashcroft's fibrosis score as: Normal (0), minimal fibrous thickening of alveolar or bronchiolar walls (1), moderate thickening of walls without obvious damage to lung architecture (2,3), increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses (4,5), severe distortion of structure and large fibrous areas; "honeycomb lung" is placed in this category (6,7), total fibrous obliteration of the field (8). The inflammatory infiltrate in airspaces was ignored but organized inflammation was treated as fibrosis[32]. The mean score of all fields was taken as the fibrosis score of that lung section with a maximum score of 8.

Determination of Hydroxyproline Levels

The right lungs were used for hydroxyproline content determination with spectrophotometrically methods as an index of collagen accumulation. The lungs were homogenized in 1 ml of phosphate buffered saline (PBS), and hydrolyzed by the addition of 1 ml of 12 N HCL at 120 °C for 16 h and dissolved in 2 ml deionized water. Samples were incubated with Chloramine T solution for 20 min at room temperature, and with Ehrlich's solution at 65 °C for 15 min. The absorbance of the Wnal reaction solutions at 550 nm were measured and amount of hydroxyproline was obtained as microgram[33].

Preparation of Lung Tissue for Biochemical Analyses

After removal, the lung tissues were washed twice with cold saline solution, placed into glass bottles, labeled, and stored at −80 °C until processing. The lung tissues were homogenized in a four volumes of ice-cold Tris-HCl buffer (pH 7.4) for 3 min at 16,000 rpm using a glass teflon homogenizer (IKA Ultra-Turrax T25 basic homogenizer, Staufen, Germany) after cutting of the lung tissues into small pieces with a scissors. Tissue malondialdehyde (MDA) and nitric oxide (NO) and reduced glutathione (GSH) levels were determined in the homogenates. The homogenates were then centrifuged at 5000 × g for 60 min to remove debris. The clear upper supernatant fluid was taken and assayed for catalase (CAT), glutathione peroxidase (GSH-Px) and myeloperoxidase (MPO) activities. After the supernatant solution was extracted with an equal volume of an ethanol/chloroform mixture (5/3, v/v) and centrifuged at 5000×g for 60 min, the upper ethanol phase was taken and used in the superoxide dismutase (SOD) assays. The protein measurements were analyzed in homogenates, supernatant and extracted samples.

Determination of MDA Levels

The thiobarbituric acid reactive substance level was determined by a method based on the reaction with TBA at 90°C to 100°C[19]. In the TBA test reaction, MDA or MDA-like substances (ie, the byproduct of lipid peroxidation process of the polyunsaturated fatty acids) and TBA react together for production of a pink pigment having an absorption maximum at 532 nm. The reaction was performed at pH 2 to 3 at 90°C for 15 minutes. The sample was mixed with 2 volumes of cold 10% (w/v) trichloro acetic acid to precipitate protein. The precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 minutes. After cooling, the absorbance was read at 532 nm (UV-1800, Shimadzu Corp., Tokyo, Japan). The results were expressed as nmol/g protein[34].

Determination of NO Levels

Direct measurement of NO, a very labile molecule, in biological samples is difficult. In aqueous solution, NO reacts with molecular oxygen and accumulates in the plasma as nitrite ($NO2^-$) and nitrate ($NO3^-$) ions. Therefore, the stable oxidation end products of NO, $NO2^-$, and $NO3^-$ can be readily measured in biological fluids and have been used *in vitro* and *in vivo* as indicators of NO production. Tissue nitrite plus nitrate concentrations as an index of plasma NO levels were determined by the method described previously[35]. Quantification of nitrite and nitrate was based on the Griess reaction, in which a chromophore with a strong absorbance at 540 nm is formed by reaction of nitrite with a mixture of naphthylethylenediamine and sulfanilamide. The absorbance was measured by a UV spectrophotometer (UV-1800, Shimadzu Corp.) to assess the nitrite concentration. For nitrate detection, a second sample was treated with copperized cadmium in glycine buffer at pH 9.7 to reduce nitrate to nitrite, the concentration of which represented the total nitrite plus nitrate. A standard curve was established with a set of serial dilutions (10–8–10–3 mol/L) of sodium nitrite. Results were expressed as μmoL/g protein.

Determination of GSH Levels

The lung tissue GSH levels were measured by the method of Beutler et al[36]. Reduced glutathione was assessed in cell lysates (prepared by mixing 0.2 mL of packed cells to 1.8 mL of Na2EDTA) using metaphosphoric acid as the precipitation agent. Samples were treated with 5,5V-dithiobis-2-nitro benzoic acid (DTNB), a compound readily reduced by sulfhydryl compounds, forming a highly colored compound which has a maximum absorbance at 412 nm. GSH concentrations were expressed as nmol/g wet tissue.

Determination of GSH-Px Activity

GSH-Px (EC 1.6.4.2) activity was measured using the method of Paglia and Valentine[37]. The enzymatic reaction was initiated by the addition of H_2O_2 to the reaction mixture containing reduced glutathione, nicotinamide adenine dinucleotide phosphate, and glutathione reductase. The change in the absorbance at 340 nm was monitored spectrophotometrically. Activity was expressed as U/g protein.

Determination of MPO Activity

Myeloperoxidase (EC 1.11.2.2) activity was determined using a 4-aminoantipyrine/phenol solution as the substrate for MPO mediated oxidation by H_2O_2 and changes in absorbance at 510 nm (A510) were recorded[38]. Data are presented as U/g protein. All samples were assayed in duplicate.

Determination of CAT Activity

CAT (EC 1.11.1.6) activity was measured according to the method of Aebi et al[39]. The principle of the assay is based on the determination of the rate constant k (dimension: s–1, k) of hydrogen peroxide (H_2O_2) decomposition. The rate constant of the enzyme was determined by measuring the absorbance changes per minute. Activities were expressed as k/mg protein.

Determination of SOD Activity

The principle of the total SOD (EC 1.15.1.1) activity method is based, briefly, on the inhibition of nitrobluetetrazolium (NBT) reduction by $O2^-$ generated by a xanthine/xanthine oxidase system[40]. Activity was assessed in the ethanol phase of the serum after 1.0 mL ethanol/chloroform mixture (5/3, v/v) was added to the same volume of serum and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. Activity was expressed as U/g protein.

Statistical Analysis

Data were analyzed by using SPSS for Windows version 22.0 (SPSS Inc., Chicago, IL, USA). Distribution of the groups was analyzed with the Shapiro-Wilk test. Groups showed normal distribution for the level of MDA, GSH and the activities of GSH-Px so that parametric statistical methods were used to analyze these data. A One-way ANOVA test was performed and post hoc multiple comparisons were made using Tukey HSD. The distribution was not normal for hydroxyproline, MPO, NO, SOD and CAT levels in groups. The results of abnormal distributed were assessed between groups using the Kruskal-Wallis test. The Mann-Whitney U test was made for the difference between 2 groups. Results are presented as mean±standard deviation (SD). $p < 0.05$ was considered to be statistically significant for normal distributed data, but $p < 0.005$ was considered to be significant for abnormal distributed data.

Results

The anesthesia and bleomycin instillation procedure were well tolerated by the rats. No adverse effects were observed in rats that received NAC and β-Glucan daily. The rats that received bleomycin without any therapeutic agent showed mild degree of systemic toxicity with not significant weight loss on day 14 compared to other groups. Food and water consumption decreased in this group day-by-day. In the bleomycin group, 1 rat died on 7th day and 1 rat died on 12th day. All of the animals from the other groups survived until the end of the experiment. The residue of β-Glucan solution was gelled in the tube at room temperature for 12 hours.

Assessment of Lung Fibrosis

Lung fibrosis was primarily assessed by measuring hydroxyproline content in lungs tissue as a marker of collagen accumulation. A comparison of hydroxyproline contents among the groups was shown in Figure 1. Bleomycin treatment resulted in a significant increase in the hydroxyproline levels (26.25 ± 4.1 mg/g dry tissue) as compared to the other groups ($p < 0.004$) after 14 days. This increase was prevented by NAC and β-Glucan, started before and after bleomycin instillation. The grades of fibrosis in five groups were also presented in Figure 1. The semiquantitative assessment of lung sections that received no treatment and received β-Glucan six hours later then bleomycin revealed normal lung histology (Figure 1). All rats in the bleomycin group had same score (7). In the bleomycin+NAC and the β-Glucan+bleomycin groups, fibrosis scores were 3.22 ± 1.86 and 3.78 ± 0.82, respectively. The β-Glucan treatment reversed the increase in the fibrosis score, the fibrosis score of the Bleomycin+ β-Glucan group, 0.22 ± 0.44, was almost equal to that of the control group. Thus it did not represent any fibrotic change (0) (Figure 1).

The lungs of control group showed normal histomorphology (Figure 2). The wide areas of fibrosis and cyst formation of the parenchyma (honeycomb lung) were seen in the Bleomycin group (Figure 2). Fibrosis was slightly decreased in the β-Glucan+bleomycin group (Figure 2). There were only mild fibrosis and minimal chronic inflammation in the peribronchial areas in the Bleomycin+β-Glucan group (Figure 2).

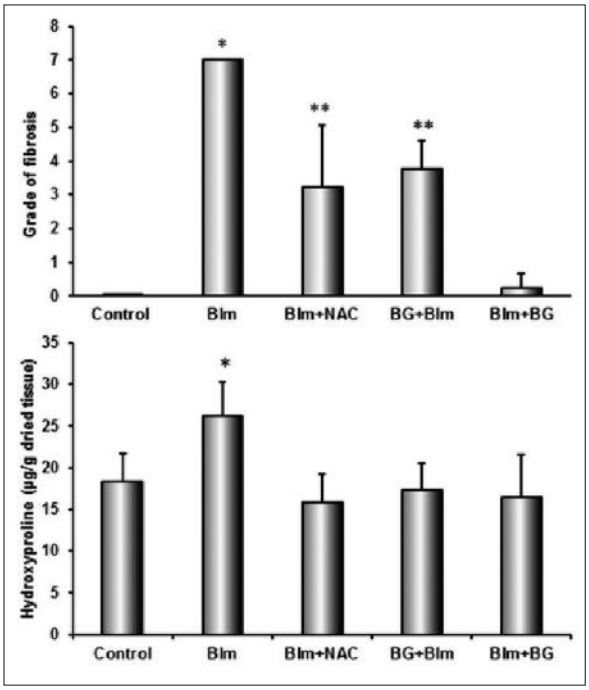

Figure 1. Effect of β-Glucan (BG) and N-acetylcysteine (NAC) on bleomycin (Blm)-induced increases in the hydroxyproline levels and the fibrosis scores in the experimental groups. An alone bleomycin administration significantly increased the hydroxyproline levels and the fibrosis scores in the lung tissue which were inhibited by the treatment with β-glucan after bleomycin administration. *Significantly lower ($p < 0.005$) when compared to the other groups. β-Glucan and NAC administration before bleomycin instillation significantly decreased the fibrosis scores versus the bleomycin group, but not returned to the levels of Control and Bleomycin+β-Glucan groups (**$p < 0.005$). Data are presented as means ± SD.

The Analysis of Oxidant Stress Markers

A significant elevation was observed in MDA level, an indicator for lipid peroxidation, in the lung tissue of rat exposed to bleomycin (58.37 ± 23.29 nmol/g wet tissue) compared with the control group (12.76 ± 3.92 nmol/g wet tissue) and the Bleomycin+β-Glucan group (12.75 ± 1.8 nmol/g wet tissue) ($p < 0.05$). The increase in MDA level of the lung tissue remained at 26.01 ± 6.98 nmol/g wet tissue in the β-Glucan+ Bleomycin group and to at 32.21 ± 15.17 nmol/g wet tissue in the Bleomycin+NAC group ($p < 0.05$) as shown in Figure 3.

The decrease in tissue GSH level and the depletion in GSH-Px activity reflect indirectly the generation of free radicals. Bleomycin produced a significant reduction in the GSH-Px activity 0.37 ± 0.12 nmol/gr wet tissue after 14 days

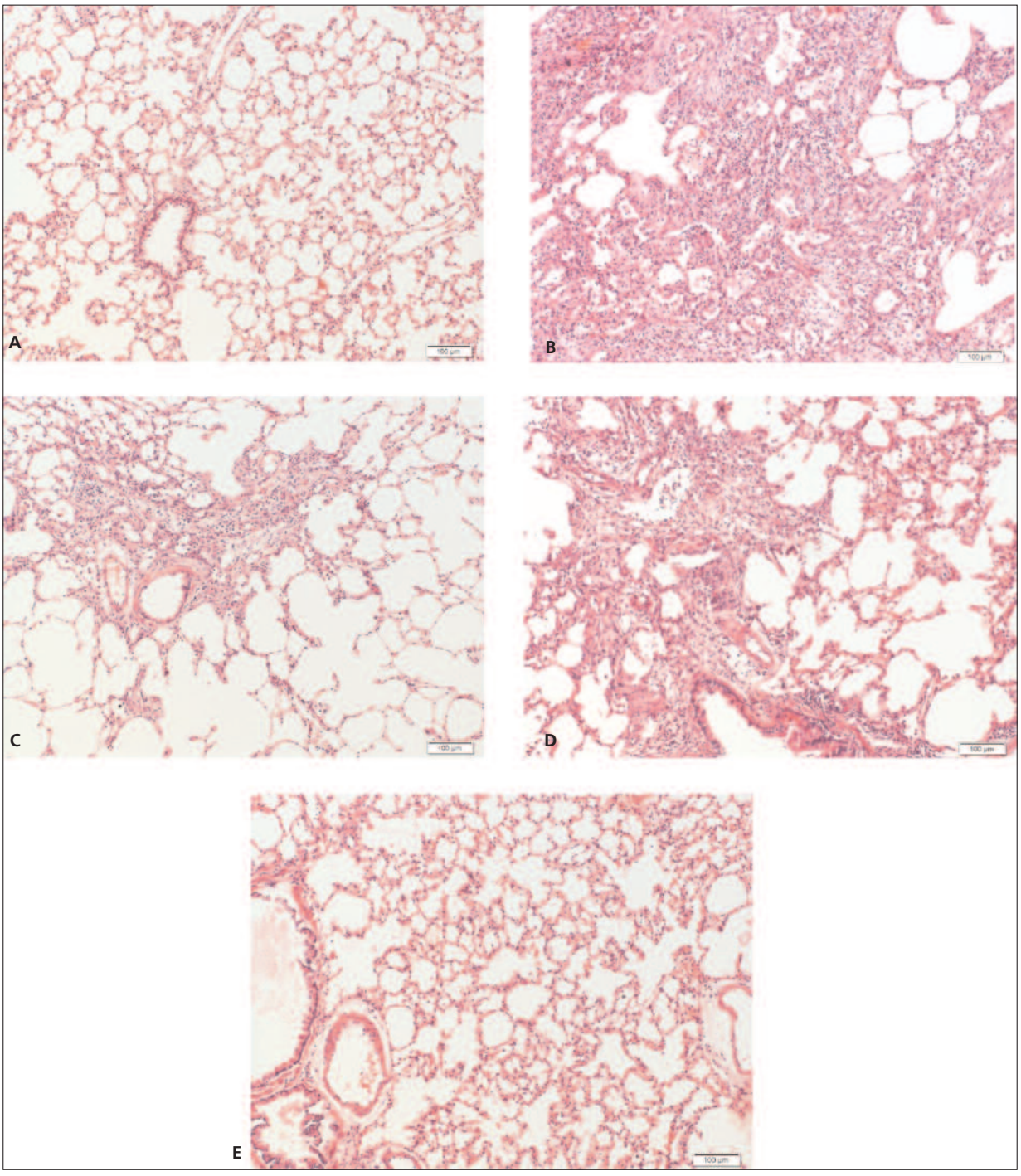

Figure 2. Morphological examination of the lungs after 14 days administration of bleomycin instillation. ***A,*** (Control): Normal lung parenchyma from saline-treated rats, score 0; ***B,*** (Bleomycin): Marked thickening in alveolar septa, collapse of alveolar spaces, a large number of leukocytes accumulated in alveolar walls and proliferation of fibroblasts, score 7; ***C,*** (Bleomycin+NAC) A mild inflammation and fibrosis, score 3; ***D,*** (β-Glucan+Bleomycin); slightly decreased inflammation and fibrosis, score 3; and ***E,*** (Bleomycin+β-Glucan): Near-normal alveoli and mild chronic inflammation of the peribronchial area was seen in the rat lung. H&E x100.

when compared with the other groups (Figure 4). Treatments with β-Glucan and NAC did not return reduced GSH-Px activity to the normal values. Additionally bleomycin produced a significant decrease in GSH level (85.62 ± 6.2 nmol/g wet tissue) versus the other groups, on the con-

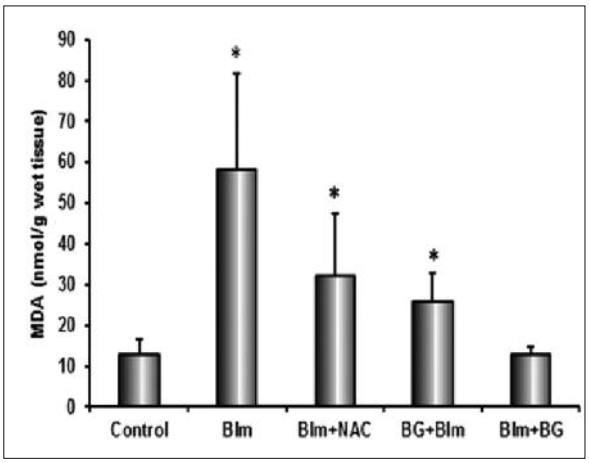

Figure 3. ***A, B,*** Effect of β-Glucan (BG) and N-acetylcysteine (NAC) on bleomycin (Blm)-induced increases in the malondialdehyde (MDA) levels in the experimental groups. Bleomycin administration significantly increased the MDA in the lung tissue, which were inhibited by the treatment with β-Glucan after bleomycin administration. Data are presented as mean ± SD. *Significantly lower ($p < 0.005$) when compared the Control and the Bleomycin+β-Glucan groups.

trary significant increase in the Bleomycin+β-Glucan group (492.7 ± 35.3 nmol/g wet tissue) versus the other groups.

The catalase and SOD activities are an important indicator for oxidant and antioxidant status. However, in this study, SOD activity was significantly decreased in β-Glucan post-bleomycin instillation 1.09±0.76 U/g protein versus β-Glucan pre-Bleomycin 0.49±0.11 U/gr protein ($p < 0.005$). The catalase activity was significantly decreased in the bleomycin+NAC group (0.43 ± 0.11k/g protein) versus the β-Glucan post-Bleomycin group (0.88 ± 0.45 k/g protein) (Figure 5).

Lung Tissue Myeloperoxidase Analysis

As shown in Figure 6, MPO content, a marker of neutrophil influx into tissues, was decreased in rats treated with β-Glucan after bleomycin instillation (47.19 ± 15.03 mU/g protein) as compared to the control rats (73.33 ± 10.77) ($p < 0.005$) (Figure 6). No significant difference among the groups related with NO levels was detected.

Discussion

In the present study, we aimed to evaluate the protective and early therapeutic effects of β-Glucan on the bleomycin-induced early lung injury and fibrosis. After administration of bleomycin, onset of an acute inflammatory response lasting up to 8 days was followed by fibrogenic changes resulting in extensive production of matrix materials and distortion of lung architecture in 28-35 days. Treatments during the first seven days would be considered as "preventive" while treatments during the later stages after 7-10th days would be considered as "therapeutic"[41]. The lung fibrosis was determined by using semiquantitative morphological indicators and measuring hydroxyproline content. Four possible explanations for the therapeutic route of β-Glucan should be the inhibition of cellular infiltration, scavenging of the reactive oxygen species produced by inflammatory cells and normal pulmonary cells, stimulated re-epithelization of alveolar system, or direct detoxification of bleomycin-induced free radicals before they damage the lung tissue.

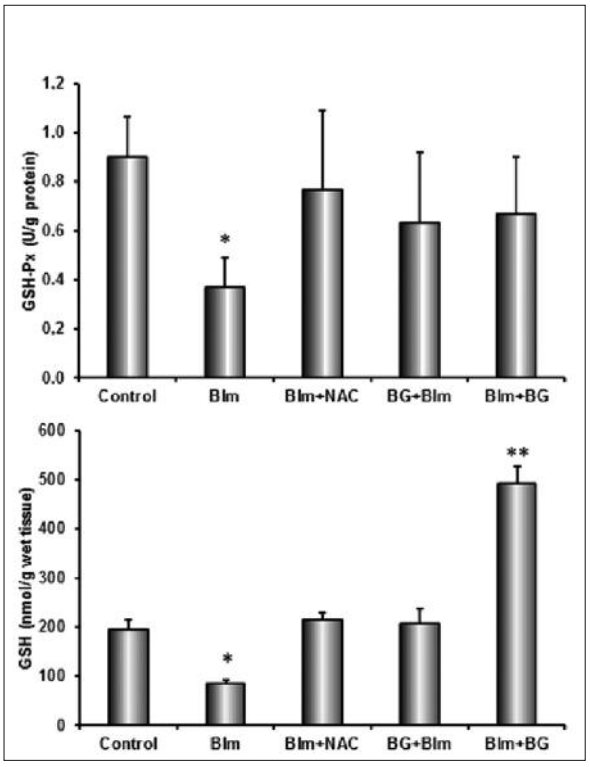

Figure 4. Effect of β-Glucan (BG) and N-acetylcysteine (NAC) on bleomycin (Blm)-induced decreases in the reduced glutathione (GSH) and glutathione peroxidase (GSH-Px) levels in the experimental groups. Bleomycin administration significantly decreased the GSH levels and GSH-Px activities in the lung tissue, which were elevated by the treatment with NAC and β-Glucan administration. Conversely, β-glucan administration after bleomycin instillation significantly elevated the GSH level versus the other groups. Data are presented as mean ± SD. *Significantly lower ($p < 0.05$); **Significantly higher ($p < 0.05$).

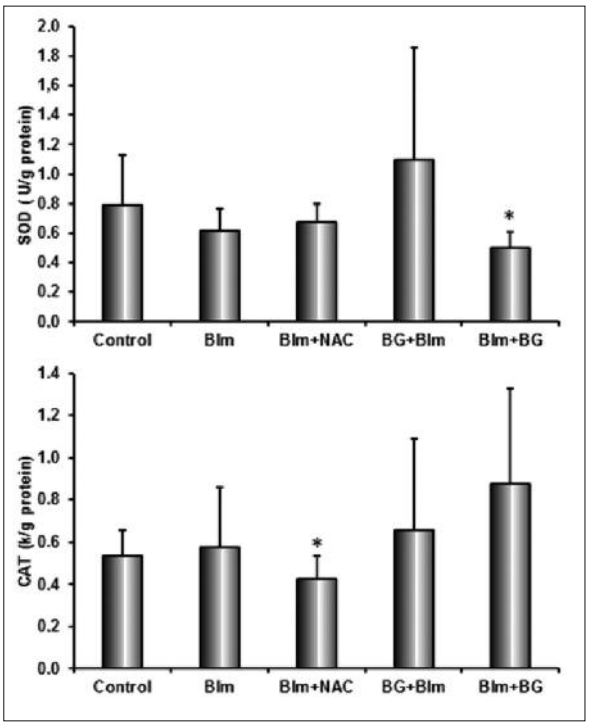

Figure 5. Effect of β-Glucan (BG) and N-acetylcysteine (NAC) on bleomycin (Blm)-induced changes in the catalase (CAT) and superoxide dismutase (SOD) activities in the experimental groups. Bleomycin induced changes were not significanton CAT and SOD activities. But, CAT activity significantly depressed in the bleomycin+NAC group versus the Bleomycin+β-Glucan group. The SOD activity significantly decreased with β-Glucan administration after bleomycin instillation versus the β-glucan+bleomycin group. Data are presented as mean ± SD. *Significantly lower ($p < 0.005$).

The free radical cascade resulting in bleomycin-induced lung fibrosis is associated with series of reactions resulting in the formation of various types of free radical species[42-44]. Inflammation is a major component in the pathogenesis of interstitial lung disease that is coordinated by, in part, endogenous and migrating leukocytes. The epithelial and endothelial pulmonary cells together with leukocytes produce a feedback circle in which stimuli derived from injury responses can activate alveolar and interstitial macrophages. Activated leukocytes further can release reactive oxygen and nitrogen species and proteases that sustain the injury and repair procedures that are considered to contribute to the bleomycin-induced fibrotic processes[45].

Lipid peroxidation, an autocatalytic mechanism mediated by free oxygen radicals, results in both DNA damage and destruction of the cell membranes containing polyunsaturated fatty acids, which are extremely sensitive to oxidation[46]. Therefore, lipid peroxidation has been suggested to be closely related to bleomycin-induced lung injury. MDA is a final product of lipid peroxidation and is widely known as a sensitive marker of the rate of lipid peroxidation[47-49]. We found significantly increased MDA level in only bleomycin but not any therapeutic agent applied rats, reflecting increased lipid peroxidation ratio due to increased oxidative stress. In many previous studies, increased tissue MDA levels were reported after bleomycin administration in rats[9-11]. In our study, bleomycin significantly increased the MDA level of lung tissue, β-glucan administration, especially after bleomycin instillation, suppressed the elevation, indicating decreased degree of oxidative stress and subsequeny lipid peroxidation. Sener et al[50] found that β-glucan significantly decreased MDA levels in kidney and bladder in nicotine-induced oxidative damage in rats. β-glucan has been reported to sig-

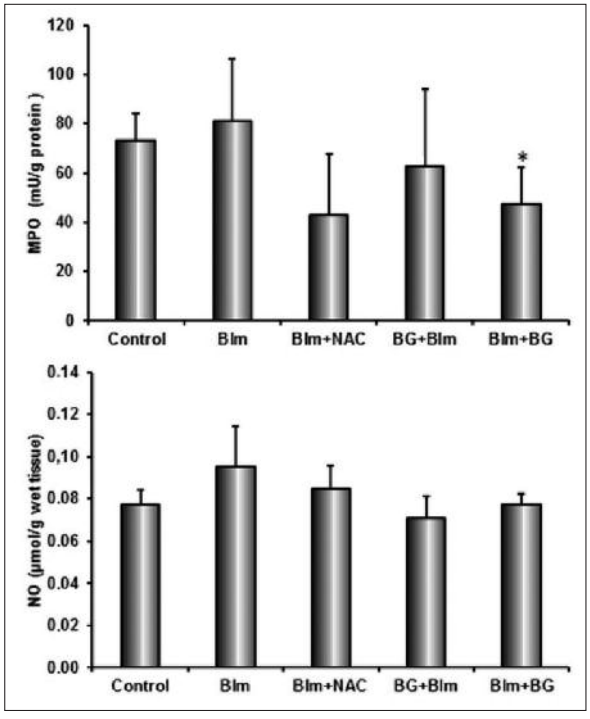

Figure 6. Effect of β-Glucan (BG) and N-acetylcysteine (NAC) on bleomycin (Blm)-induced changes in the nitric oxide (NO) levels and myeloperoxidase (MPO) activities in the experimental groups. The NO level changes were not significantin the all groups. But, MPO activity significantly decreased in the Bleomycin+β-Glucan group versus the control group. Data are presented as mean±SD. *Significantly lower ($p < 0.005$).

nificantly suppress MDA levels in lung tissue during the oxidative stress in burn injury and its remote organ injury[51]. In another study, it has been reported that β-glucan attenuates lung injury induced by aortic ischemia-reperfusion in rats[52]. The authors commented the beneficial effect of β-glucan with reduced systemic inflammatory response, oxidative stress and lipid peroxidation and inhibition of leukocyte migration in the lung tissue[51]. β-glucan immunostimulation is active over abroad spectrum of biological species. β-Glucans are recognized in the major component of bacterial and fungal cell walls as the first defense mechanisms[53]. Detailed analysis of the interaction of human cells with β-Glucan has demonstrated that the complement receptor type 3[54], and novel Dectin-1 receptors are primarily responsible for both the binding and biological effects of β-Glucan[53,55,56]. Human monocyte scavenger receptor, another β-Glucan receptor belonging to the family of pattern recognition receptors, was found on the immunocompetent cells[57].

The soluble β-glucan has a potential in the treatment of chronic diabetic foot and leg ulcers. β-glucan appears to induce a rapid onset of action, and promotes a shorter time healing compared with standard wound care[58]. Apparently, β-glucan treatment promotes ulcer healing more rapidly than observed in the becaplermin studies, and the relative ulcer size reduction seems to be even more pronounced during the very first weeks of treatment. Thus, targeting and modulating macrophage function by β-glucan, seems to be a promising approach to promote healing of diabetic ulcers. It is known that β-glucan stimulates the secretion of an array of cytokines from human monocytes that modulate inflammation[59]. Macrophages are not only important during the inflammatory phase[60], but also coordinate later events in wound healing, suggesting that soluble β-glucan might have a positive impact on several stages of the process. Indeed, a soluble β-glucan from the medical mushroom, *Sparassis crispa*, has been reported to promote wound healing after oral administration in rats with streptozotocin-induced diabetes[61]. Furthermore, supernatants from macrophages stimulated in vitro with a soluble β-glucan from *Saccharomyces cerevisiae* induced cross-linking of collagen after topical administration to wounds in rats[62]. β-glucan can also directly induce production of collagen by dermal fibroblasts *in vitro*[63], suggesting that β-glucan might work through fibroblasts as well. Thus, soluble β-glucan might possibly exert its effect through action on several cell types and processes in the wound bed. Ozkan et al[64] proposed that β-glucan has therapeutic effect for prevention and/or treatment of acetylsalicylic acid induced gastric ulcers. Additionally, Bayindir at al[65] showed that β-glucan amikacin-induced hearing loss in rats may be limited to some extent by concomitant use of β-glucan. Amikacin-induced hearing loss is originated from damaged epithelial cell of the Corti organ, an inner part of ears.

The concentration of intracellular GSH is essential for the protection of thiol and other nucleophilic groups in proteins from the toxic metabolites[66]. NAC is a precursor in the formation of the antioxidant reduced glutathione and possesses the ability of reducing free radicals. This drug has been inclinical used as mucolytic therapy in a variety of respiratory diseases, in the management of acetaminophen overdose, and in the prevention of radiocontrast-induced nephropathy by augmenting reduced glutathione reserves for binding of toxic metabolites. In the context of pulmonary fibrosis,it has shown effectiveness as preventive medication in the bleomycin-induced lung fibrosis[8,51] that there is repaired GSH level in an acetaminophen induced liver toxicity. In this study, bleomycin administration significantly decreased GSH level and NAC and β-glucan started before bleomycine, prevented from these elevations. Controversially, β-glucan post-bleomycin instillation, resulted in significant elevation of GSH in rats.

In the present study, although β-glucan, started before bleomycin instillation, could not completely prevent the increases in MDA levels and fibrosis score, it totally prevented the increase in hydroxyproline content of the lung tissue and decrease in GSH level and GSH-Px activity. These findings suggest the protective and early therapeutic effects of β-glucan. We probably found another additional mechanism rather than as mentioned above. Based on these findings, one could speculate that β-glucan may have direct inhibitor activity on fibroblasts or collagen synthesis, or effects of β-glucan used after triggered inflammation may differ from un-triggered inflammation.

Conclusions

We showed the protective and early therapeutic effect of β-glucan administration on the bleomycin induced pulmonary fibrosis by

histopathological evaluation and measurement of lung hydroxyproline content in rats. This effect may be due to the inhibition of leukocyte accumulation into the lungs, to scavenging of reactive oxygen radicals or enhanced epithelized effects by β-glucan itself. Our data suggest that β-glucan either pre or post-bleomycin might be a promising new neoadjuvant therapeutic agent for IPF or at least for preventing the development of bleomycin-induced lung fibrosis during antineoplastic therapy.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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