Combined Effect of N-Acetyl Cysteine and Clarithromycin on Bleomycin Induced Pulmonary Fibrosis

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Combined therapy with clarithromycin (CLTR) and N–acetyl cysteine (NAC) may be useful in diseases with impaired oxidant-antioxidant balance, fibroblast proliferation, and collagen deposition such as pulmonary fibrosis. Activated inflammatory cells which accumulate in the lower airways may release increased amounts of reactive oxygen species (ROS) when accompanied with a deficiency in glutathione, the major component of the lung antioxidant defense system, leading to lung injury and fibrosis. The aim of this study was to examine the combined effect of CLTR, and NNAC on bleomycin-induced lung fibrosis in rats. Bleomycin was administered by single intra tracheal instillation to Wistar rats to induce lung fibrosis. Rats under study were orally administered with NAC (3 mmol/Kg), and CLTR (20 mg/Kg) from day 4 to 21, after a single intra tracheal instillation of bleomycin (2.5 U/Kg) or saline on day 1. Combined treatment with CLTR and NAC significantly decreased the augmented collagen deposition in bleomycin exposed rats (P< 0.05). Hydroxyproline content was 1.711±0.94 mg/g/tissue, and 1.055±1.83 mg/g/tissue in bleomycin-treated (positive control), and CLTR + NAC treated rats, respectively. CLTR and NAC combined therapy resulted in a significant increase (P< 0.05) in GSH (22%), significant decrease (P< 0.05) in MDA (14%), and significant decrease (P< 0.05) in total protein levels (39%) when compared to positive control rats. The histological assessment using a semi quantitative score showed less collagen deposition, and inflammatory cells in CLTR + NAC treated rats compared to those receiving bleomycin alone. Additionally, hematological and clinical chemistry parameters of blood did not revealed any signs of toxicity of combined treatment. These results indicate that combined treatment with NAC and CLTR improves the pulmonary antioxidant protection, collagen deposition, and thus might be useful in reducing lung damage produced by bleomycin.

Keywords: Pulmonary fibrosis, bleomycin, N–acetyl cysteine, clarithromycin

Pulmonary fibrosis is the pathological hallmark of a heterogeneous group of disorders in which fibroblast proliferation, excessive collagen accumulation, and extensive other extracellular matrix deposition result from a variety of insults to the lung. Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive lung disease associated with interstitial pneumonia of unknown cause (1-3). Although corticosteroids, immunosuppressive agents, cytotoxic drugs, and anti-fibrotic agents have been used for the treatment of IPF over the past decades, however, available therapies has shown no particular effects on survival rate improvement in IPF patients (4, 5).

Bleomycin (BLM) is a chemotherapeutic agent that induces alveolar injury, which leads to subsequent fibrotic extracellular matrix remodeling in a number of animal species. Intra tracheal

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instillation of BLM into the lungs of animal models, results in an alveolar epithelial cell damage, inflammatory response, fibroblast proliferation, and subsequent extracellular collagen deposition, which resembles human fibrotic lung disease, both histologically and physiologically (6).

The aim of the present study was to evaluate the protective effect of N–Acetyl cysteine (NAC) a well-known free radical scavenger, and clarithromycin (CLTR) a neutrophil chemo attract-tant, combined treatment on the BLM induced pulmonary fibrosis.

Materials and methods

Animals

Pathogen-free male and female Wistar rats (8 - 10 weeks), weighing 180 - 220 g at the start of experiments, were obtained from Animal Breeding Facility (Jai Research Foundation, India). The animals used in this study were handled and treated in accordance with the strict guiding principles of the National Institutes of Health guide for the care and use of laboratory animals, and association for assessment and accreditation of laboratory animal care (AAALAC).

Experiments were carried out according to institutional animal ethical committee (IAEC), and were approved by CPCSEA (Proposal N°. JRF-IAEC/2008-09/592 approved on December 27, 2008). Rats were fed with pellet feed (Tetragon Chemic Pvt. Ltd.), and UV sterilized filtered water through reverse osmosis water filter system which was provided *ad libitum*. The rats were kept in a controlled environment (temperature: 22 ± 3 ºC, and relative humidity: 30 to 70%) with a 12 h light / dark cycle.

Experimental groups

Eighty 7-8 week old Wistar rats (40 male + 40 female) were randomly allocated to eight groups of 5 males and 5 females. Female rats were nulliparous, and non-pregnant. Rats were acclimatized to the experimental room conditions for a period of five days, and were randomized into five groups viz.

vehicle control group (G1), BLM control group (G2), BLM + NAC group (G3), BLM + CLTR group (G4), and BLM + (NAC + CLTR) group (G5), before induction.

Experimental procedure

To produce pulmonary fibrosis, BLM at the dose of 2.5 U/Kg of body weight was administered by intra tracheal instillation on day 1 in respective group of animals. NAC, and CLTR were administered orally at the dose of 3 mmol/Kg, and 20 mg/Kg of body weight, respectively from day 4 to 21. Vehicle group was treated with normal saline on day first by intra tracheal instillation followed by oral administration of normal saline from day 4 to 21.

Prior to sacrifice, the blood was collected from orbital plexus of all rats on day 22, and day 23 for hematological, and clinical parameter estimation, respectively. Rats were sacrificed by carbon dioxide asphyxiation. All animals were subjected to gross necropsy. The organ weights were recorded, and subjected to histopathological examination. For the estimation of biochemical parameters of rat lungs, the right lung of 6 rats (3 male, and 3 female), and for histopathology evaluation, the left lung of 6 rats (3 male, and 3 female) were randomly selected from each group. For the estimation of total cell count, bronchoalveolar lavage fluid was collected from lungs of 4 rats (2 male, and 2 female).

All animals were observed twice a day for mortality, and any sign of morbidity. Body weights were recorded individually for all animals at randomization, start of dosing, and four days interval thereafter. The food consumption was calculated at weekly intervals throughout the study period.

Blood analyzes

At the end of the experimental period, blood samples were collected from all the surviving animals for hematological, and clinical estimation. Blood samples were collected by retro orbital plexus puncture with the help of a fine capillary tube under light anesthesia (Isoflorane). Prior to blood

collection, all animals were fasted overnight with free access to water. Approximately 0.5 ml of blood was collected in vials containing each 4% EDTA, and 3.2% sodium citrate for hematological parameters analysis. Hematological parameters (white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT)) were estimated in blood by using hematology analyzer. Clinical parameters (alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, total protein) were estimated in blood by using BCT 2000 analyzer.

Bronchoalveolar lavage fluid (BALF)

BALF was obtained by washing the right lung four times with 4 mL aliquots of saline through a tracheal cannula. Cell suspensions were concentrated by low speed centrifugation, and the cell pellet was resuspended. Total cell counts were made in a hemocytometer. Differential cell counts were determined from cytospin preparations by counting 200 cells stained with Leishman reagent or May-Grunwald-Giemsa.

Biochemical analyzes

Oxidative stress was assessed by measuring the amount of thio-barbituric acid reactive substances (TBARS), and reduced glutathione (GSH) as the non-enzymatic antioxidant marker. All biochemical parameters were assessed on a 10% homogenate which was prepared from 0.5 g of lung tissue homogenized in 5 ml of homogenizing buffer (250 mM sucrose, and 12 mM Tris-HCl), and then centrifuged at 3,000xg for 10 min. The supernatant was collected, and stored at -80 °C.

Total protein estimation

Lung's total protein was estimated by Lowry's method (7) with slight modifications. Briefly, 0.2 ml of supernatant was taken in a test tube, volume was adjusted to 1 ml with distilled water (DW), and then 5 ml of Lowry's reagent (freshly prepared mixture of 0.5% w/v copper sulphate in 1% w/v sodium potassium tartrate solution, and 2% w/v alkaline

sodium carbonate in 0.1 N NaOH in the ratio of 1:50, respectively) was added, and mixed thoroughly. The mixture was allowed to stand for 10 min at room temperature, and then 0.5 ml of 1:1 v/v diluted Folin Ciacalteu reagent was added. Contents were vortexed, and incubated at 37 °C for 30 min. Then, absorbance was determined spectrophotometrically at 660 nm (UV Pharmaspec 1700, Shimadzu, Japan) against suitably prepared blank. A standard curve using 10-40 μg of bovine serum albumin (BSA) was plotted. The amount of total protein was expressed in mg%.

TBARS estimation

The whole lung TBARS level was measured by the method of Okhawa et al. (8) with slight modifications. Briefly, 0.2 ml lung homogenate was added into a test tube, followed by addition of 0.2 ml sodium dodecyl sulphate (SDS), 1.5 ml of 30% acetic acid (pH 3.5), 1.5 ml of 0.8% thiobarbituric acid (TBA), and the volume was adjusted to 4.0 ml with DW. The test tubes were incubated at 95 °C for 60 min, and then cooled. 1.0 ml of DW and 5.0 ml of n-butanol: Pyridine (15:1 v/v) mixture were added to the test tubes, and centrifuged at 4,000xg for 10 min. The absorbance of developed color in organic layer was measured spectrophotometrically at 532 nm (UV Pharmaspec 1700, Shimadzu, Japan). The amount of TBARS was calculated using a molar extinction coefficient of 1.56×10^5 M/cm and the results were expressed as nmol of TBARS / mg total protein.

GSH estimation

The whole lung GSH level was measured by the method of Sedalk and Lindsay (9) with slight modifications. Briefly, the proteins were precipitated from the tissue homogenate with 50% w/v chilled trichloroacetic acid. Samples were kept in ice bath, and were centrifuged at 3,000xg for 15 min at 4 °C. GSH levels were measured in the supernatant by adding 0.05 ml of 10 mM of DTNB {5,5'-dithiobis (2-nitro benzoic acid)} freshly prepared solution (3.96 mg/ml in 0.1 M potassium phosphate buffer pH 7.4) just before measuring the absorbance

spectrophotometrically at 540 nm (UV Pharmaspec 1700, Shimadzu, Japan). Different concentrations of GSH standard were also processed similarly to prepare a standard curve. Results were expressed as nmol of GSH / g of protein.

Estimation of hydroxyproline

Lung hydroxyproline content was measured by homogenizing lung samples in 6 N HCL for 18 h at 110 °C. The hydrolysate was then neutralized with 2.5 M NaOH. Aliquots (2 ml) were analyzed for hydroxyproline content after the addition of 1 ml of chloramine T, 1 ml of perchloric acid, and 1ml of dimethylaminobenzaldehyde. Samples absorbance was read at 550 nm in a spectrophotometer. Results were expressed as mg of hydroxyproline per lung.

Pathology and histopathological examination

All animals were euthanized by carbon dioxide asphyxiation, and subjected to a full gross necropsy under the direct supervision of a veterinary pathologist at the end of the treatment, and recovery periods. The animals were examined carefully for external abnormalities. The thoracic, and abdominal cavities were cut open, and a thorough examination of the organs was carried out to detect abnormalities. Lungs were removed, examined and weighed where appropriate, and placed in 10% neutral formalin for future histopathological examination.

Histopathological examination was performed for lungs. After dehydration, and embedding in paraffin wax, sections of tissues were cut at 3-5 µm thickness with microtome, and stained with hematoxylin and eosin. Histopathological examination was performed in all preserved lung tissues of all animals.

Organ weight was determined for lungs and relative organ weight was calculated.

Statistical analysis

The raw data were processed by using GraphPad Prism version 5 statistical software for statistical calculation to get group means and standard deviations with significance between the control and treated groups. Statistical methods (one way ANOVA followed by Dennett's- t test) were employed to assess the significance among different groups with significance level of $P < 0.05$.

Results

Body weight and feed consumption

Single intra tracheal administration of BLM (2.5 U/Kg) resulted in a marked decrease in body weight on days 4, 8 and day 12 as compared to the saline-treated control group. However, body weight of NAC + CLTR treated rats remained comparable to the control group throughout the period of experiment (Figures 1-4).
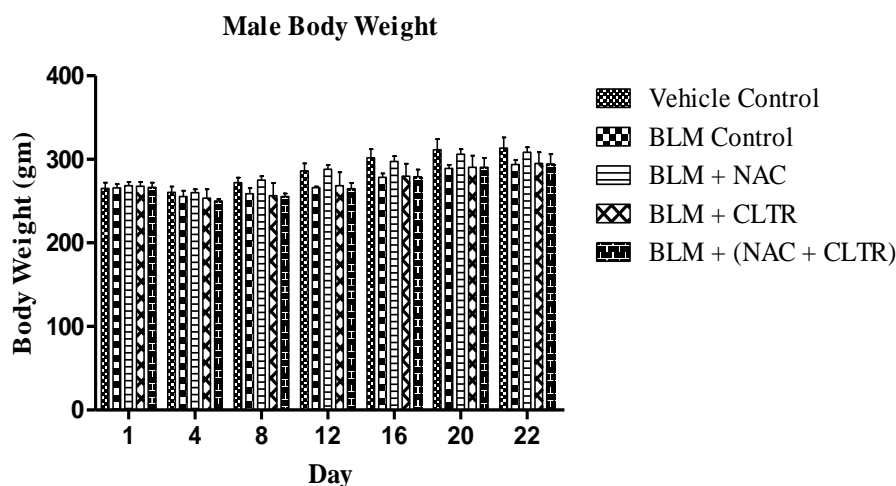

Figure 1. Male rat's body weight. Values are represented as Mean±SEM (N= 5), and differences between all groups and vehicle control group were non-significant (P> 0.05).
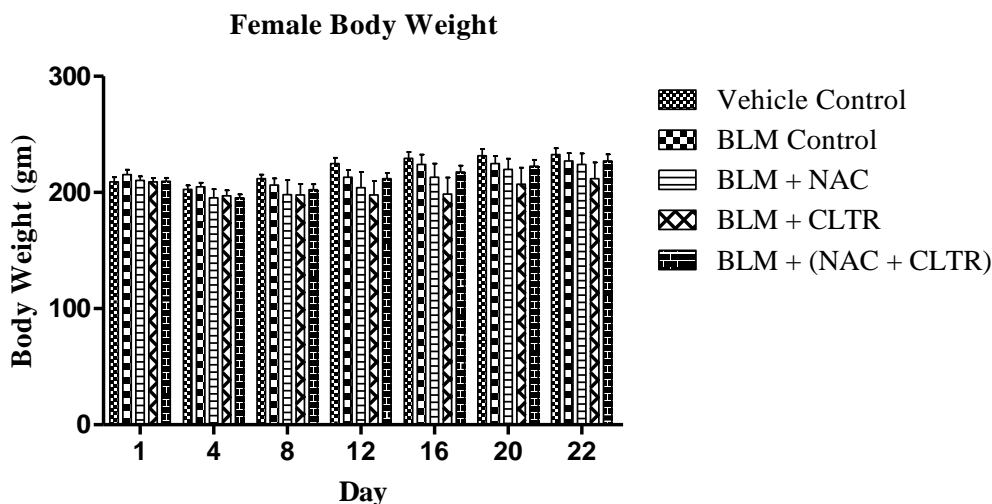
Female Body Weight

Figure 2. Female rat's body weight. Values are represented as Mean±SEM (N= 5), and differences between vehicle control group and all test groups (except BLM + CLTR) were non-significant (P> 0.05).

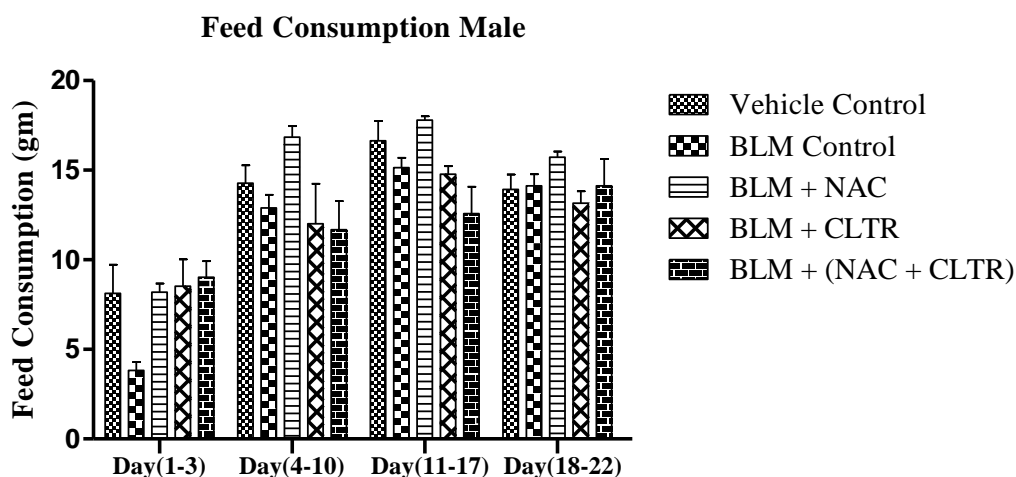## Feed Consumption Male

Figure 3. Male rat's feed consumption. Values are represented as Mean±SEM (N=5), and differences between all groups and vehicle control group were non-significant (P> 0.05).
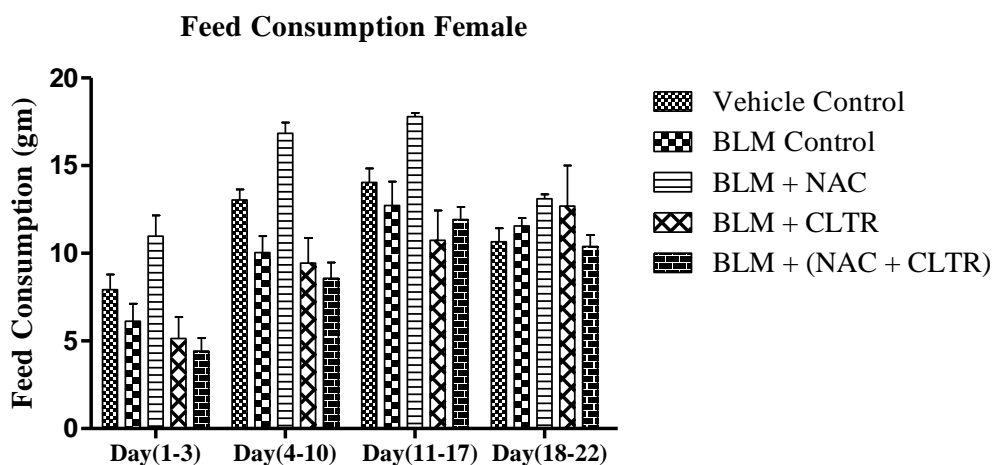
Feed Consumption Female

Figure 4. Female rat's feed consumption. Values are represented as Mean±SEM (N=5), and differences between all groups and vehicle control group were non-significant (P> 0.05).

Hematology analyzes

No significant difference was observed in any of the hematological parameters when compared with vehicle control group in both male and female rats (Tables 1 and 2).

Clinical parameters analysis

Significant differences were observed in clinical parameters in some groups when compared with vehicle control group in both male (Table 3) and female rats (Table 4).

In male rats, a significant decrease was observed in ALT levels in G2, and in AST levels in G5 (P< 0.05). However, no significant difference was observed in creatinine and total protein levels.

In female rats, a significant decrease (P<0.05) in AST levels was observed in G3. No significant difference was observed in ALT, creatinine, and total protein levels in all groups of female rats.

Total and differential cell count in bronchial-veolar lavage fluid

Typically, total fluid recovery exceeded 80%, and the percentages of fluid recovered did not significantly differ among experimental groups. The pulmonary inflammation response after BLM administration, as reflected by the cells recovered in BALF, is shown in Table 5.

Significant decrease in macrophages (P<0.05),

and increase in lymphocyte, and neutrophil count was observed in G2 and G3 (P< 0.05). All treated groups exhibited significant decrease in total cell count when compared to the vehicle control group (P< 0.05).

Biochemical analyzes of lung tissues

Significant differences were observed in biochemical parameters in some groups when compared with vehicle control group in both male and female rats (Table 6).

Significant decrease in GSH levels (P< 0.05), and increase in total protein, and hydroxyproline levels was recorded in G2 (P< 0.05). Moreover, a significant increase in MDA/TBARS levels was observed in all treated groups when compared with vehicle control group (P< 0.05).

Pathology and histopathological examination

No external and internal gross finding was observed in any of the groups in both male and female rats.

There was a significant increase in lung weight in G2 when compared to vehicle control group.

Histopathology examination of lungs in groups G2 to G5 revealed various pathological lesions viz., increase in cellular thickening of intracellular septa, mononuclear cells/ peripheral blood mononuclear cells/ macrophages infiltration, fibrosis, tissue

Table 1. Male rats' hematological parameters					
Blood Parameter	**G1**	**G2**	**G3**	**G4**	**G5**
WBC (x 10³ /µL)	6.74±0.94	7.80±1.08	6.68±1.19	8.02±3.47	8.40±2.61
RBC (x 10⁶/ µL)	8.10±0.55	7.95±0.52	7.66±0.22	6.44±1.46	7.16±1.59
HGB (g/dL)	15.90±0.96	15.98±0.80	15.52±0.33	13.42±2.49	14.16±2.93
HCT (%)	42.02±2.70	42.24±2.77	40.80±1.61	34.84±7.12	37.30±8.07
MCV (fL)	51.86±0.38	53.14±0.27	53.26±0.59	54.32±2.97	52.18±1.16
MCH (pg)	19.62±0.30	20.12±0.56	20.28±0.53	21.00±1.47	19.84±0.47
MCHC (g/dL)	37.82±0.38	37.88±1.01	38.10±1.29	38.66±1.13	38.04±0.62
PLT (x 10³/ µL)	756.±174.92	757±149.37	633.4±259.4	608.2±245.7	707.6±380.3

G1: vehicle control group; G2: BLM control group; G3: BLM + NAC; G4: BLM + CLTR group; G4: BLM + (NAC + CLTR) group; G5: before induction; WBC: white blood cells; RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet. Values are represented as Mean±SEM (N=5), and differences between all groups and vehicle control group were non-significant (P> 0.05).

proliferation in most of the animals. Focal perivascular edema was also seen in one case from G2. The lesions were mild to severe, and more prominent in G2, indicative of the induction of proliferative infiltration in lungs. Groups treated

with drugs post induction, i.e. G3 and G4, showed mild to moderate lesions, whereas G5 animals showed better recovery with very mild degree of above lesions (Figure 5 A-E).

Table 2. Female rats' hematological parameters

Blood Parameter	G1	G2	G3	G4	G5
WBC (x 10^3 /μL)	6.74±0.94	7.80±1.08	6.68±1.19	8.02±3.47	8.40±2.61
RBC (x 10^6/ μL)	8.10±0.55	7.95±0.52	7.66±0.22	6.44±1.46	7.16±1.59
HGB (g/dL)	15.90±0.96	15.98±0.80	15.52±0.33	13.42±2.49	14.16±2.93
HCT (%)	42.02±2.70	42.24±2.77	40.80±1.61	34.84±7.12	37.30±8.07
MCV (fL)	51.86±0.38	53.14±0.27	53.26±0.59	54.32±2.97	52.18±1.16
MCH (pg)	19.62±0.30	20.12±0.56	20.28±0.53	21.00±1.47	19.84±0.47
MCHC (g/dL)	37.82±0.38	37.88±1.01	38.10±1.29	38.66±1.13	38.04±0.62
PLT (x 10^3/ μL)	756.±174.92	757±149.37	633.4±259.4	608.2±245.7	707.6±380.3

Gl: vehicle control group; G2: BLM control group; G3: BLM + NAC; G4: BLM + CLTR group; G4: BLM + (NAC + CLTR) group; G5: before induction; WBC: white blood cells; RBC: red blood cells; HGB: hemoglobin; HCT: hematocrit; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet. Values are represented as Mean±SEM (N=5), and differences between all groups and vehicle control group were non-significant (P> 0.05).

Table 3. Male rats' clinical parameters

Clinical Parameter	G1	G2	G3	G4	G5
ALT	48.30±3.60	39.57±6.63*	60.29±41.57	51.91±6.79	47.97±4.38
AST	160.88±23.01	171.45±63.14	149.08±27.98	166.82±55.86	116.02±18.6*
Creatinine	0.69±0.1	0.62±0.12	0.62±0.14	0.71±0.04	0.69±0.06
Total protein	7.48±.014	7.34±0.54	7.34±0.34	7.55±0.43	7.00±0.47

Gl: vehicle control group; G2: BLM control group; G3: BLM + NAC; G4: BLM + CLTR group; G5: BLM + (NAC + CLTR) group; AST: alanine aminotransferase; ALT: aspartate aminotransferase. Values are represented as Mean±SD (N=5). *: P< 0.05 compared with the vehicle control group.

Table 4. Female rats' clinical parameters

Clinical Parameter	G1	G2	G3	G4	G5
ALT	45.47±8.91	41.80±5.52	46.46±12.53	46.82±13.43	45.50±7.47
AST	160.68±13.37	138.99±17.23	126.51±22.7*	138.55±22.45	140.79±14.71
Creatinine	0.71±0.13	0.66±0.08	0.63±0.08	0.61±0.14	0.46±0.26
Total protein	8.22±0.49	7.59±0.42	7.55±0.56	7.65±0.98	7.87±0.74

Gl: vehicle control group; G2: BLM control group; G3: BLM + NAC; G4: BLM + CLTR group; G5: BLM + (NAC + CLTR) group; AST: alanine aminotransferase; ALT: aspartate aminotransferase. Values are represented as Mean±SD (N=5). *: P< 0.05 compared with the vehicle control group.

Table 5. BALF parameters of male and female rats

Clinical Parameter	G1	G2	G3	G4	G5
Neutrophils %	2.375±0.48	29.9±1.31*	27.50±1.69*	7.125±1.0	4.63±1.11
Macrophages %	82.25±1.26	65.75±1.55*	68.50±1.47*	80.5±1.68	83.5±1.29
Lymphocytes %	15±1.41	4.125±1.974*	5.50±0.791*	12±2.1	11.62±1.49
Total cells*10^5/ml	1.28±0.06	5.66±0.28*	4.55±0.18*	2.39±0.09*	2.125±0.10*

G1: vehicle control group; G2: BLM control group; G3: BLM + NAC; G4: BLM + CLTR group; G5: BLM + (NAC + CLTR) group. Values are represented as Mean±SD (N= 4). *: P< 0.05 compared with the vehicle control group.

Table 6. Biochemical parameters of male and female rats

Biochemical Parameter	G1	G2	G3	G4	G5
GSH (mg/g/tissue)	8.278±0.42	7.037±0.42*	8.685±1.128	8.685±0.657	8.63±0.769
MDA/TBARS (nmol/g/tissue)	15.278±1.40	19.85±0.43*	17.927±0.6*	18.259±0.3*	17.019±0.28
Total protein (mg/ml)	3.33±0.353	5.642±0.25*	3.197±0.48	3.794±0.556	3.437±0.396
Hydroxyproline (mg/g/tissue)	1.069±.0839	1.711±.094*	1.114±.0804	1.095±.221	1.055±.183

GSH: gluthatione; MDA: malondialdehyde; TBARS: thio-barbituric acid reactive substances. Values are represented as Mean±SD (N= 6). *: P<0.05 compared with the vehicle control group.
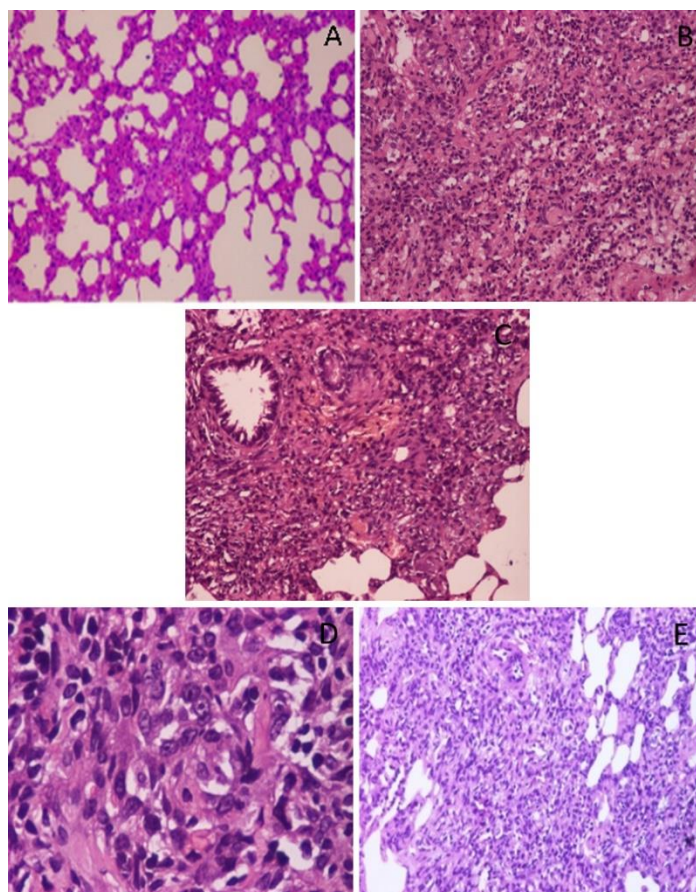

Figure 5. Haematoxyline and Eosin staining of lung tissues. A: G1; B: G2; C: G3; D: G4; and E: G5. X40 magnification.

Discussion

BLM, a glycopeptide antibiotic, is generally used for the treatment of lymphomas, squamous cell carcinoma, and testicular tumors (10). Due to its toxic effect on epithelial cells, BLM is also used for the treatment of malignant pleural or pericardial effusions. However, the effective use of BLM in chemotherapy is greatly limited, since it causes a dose-dependent interstitial pneumonitis that often progresses to IPF. In addition, several studies have demonstrated that BLM administration in rats decreased the antioxidative capacity, and increased the oxidative stress in the lung tissue, which aggravated pulmonary fibrosis (11, 12). Based on these evidences, we employed a BLM rat model to investigate the putative protective effect of NAC and CLTR against pulmonary fibrosis.

It is well known that oxygen free radicals can injure lipids, proteins, and DNA, and thus may contribute to the loss of enzymatic activity or structural integrity. Oxidant induced lipid peroxidation causes a loss of membrane stability, and integrity leading to increased trans epithelial permeability (13). Our observation of increased cell count in BALF supports this hypothesis since there was also a significant increase in the MDA levels, an index of lipid peroxidation in the lung tissue. Several studies have also shown the elevated lipid peroxidation products in the BALF and lung tissue of BLM-treated rats (13-15). Moreover, in the present study NAC prevented BLM induced lipid peroxidation; this caused a dramatic decrease in cell count, suggesting a membrane protective effect of this antioxidant agent. In accordance with the increased oxidant production and lipid peroxidation, GSH levels were decreased. Because GSH is essential for the protection of thiol and other nucleophilic groups in proteins, from the toxic oxygen radicals, the concentration of intracellular GSH, is therefore the key determinant of the extent of BLM-induced lung injury (16, 17). It has been demonstrated that cellular GSH concentration can be influenced by exogenous administration of antioxidants (13-15). In the present study, depletion of GSH stores following BLM administration was restored by NAC, and CLTR treatment. Considering the mechanism of antioxidant activity, NAC which is a well-known free radical scavenger, and a stimulator of several antioxidative enzymes (e.g. superoxide dismutase, glutathione peroxidase, and glutathione reductase) may be important in determining GSH homeostasis within the cell, and may also determine the total amount of GSH within the cell (18). Thus, modulation of GSH metabolism by NAC, and CLTR might present a useful adjuvant therapy in the BLM treatment.

Observations suggest that reactive oxygen species (ROS) play a role in the recruitment of neutrophils into damaged tissue, but activated neutrophils are also a potential source of ROS (19). Although it is not certain whether neutrophil accumulation, and activation are the causes or the results of injury, increasing evidences suggest that mesengial cells, and neutrophils release chemotactic substances (e.g., interleukin 8), which further promote neutrophil migration to the tissue, activate neutrophils, and increase the damage (20). CLTR which is 14 membered macrolide, acts as a neutrophil chemo attractant, resulting in attenuation of neutrophil migration into inflamed tissues, and inhibits the expression of adhesion molecules. It has been hypothesized that activated inflammatory cells which accumulate in the lower airways release harmful amounts of ROS, that results in lung injury, and proliferation of fibroblasts in alveolar walls (21). Our results together with biochemical, and morphological data agree with this hypothesis, since BLM-induced increase in fibrotic activity (as assessed by the lung collagen content) is also reduced by NAC, and CLTR treatment. This finding suggests that NAC, and CLTR combined therapy have an additional protective effect on inflammation-induced production, and deposition of extracellular matrix components, and thus reduces/prevents the development of pulmonary fibrosis.

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Conflict of interest

The authors declare that they have no competing interest.

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