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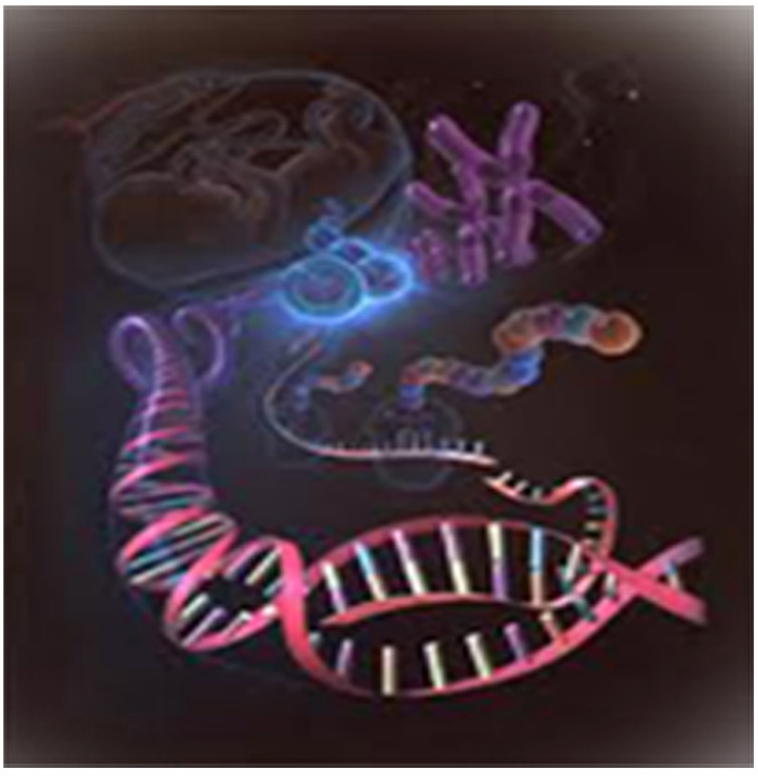

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Research Paper

ORAL ADMINISTRATION OF BOSENTAN ATTENUATES THE BLEOMYCIN INDUCED PULMONARY FIBROSIS IN MICE

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Idiopathic Pulmonary Fibrosis (IPF) is characterized by alveolitis with epithelial, endothelial damage leading to fibrosis. As signaling of endothelin-1 was involved in IPF, the effect of bosentan a non-specific endothelin receptor antagonist was determined in a mouse model of bleomycin induced pulmonary fibrosis. In the present study, mice were instilled with intra-tracheal instillation of bleomycin (0.05U) and were administered with bosentan at 100 mg/kg body weight. The treatment with bosentan significantly ($p \leq 0.05$) prevented bleomycin induced mortality and loss of body weight. On day 7, bosentan significantly ($p \leq 0.05$) attenuated bleomycin induced increase of total and differential inflammatory cell counts, total proteins, edema, MPO activity and inflammatory cell infiltration in lung tissue. The activities of superoxide dismutase and catalase were restored by bosentan treatment which lowered in bleomycin administered mice. Bosentan treatment significantly attenuated bleomycin induced increase in fibrosis score, collagen deposition and hydroxyproline levels. On day 21, treatment with bosentan significantly attenuated α-smooth muscle actin and collagen-I gene expression levels and matrix metalloproteinases 2 and 9 activities. The results revealed that bosentan exerted enhanced protection against bleomycin induced inflammation and fibrosis.

Keywords: Pulmonary fibrosis, Endothelin-1, Bosentan, Collagen

INTRODUCTION

Idiopathic Pulmonary Fibrosis (IPF) is one of the most common forms of interstitial lung disease and is characterized by alveolar damage and increased scar tissue production that results in fibrous destruction of lung parenchyma and subsequent lung dysfunction (Wilson and Wynn, 2009). Currently 5 million people worldwide are affected by the IPF with over 200,000 patients in United States and median survival time is approximately 3-5 years (Raghu *et al.*, 2006). Although etiology of IPF has not been clearly under stood, its principal pathologic characteristics include epithelial and endothelial damage, inflammation followed by the proliferation of type II pneumocytes and fibroblasts

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leads to collagen deposition. The pathogenesis of IPF is complex, involving mediators, cytokines and growth factors produced by a variety of cells within the lung (Crystal *et al*., 1984). One potential mediator is endothelin-1 (ET-1), a vasoactive peptide first isolated and purified from the culture medium of porcine aortic endothelial cells (Yanagisawa *et al.*, 1988; Inoue *et al*., 1989). The effects of ETs are mediated by two principal specific receptors, ET-A, which has a substantially greater affinity for ET-1 than ET-2 and ET-3 (Arai *et al.*, 1990), and ET-B, with a similar affinity for ET-1, -2, and -3 (Sakurai *et al.*, 1990). ET-1 is well known for the potent vasoconstrictor activity, but it also serves as a bronchoconstrictor, chemoattractant, fibroblast cell mitogen, and stimulant of collagen synthesis (Fagan *et al*., 2001). ET-1 has been extensively implicated in the pathogenesis of pulmonary fibrosis. Patients with scleroderma have elevated levels of ET-1 in plasma, Bronchoalveolar Lavage Fluid (BALF) and lung tissue (Kadono *et al.*, 1995; Cambrey *et al.*, 1994). In IPF patients, plasma ET-1 levels are elevated (Uguccioni *et al.*, 1995) and histological expression correlates with disease severity (Uguccioni et al., 1995; Saleh et al., 1997). Intra-tracheal administration bleomycin increases (Mutsaers et al., 1998; Hocher et al., 2000). ET-1 in the lung may be important in the initial events in lung injury by activating neutrophils to aggregate, release of oxygen radicals and cytokine production (Helset *et al*., 1996; Ishida *et al.*, 1994; Helset *et al.,* 1994). Transforming growth factor-β1 (TGF- β1) and tumor necrosis factor-α (TNF-α) are key profibrotic cytokines induced by ET-1 (Khalil *et al.*, 1989; Piguet *et al.*, 1989). It is also profibrotic by stimulating fibroblast replication, migration, collagen synthesis and conversion of fibroblasts into contractile myofibroblasts (Shahar *et al.*, 1999). ET-1 may thus play an important role in the initial injury, inflammatory and eventual fibrotic reparative process of lung. We hypothesized that ET-1 is an important mediator in pulmonary fibrosis, hence present study was designed to investigate the potential protective effect of bosentan-non specific ET receptor antagonist in bleomycin induced pulmonary fibrosis in mice

MATERIALS AND METHODS

Reagents

Bleomycin sulfate and bosentan were purchased from Sigma, USA, and all other chemicals used for the present study were purchased from Himedia (Mumbai, India).

Experimental Animals and Dosing

Healthy, female C57BL/6J mice weighing 20-23 g were purchased from National Institute of Nutrition (Hyderabad, India). All the animals were housed in polypropylene cages in pathogen free experimental rooms and were maintained at a room temperature of 22 ± 3^{o}C and relative humidity of 55 ± 6% with 12:12 h light/dark cycles for one week prior to experiment. Food and water were provided *ad libitum*. All the experiments were performed in accordance with the Institutional Animal Ethics Committee (IAEC). After randomization, the animals were divided into three groups and were anesthetized with intra-peritoneal injections of ketamine and xylazine at100 and 10 mg/kg body weight respectively. Using aseptic techniques, a single incision was made at the neck, and the muscle covering the trachea was snipped to expose the tracheal rings. A single intratracheal instillation of 0.05 U bleomycin sulfate in 50 µL of sterile 0.9% NaCl was performed using a 27-gauge needle for each

animal. Following were set of groups for the present study; Group- I animals were received intra-tracheal instillation of sterile 0.9% NaCl at day 0 and was given vehicle - 0.5% carboxymethyl cellulose through oral gavage. Group- II animals were received intra-tracheal instillation of bleomycin at day 0 and were given vehicle alone. Group- III animals were received intra-tracheal instillation of bleomycin and were administered with bosentan at 100 mg/kg b.wt in vehicle by oral gavage at dosage volume of 10 mL/kg b.wt three days prior to intratracheal bleomycin instillation and continued daily once up to 21 days after bleomycin instillation. Appropriate numbers of mice were sacrificed on day 7 and 21 after bleomycin administration. BALF was collected for cell analysis and the remaining supernatant was stored at -20°C for further analysis. Right lung was excised and stored at -80°C for biochemical assays. Left lung was collected and stored in 10% neutral buffered formalin for histological analysis.

BALF Cell Analysis

Briefly, on day 7 of post bleomycin instillation the mice were euthanized and the trachea was immediately cannulated with intravenous polyethylene catheter equipped with 24-gauge needle attached to 1 mL syringe. The lungs were lavaged with 0.8 mL of PBS each time for four times and withdrawn from the lungs via an intratracheal cannula. The recovery volume was approximately 0.7 mL each time. The lavage fluids were pooled and centrifuged at 1000 rpm for 10 min at 4°C and the supernatant was collected and stored at –20°C until further use. The cell pellets were re-suspended in 1 mL of PBS, and 10 or 20 µL of cell suspension was used to enumerate cell population using hemocytometer in the presence of trypan blue. The microscopic slides were prepared manually and stained with 5% Giemsa. Differential counting was performed by counting at least 400 cells from randomly chosen areas for each sample and the results were expressed as total number of cells/mL (Furonaka *et al*., 2009).

Measurement of Fluid Content in Lung

The right lung was carefully excised, and wet weight was measured. Subsequently, the lung was dried for 24 h at 60°C, and its dry weight was measured. The ratio between wet and dry lung weight is the measure of edema formation.

Superoxide Dismutase (SOD) Assay

SOD activity was measured based on the ability of the enzyme to inhibit superoxide anion dependent autoxidation of pyrogallol. The 10% homogenate of lung tissues was prepared in 50 mM sodium phosphate buffer (pH 7.2) using mortar and pestle. Cytosolic extracts were isolated from homogenates by employing centrifugation at 12,000 rpm for 30 min at 4°C. To 0.1 mL of cell extract (400 µg of protein) prepared in 50 mM sodium phosphate buffer (pH 7.2), 0.7 mL of 50 mM Tris (pH 8.0) 0.1 mL of 10 mM diethylaminetriamine pentaacetic acid and 0.1 mL of 10 mM pyrogallol were added, increase in optical density was monitored for 3 min at 420 nm with UV Visible spectrometer (Shimadzu, Japan). One unit of SOD is defined as the amount required to inhibit 50% pyrogallol auto oxidation per min under defined assay systems (Manikonda *et al.*, 2012). The results were expressed as units/gram tissue.

Catalase Assay

The reaction mixture containing 20 mM hydrogen peroxide in 50 mM sodium phosphate buffer (final

volume 3 mL) was added to the cytosolic extract (0.2-0.4 mg protein). The change in absorbance was monitored after 5 min at 240 nm in UV Vis spectrophotometer (Shimadzu, Japan). One unit of catalase activity is defined as the activity required for decomposing 1 μmol of hydrogen peroxide per min per mg protein at pH 7.0 and at 25°C (Manikonda *et al.*, 2012). The results were expressed as units/gram tissue.

Myeloperoxidase (MPO) Assay

Ten percent homogenates of lung tissues were prepared in 50 mM phosphate buffer (pH 6.0) containing 0.5% of hexadecyltrimethyl ammonium bromide using mortar and pestle. The homogenate samples were subjected to three freeze-thaw cycles and centrifuged at 15,000 rpm for 20 min. 0.1 mL of the supernatant combined with 1.8 mL 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/mL O - dianisidine hydrochloride and 0.0005% of hydrogen peroxide. The change in absorbance for 5 min at 465 nm was measured with spectrophotometer (Shimadzu, Japan). One unit of myeloperoxidase activity is defined as that degrading 1 μmol of peroxide per minute at 25°C (Yan *et al.*, 1998). The results were expressed as u/mg tissue.

Hydroxyproline Assay

Hydroxyproline content of lungs was determined as a biochemical quantitative measure of collagen deposition, was determined as previously described (Wossner *et al.*, 1961). The 10% homogenates in PBS (pH 7.4) were hydrolyzed with 6 N hydrochloric acid (HCl) at 120°C for 8 h. One milliliter of 0.05 M chloramine -T solution was added to acid extract and incubated for 20 min at room temperature and 1 mL of Ehrlich's solution was added and incubated for 20 min at 65°C. The samples were cooled and the OD was read at 550 nm. The data was expressed as μg of hydroxyproline/g tissue as determined by the standard curve.

Reverse Transcriptase - PCR

For determining the steady state levels of mRNA expression, a semiquantitative-PCR was performed. Total RNA was extracted from the lung tissues with TRIzol reagent (Fermentas) according to the instructions of the manufacturer. The concentration of total RNA was calculated by measuring OD at A260. The first strand of cDNA was synthesized using 2 μg of RNA in 20 μL of reaction buffer by reverse transcription using RevertAid M-MuLV Reverse Transcriptase and oligo (dT) primer (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific). Aliquots of total RNA (1.0 μg) from each sample was transcribed into cDNA by revertide cDNA synthesis kit (Fermentas) according to the instructions of manufacturer. PCR was carried out using a standard PCR kit on 2 μL aliquots of cDNA and Taq polymerase using gene specific primers. About 20 to 25 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for amplification in the linear range were used, followed by a final extension step at 72° C for 7 min. The PCR products were size fractionated on agarose gels and detected by ethidium bromide staining. Band intensities were quantified by the densitometry using Image J software (NIH, Bethesda, MD, USA). The sequences of primer sets are specified as follows, collagen-I 5' GAGCGGAGA GTACTGGATCG 3' (sense) and 5'TACTCGAAA CGGGAATCCA 3'(antisense); α-SMA 5'CAGGGA GTAATGGTTGGAAT 3' (sense) and 5'TCTCAAA CATAATCTGGGTCA 3'(antisense); β-actin 5'TGCGTGACATCAAAGAGAAG 3' (sense) and 5'GATGCCACAGGATTCCATA 3'(antisense).

Measurement of MMPs by Gelatin Zymography

MMPs have been shown to actively participate in the pathogenesis of IPF through ECM degradation and basement disruption facilitating proliferation and migration of inflammatory cells and fibroblasts. MMP-2 and MMP-9 are the two major gelatinases elevated in IPF patients (Pardo and Selman *et al*., 2006). To determine the MMP-2 and MMP-9 activities gelatin zymography was performed as described (Sumii *et al.*, 2002). Briefly, 10% homogenates of lung tissues was prepared in lysis buffer (50 mM Tris HCl, 150 mM NaCl and 1% Triton X-100, pH 7.4). The total protein concentration of clear supernatants was determined by the Bradford reagent (Bio-Rad Laboratories). Homogenate containing 20 µg of total protein was mixed with 2X non-reducing SDS PAGE sample buffer (126 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.005% bromophenol blue) and electrophoresed in 10% polyacrylamide gel containing 2 mg/mL gelatin. Following electrophoresis, gels were washed twice for 20 min with 2.5% Triton X-100 in Tris buffered saline (50 mM Tris HCl, pH 7.5, 150 mM NaCl) buffer to allow protein to renature. Gels were then incubated for 18-20 h at 37°C in incubation buffer (50 mM Tris HCl, pH 8, 10 mM $CaCl_2$, and 0.02% NaN_3), stained with Coomassie R 250 for 30 min and destained for 1 h. Gelatin-degrading enzymes were visualized as clear bands, indicating proteolysis of the substrate protein. Band intensities were quantified using Image J software analysis (NIH, USA).

Histological Assessment

Lung tissues were collected on day 7 and 21 after bleomycin instillation. Tissues were fixed for one week in 10% (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded alcohol and embedded in paraffin wax. After the tissues were embedded in paraffin and tissue sections were prepared and stained with hematoxylin and eosin for morphometric analysis, trichrome staining and picrosirus red staining were performed to assess the severity of fibrosis. The severity of fibrosis was semiquantitatively assessed according to the method described by Ashcroft *et al.* (1988).

RESULTS

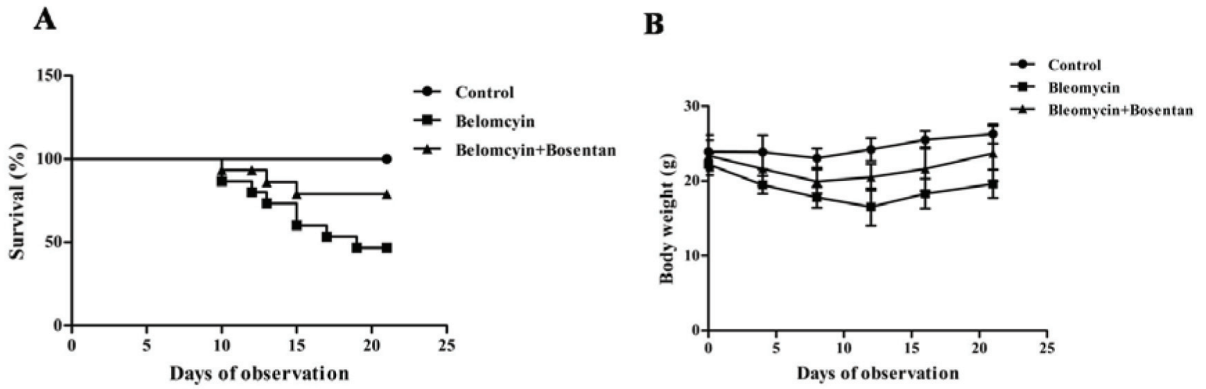
Effect of Bosentan on Body Weights and Mortality of Mice in Bleomycin Induced Pulmonary Fibrosis

The severe lung injury caused by the bleomycin instillation was associated with high mortality and reduction of body weights. Bleomycin instillation reduced the survival rate (47%; 7 survived out of 15 animals) of mice (Figure 1A). In contrast, bosentan administration significantly ($p \leq 0.05$) increased survival rate by 80% in bleomycin induced mice. Further, the body weights of control animals were increased during 21 days. Conversely, the body weights of bleomycin induced pulmonary fibrotic mice were decreased during 21 days of study (Figure 1B). However, the loss of body weights was increased by the bosentan treatment.

Effect of Bosentan Administration on Bleomycin Induced BALF Inflammatory Cells in Mice

To evaluate the effect of bosentan in bleomycin induced inflammation, the number of inflammatory cells in BALF at day 7 after bleomycin instillation was measured. The total number of inflammatory cells in BALF were significantly ($p \leq 0.05$) increased by 4.8 fold in

Figure 1: Effect of bosentan on body weight loss and survival rate. Pulomnary fibrosis was induced with 0.05 U bleomcyin/mice. 100 mg/kg b.wt of bosentan was adminstered orally for 21 days post bleomcyin instillation. The survival rate (A) and body weight (B) were measured in each group. Bleomycin induced group was compared with respective control as well as bosentan adminstered group. Results were expressed as Mean ± SD obtained from survival animals (initial number of animals n=15) per group ($p \leq 0.05$)



bleomycin instilled mice when compared to respective controls (Figure 2A). However bosentan administration significantly ($p \leq 0.05$) decreased the inflammatory cells by 43.7% compared to belomycin group. In addition, the differential count of cells present in BALF has shown a drastic increase of lymphocytes (6.5 fold), macrophages (3.0 fold) and neutrophils (13.5 fold) ($p \leq 0.005$) in bleomycin induced pulmonary fibrotic mice compared to respective controls (Figure 2B, 2C and 2D). Where as the treatment with bosentan significantly ($p \leq 0.05$) decreased the lymphocytes, macrophages and neutrophils by 40%, 42.3% and 53%, respectively. In addition to the inflammatory cells, the total protein levels in BALF have significantly ($p \leq 0.05$) increased by 3.0 fold in bleomycin instilled mice compared to respective controls. These elevated levels of protein significantly decreased ($p \leq 0.05$) by 41% in bosentan treated mice compared to bleomycin instilled mice (Figure 3C).

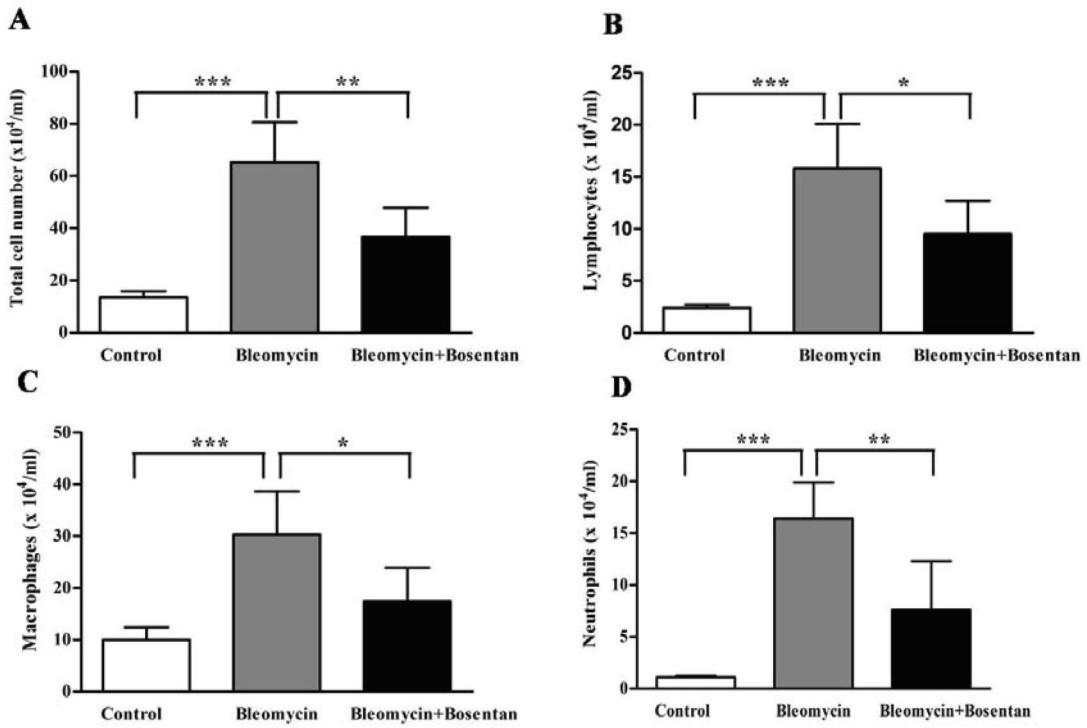
Effect of Bosentan on Oxidative Stress Induced by Bleomycin

The present investigation focussed on oxidative stress indicators such as SOD, and catalase in bleomycin induced lung fibrotic mice. As shown in Figure 3A, the SOD activity was significantly ($p \leq 0.05$) decreased by 47% in bleomycin instilled mice compared to controls. However, the SOD activity was restored by 31.5% in bosentan treated mice compared bleomycin group. Similarly way the catalase activity was significantly decreased by 59% in bleomycin instilled mice compared to saline instilled mice. In contrast bosentan administration significantly ($p \leq 0.05$) increased the catalase activity by 78% compared to bleomycin group (Figure 3B).

Effect of Bosentan Administration on Inflammatory Cells Infiltration in Lung Tissues of Bleomycin Induced Mice

Hematoxylene and eosin staining of lung tissues revealed that a well alveolized normal lung structure was seen in control mice. In contrast

Figure 2: Effect of bosentan on bleomycin induced inflammation in BALF. Pulomnary fibrosis was induced with 0.05 U bleomcyin/mice. BALF fluid was collected on day-7, total cell count (A), lymphocytes (B), macrophages (C) and neutrophils (D) differential cell counts were performed. Bleomycin induced group was compared with respective control as well as bosentan adminstered group. Results were expressed as Mean ± SD, n=5-6 and* indicates the statistical significance ($p \leq 0.001$) between control and bleomycin groups. * indicates the statistical significance ($p \leq 0.05$) between bleomycin group and treatment groups**



bleomycin induced mice showed evident tissue damage, and extensive infiltration of inflammatory cells on day 7 (Figure 4A). However, this massive infiltration of inflammatory cells was reduced in bosentan administered mice. Severe epithelial degeneration, excessive deposition of extracellular matrix proteins and distorted alveolar architecture were observed in the lungs of bleomycin instilled mice on day 21 (Figure 4B). Whereas in lungs of bosentan administered mice showed reduced epithelial degeneration, extracellular matrix deposition and restored alveolar architecture when compared to the mice administered with bleomycin alone.

Since, myeloperoxidase (MPO) activity is the marker of lung infiltration with polymorphonuclear neutrophils, the MPO activity was measured in the lungs of bleomycin induced pulmonary fibrotic mice. Bleomycin caused 8.9 fold increase in MPO activity significantly ($p \leq 0.05$) in lung tissues compared to the saline instilled mice (Figure 4C). However, the administration of bosentan in bleomycin induced pulmonary fibrotic mice decreased the MPO activity by 31% compared to the bleomycin group (Figure 4C).

Lung wet/dry weight ratio is an indicator of tissue edema (inflammatory response) which

Figure 3: Effect to bosentan on SOD and catalase activities changes by bleomycin administration and total protein level in BALF fluid. SOD & catalase activity in control and bleomycin instilled mice with and with out bosentan adminstration (A). Total protein levels in control and bleomycin instilled mice with and with out bosentan adminstration (C). Data represented as mean SD± values, n=5-6. * indicates the statistical significance ($p \leq 0.001$) between control and bleomycin groups. * indicates the statistical significance ($p \leq 0.05$) between bleomycin group and treatment groups**

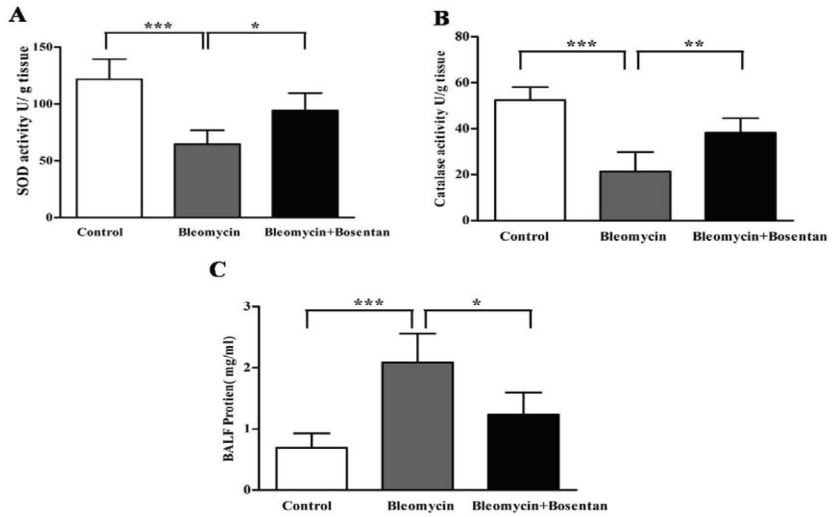
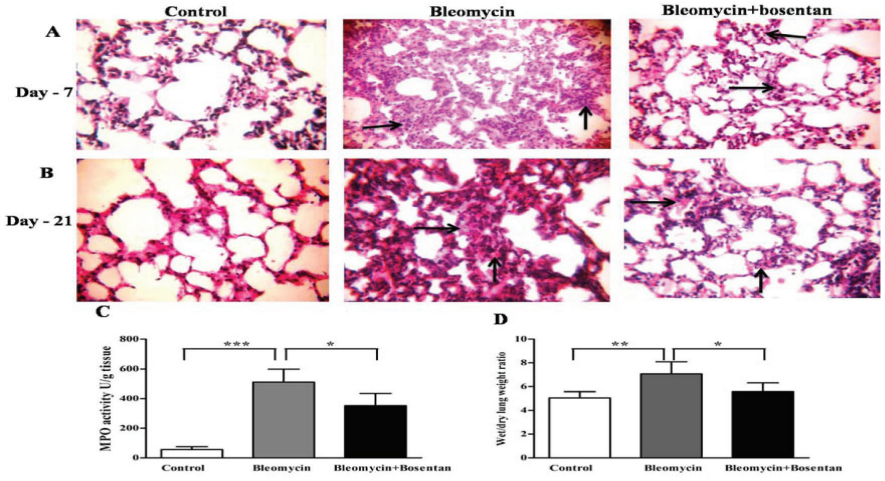


Figure 4: The H&E staining images on day-7 (A) showed that extensive inflammatory cell infiltration in bleomycin instilled mice where as it was decreased by bosentan administration. H&E staining images at day-21(B) revealed that higher deposition of collagen in mice instilled with bleomycin alone where as it was decreased in mice treated with bosentan. All Images were represented as 40X magnifications. Bleomycin instilled mice elicited significant increase in MPO activity in lung tissues (C) and wet/dry lung weight ratio (D). Bosentan significantly decreases the MPO and wet/dry lung weight ratio on day-7 after bleomycin instillation. Data represented as mean SD± values, n=5-6. * indicates the statistical significance ($p \leq 0.001$) between control and bleomycin groups. * indicates the statistical significance ($p \leq 0.05$) between bleomycin group and treatment groups. Black arrow indicates inflammatory cells in (A) and collagen deposition in (B)**



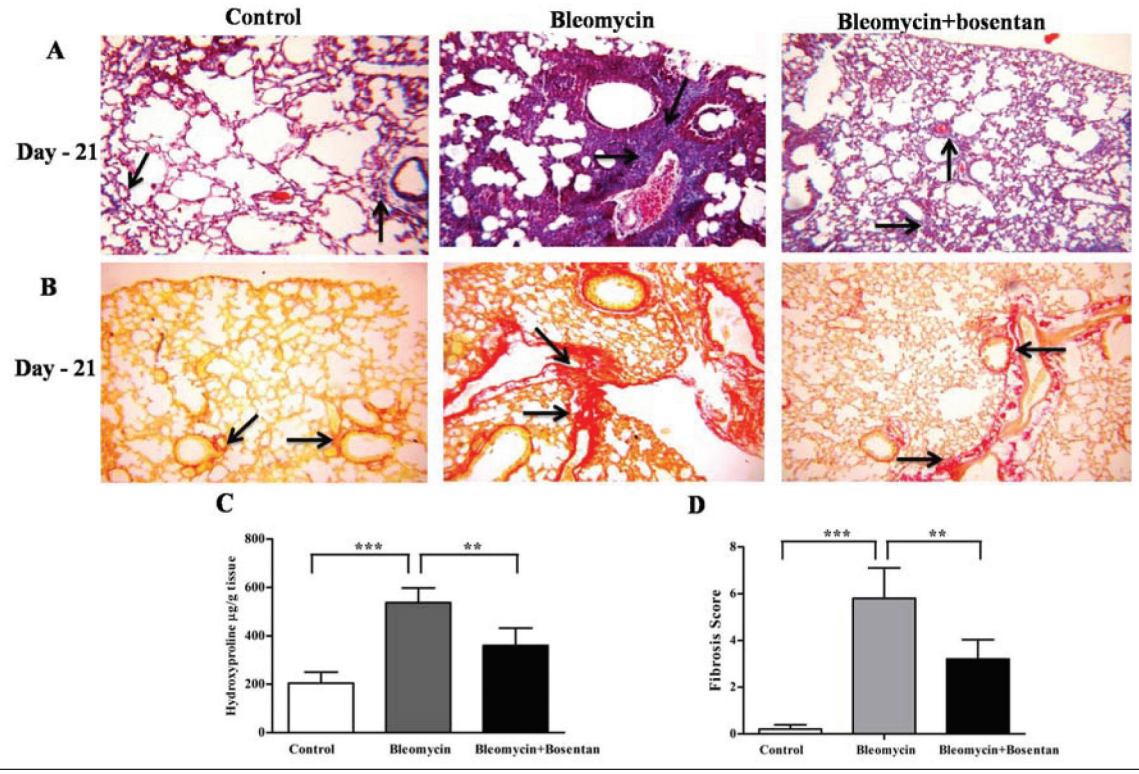
was characterized by the accumulation of water in lungs. Intratracheal bleomycin instilled mice showed a 40% increase in lung wet/dry weight ratio compared to saline instilled mice. However, treatment with bosentan at 100 mg/kg significantly decreased the lung wet/dry weight ratio by 21% compared to bleomycin instilled mice (Figure 4D).

Effect of Bosentan on Hydroxyproline Levels and Tissue Fibrosis Elevated by Bleomycin Instillation

Hydroxyproline is a modified amino acid uniquely found at high percentage in collagen. Therefore, the tissue hydroxyproline content of lungs was assessed as a biochemical quantitative measure of collagen deposition. Hence, we have analyzed the hydroxyproline content in mice lungs at day 21. As shown in Figure 5C the hydroxyproline content was significantly ($p \leq 0.05$) increased by 2.6 fold in bleomycin instilled mice compared to saline instilled mice. However in mice administered with bosentan at 100 mg/kg showed significant ($p \leq 0.05$) decrease in collagen content by 33.1%.

Pulmonary fibrosis was evaluated by light microscopy using sections stained with Masson's trichrome stain or picrosirus red stain. As shown

Figure 5: Effect of bosentan on the collagen accumulation induced by bleomycin. The trichrome staining images (A) and picrosirus red staining images (B) on day-21 showed the excessive deposition of collagen in lung tissue sections of bleomycin instilled mice where as it was decreased by bosentan administration. All Images were represented as 10X magnifications. Bleomycin instilled mice demonstrated significant increase in lung hydroxyproline levels (C) and fibrosis score (D). Bosentan significantly decreases the collagen and fibrosis score on day-21 after bleomycin instillation. Data represented as mean SD± values, n=5-6. * indicates the statistical significance ($p \leq 0.001$) between control and bleomycin groups. * indicates the statistical significance ($p \leq 0.05$) between bleomycin group and treatment groups**



in Figure 5A, Masson's trichrome staining of lung tissues showed an excessive deposition of collagen which was stained as blue color in mice instilled with bleomycin compared to saline-instilled mice. Such observations were decreased in bosentan treated mice at dose level of 100 mg/kg indicating the protective role of bosentan in pulmonary fibrosis. The scores of fibrosis significantly increased in bleomycin instilled mice compared to those of normal control mice. However, bosentan administration significantly ($p \leq 0.05$) lowered the fibrosis score. Similarly, as shown in Figure 5B, picrosirus red staining also showed a similar pattern of collagen deposition in bleomycin treated animals, while this collagen deposition was reduced in mice treated with bosentan.

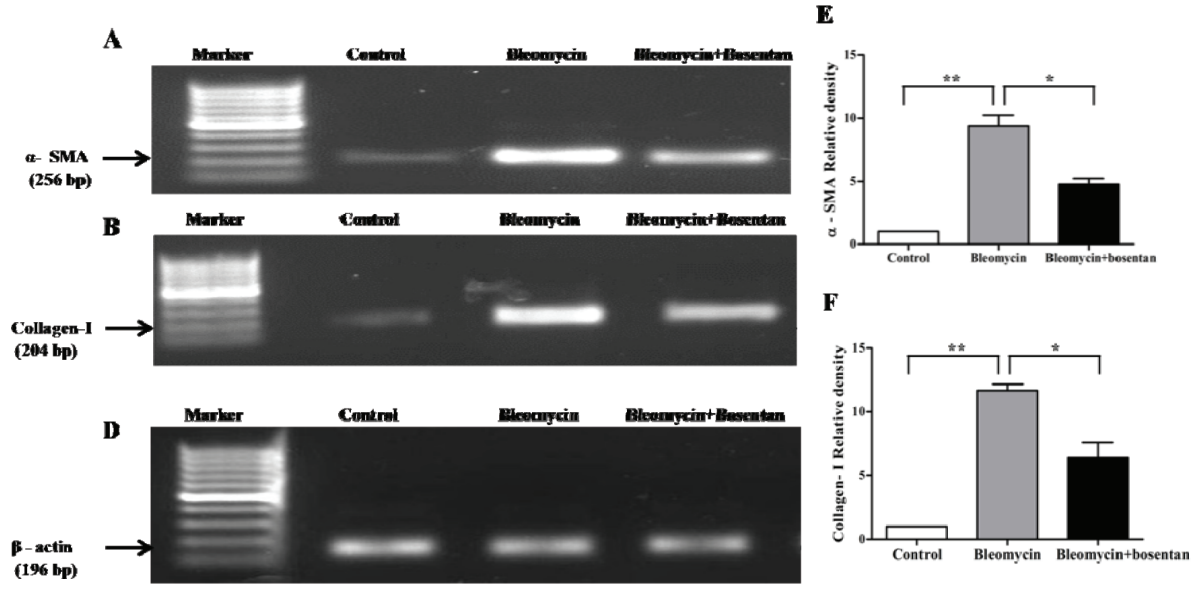
Bosentan Administration Attenuated α-SMA and Collagen-I Gene Expression in Lung Tissue of Bleomycin Induced Mice

RT- PCR analysis showed that the expression level of collagen-I and α-SMA genes were significantly increased by 12.1 fold and 10.2 fold ($p \leq 0.05$) in bleomycin instilled mice compared to saline-instilled controls. The administration of bosentan at 100 mg/kg significantly decreased the expression levels of α-SMA and collagen-I genes by 38% and 49% respectively when compared to bleomycin instilled mice (Figure 6).

Effect of Bosentan on the MMP-2 and MMP-9 Activities in Fibrotic Mice Lungs Induced by Bleomycin

To investigate the activity of MMP-2 and MMP-9

Figure 6: Effect of bosentan on the gene expression levels of collagen-I and α-SMA. Pulmonary fibrosis was induced with 0.05 U bleomcyin/mice and bosentan was administred for period of 21 days. Lung tissues will be collected on day-21 total RNA was isolated and converted to C-DNA. Semiquantitative RT-PCR was performed to measure the mRNA levels of collagen-I and α-SMA and data were expressed as a relative change compared to control for each gene. β- actin gene products were used to control for quality and equal loading of c-DNA. * indicates the statistical significance ($p \leq 0.01$) between control and bleomycin groups. * indicates the statistical significance ($p \leq 0.05$) between bleomycin group and treatment groups**

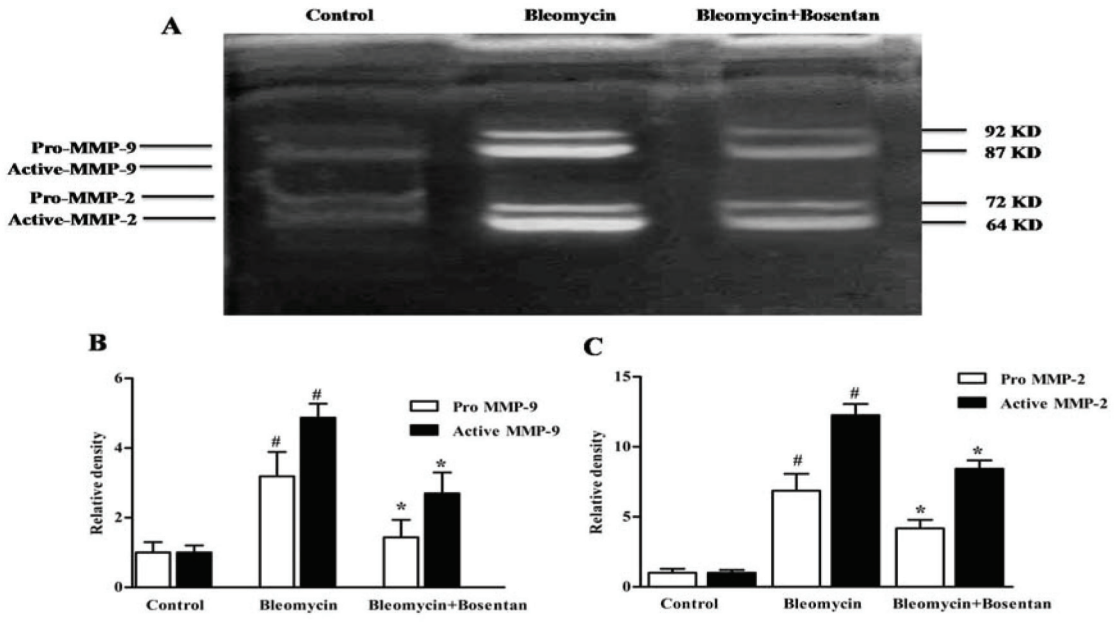


in the lungs of mice we have performed gelatin zymography of extracted proteins from lungs on day 14. As shown in figure, the mice instilled with bleomycin exhibited increased relative density of Pro MMP-2 as well as active MMP-2 by 6.8 and 12.2, respectively when compared to saline-instilled mice. Pro MMP-9 as well as active MMP-9 activities were increased by 6.8 and 12.2 respectively when compared to saline-instilled mice. However the administration of bosentan decreased ($p \leq 0.05$) relative density of Pro MMP-2 as well as active MMP-2 to 1.4 and 2.6, respectively compared to saline-instilled control. Where as the relative densities of Pro MMP-9 as well as active MMP-9 activities were decreased ($p \leq 0.05$) to 4.1 and 8.4 respectively when compared to saline-instilled mice (Figure 7).

DISCUSSION

The main findings of the present study are that the intervention with ET-1 receptor antagonist bosentan suppresses the development of pulmonary fibrosis induced by the bleomycin challenge. Bosentan reduced the body weight loss, mortality, suppressed the early inflammation, improved antioxidant ability, reduced the MMP-2 and MMP-9 activities, and finally attenuated the fibrosis of lung tissue. The paracrine lung ET system is thought to be involved in the pathogenesis of several lung diseases such as primary and secondary pulmonary hypertension (Giaid and Saleh, 1995; Lutz *et al*., 1999) pulmonary fibrosis (Mutsaers *et al.*, 1998) asthma and chronic obstructive lung

Figure 7: Identification of gelatinolytic activity activities in lung tissues (A). Supernatants from lung tissue extracts derived from bleomycin instilled, administred bosentan after bleomycin instillation and control animals were resolved by SDS PAGE (10%) containing 3mg/ml gelatin. Zone of genolytic activity appear as a clear bands over dark background. Densitometric analysis were performed data expressed as a relative change compared to control for each gene. # and * indicates the statistical significance of control ($p \leq 0.05$) compared to the bleomycin groups and bleomycin compared to treatment groups respectively



disease (Hay, 1999) and bronchiolitis obliterans (Takeda *et al*., 1997; Schersten *et al*., 1996). Activated Paracrine ET-1 caused increased pulmonary matrix synthesis and a chronic inflammatory lung disease characterized by an increased infiltration of CD-4 positive cells (Hocher *et al.*, 2000). Several pro-inflammatory mediators like interleukin (IL)-1α, IL-1β and tumor necrosis factor-α are known to stimulate the ET system (Hocher *et al*., 1997, Nakano *et al*., 1994). It has also been postulated that ET-1 it self causes recruitment of inflammatory cells (Zouki *et al.*, 1999). The molecular mechanisms leading to a recruitment of CD4-positive lymphocytes are un known so far. Interestingly, it has been shown that ET-1 induces the expression of E-selection and intracellular adhesion molecule-1, facilitating the migration of inflammatory cells (Zouki *et al.,*1999). In the present study we have demonstrated that administration of bosentan significantly suppressed the severity of the inflammatory infiltration in BALF and lung tissue. Bosentan significantly suppressed the number of total cells, macrophages, lymphocytes and neutrophils in BALF. Significant decrease in MPO activity in lung tissue, total protein content in BALF, wet/dry lung weight ratio (edema marker) and decreased infiltration of inflammatory cells in lung tissue sections on day 7 of post bleomycin instillation supported these findings. Therefore the inhibited inflammatory cell infiltration was partly accountable for the preventive effect of bosentan on bleomycin induced pulmonary fibrosis.

A number of previous studies reported that the cellular imbalance between the Reactive Oxygen Species (ROS) and antioxidant enzymes are involved in pathogenesis of IPF (Kinnula *et al.*, 2005). The lung injury caused by the bleomycin involves the generation of oxidant species and further damage is probably elicited by the generation of ROS produced by activated inflammatory cells, which accumulate in bleomycin induced pulmonary lesions (Li *et al.*, 2002). ET-1 may also participate in the pathogenesis of lung fibrosis through its effects on cellular apoptosis and oxidant/antioxidant imbalance. ET-1 was reported to induce reactive oxygen species in several cell types, including vascular smooth muscle cells and pericytes (Fonseca *et al.,* 2011). Recently, it was reported that endothelin receptor antagonist (Bosentan) increased the serum antioxidant activity in cigarette smoke extract induced emphysema in a rat model (Chen *et al*., 2010). Interestingly, emphysema and lung fibrosis are likely to share many pathogenetic pathways, as suggested by the strong link with smoking in IPF (Baumgartner *et al*., 1997). Bosentan attenuated oxidative stress and restored cellular antioxidant defense mechanism (SOD and catalase enzymes) in experimental myocardial ischemia and reperfusion (Gupta *et al.*, 2005). In the present study, we have demonstrated that there was a significant decrease in SOD and catalase activities in bleomycin challenged mice which was restored in bosentan treated mice on day 7 of post bleomycin challenge.

ET-1 is also profibrotic by stimulating fibroblast replication, migration, contraction, and collagen synthesis and secretion while decreasing collagen degradation . ET-1 was shown to promote the wound-healing response by stimulating the production of collagens IV, V, and VII, involved in reconstitution of the basement membrane and appropriate wound closure (Fonseca *et al.*, 2011) . ET-1 may thus play an important role in the initial injury, inflammatory events and eventual fibrotic reparative process in lung. The results of RT-PCR

studies demonstrated that blockade of ET-receptor with bosentan significantly decreased the collagen-I gene expression levels in bleomycin challenged mice. Significant decrease in hydroxyproline level, decreased collagen deposition in Masson's trichrome and picrosirus red stained lung tissue sections supported these findings. ET-1 enhances the conversion of fibroblasts into contractile myofibroblasts. ET-1 promotes myofibroblast differentiation and contraction to contribute to the effective matrix remodelling during tissue repair (Fonseca *et al.,* 2011). α-SMA is the marker for myofibroblasts, the findings of gene expression studies revealed that treatment with bosentan significantly decreased α-SMA gene expression. This findings suggested that bosentan inhibits conversion of fibroblasts into myofibroblasts.

Several studies have implicated MMPs in human and experimental interstitial lung disease. In humans IPF levels of MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are elevated in lungs and BALF fluid of affected patients. MMP-2 and MMP-9 proteolytically cleave the denatured collagen and have the ability to cleave the type IV collagen, the key structural components of basement membrane (Roderick *et al.,* 2000). Felix *et al*. (2006) reported that ET-1 enhances the MMP-2 and MMP-9 activity in osteosarcoma cells and human osteosarcoma tissue. The present study demonstrated that inhibition of ET-1 by bosentan significantly reduces the MMP-2 and MMP-9 activities which were elevated by bleomycin administration.

CONCLUSION

In summary, ET-1 signaling involved in pathogenesis of bleomycin induced pulmonary fibrosis in rats resulted in oxidative stress, inflammation, MMP activation and extracelluar matrix deposition. In the present study, we found that the blockade of ET-1 signaling with bosentan inhibits the inflammation, oxidative stress, MMP activation and finally extracelluar matrix deposition. Bosentan significantly abrogates the fibrosis suggests that it is useful therapeutic tool in human IPF.

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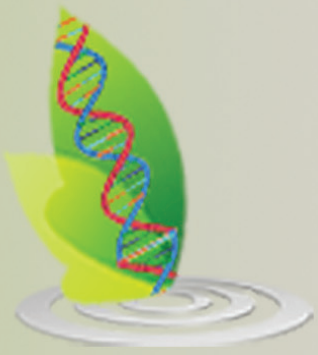

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