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RESEARCH ARTICLE

Amelioration of bleomycin-induced epithelial-mesenchymal transition in pulmonary fibrosis by baicalein in mice

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ABSTRACT

Epithelial-mesenchymal transition (EMT) play a vital role in pulmonary fibrosis. The present study investigates the ameliorative effect of baicalein, a bioactive flavonoid present in the dry roots of *Scutellariabaicalensis Georgi*, on bleomycin-inducedpulmonary fibrosis and subsequent EMT. Mice were given a single intratracheal instillation of saline containing bleomycin @ 1 mg/kg b.wt. Baicalein in different doses of 0.1, 1.0 and 10 mg/kg b.wt was given intraperitoneally daily for 8 weeks. Baicalein was effective in attenuating the BLM induced Pulmonary fibrosis through suppression of oxidative stress, inflammation, histological as well as ultrastructural damages and EMT, especially during the chronic stage injury. This study will provide additional knowledge on the ameliorative effect of baicalein during the later stages of lung injury where the EMT starts and this may help in the therapeutic or management of clinical chronic lung injury associated pulmonary fibrosis.

Keywords: Baicalein, bleomycin, chronic lung injury, epithelial-mesenchymal transition

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive and fatal lung disease of unknown etiology. Its prognosis is poor and the outcome is even worse than in many malignant diseases, and the exact pathophysiological mechanisms for the development of which are not fully understood[1]. There is a general consensus that this disease is due to the combination of alveolar epithelial cell injury which is followed by the release of proinflammatory/fibrotic cytokines, inflammatory cell infiltration, epithelial-mesenchymal transition (EMT) and excessive deposition of extracellular matrix in the lung[2]. Most of patients present at an advanced stage of the disease. Treatment options for pulmonary fibrosis are limited. Anti-inflammatory drugs such as prednisone may carry symptomatic relief, but they do not appear to halt progression of fibrosis, and their beneficial effects in IPF remain in question. Cytotoxic drugs (cyclophosphamide, azathioprim, etc.) have not been shown to improve lungs function or life expectancy and may be associated with harmful side effects.

Animal models play an important role in the investigation of diseases, and many models are established to examine pulmonary pathobiology. Chronic diseases are more difficult to model. The situation with IPF is even more complicated since the etiology and natural history of the disease is unclear and no single trigger is known that is able to induce "IPF" in animals. Different models of pulmonary fibrosis have been developed over the years. Most of them mimic some, but never all features of human IPF, especially the progressive and irreversible nature of the condition. Common methods include radiation damage, instillation of bleomycin, silica or asbestos, and transgenic mice or gene transfer employing fibrogenic cytokines. So far, the standard agent for induction of experimental pulmonary fibrosis in animals is bleomycin. Bleomycin is a chemotherapeutic antibiotic, produced by the bacterium "*Streptomyces verticillus*". Its use in animal models of pulmonary fibrosis is based on the fact that fibrosis is one of the major adverse drug effects of bleomycin in human cancer therapy. This happens by chelation of metal ions, and reaction of the formed pseudoenzyme with oxygen, which leads to the production of DNA-cleaving superoxide and hydroxide free radicals. An overproduction of reactive oxygen species can lead to an inflammatory response causing pulmonary toxicity, activation of fibroblasts and subsequent fibrosis. Bleomycin as an agent to induce experimental lung fibrosis has been reported long ago in laboratory animals[3].

The last two decades have markedly improved the knowledge about the underlying mechanisms of pulmonary fibrosis and helped to identify potential targets for novel therapies. Various therapeutic agents like

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antibiotics, corticosteroids, and antioxidants have been used as therapeutic agents, but they lead to other problem like antibiotic sensitivity, immunosuppression etc. Hence, nowadays herbal drugs are preferred which have no side effects. Herbal medicines can be used as one of the dietary supplement. There are many different systems of traditional medicine, and the philosophy and practices of each are influenced by the prevailing conditions, environment, and geographic area within which it first evolved, however, a common philosophy is a holistic approach to life, equilibrium of the mind, body, and the environment, and an emphasis on health rather than on disease. Traditional Chinese medical herbs are the most important components of the traditional Chinese medicine (TCM) system, which have been reported to cure infectious diseases, in the form of hot water extracts, for almost 2,000 years. In traditional Chinese herb medicine, the root of *Scutellariabaicalensis Georgi* was usually gathered before Tomb-Sweeping Day and decocted for the purpose of "cleansing heart" and "removing toxins," for example, cough with yellow sputum, jaundice, swelling and pain of eye, and so on. Baicalein (BAIC) is a bioactive flavonoid, which is widely used in Chinese herbal medicine. Evidence has shown that BAIC has many pharmacological effects, including antiallergic, antioxidant, antiapoptotic, antiviral, anti-inflammatory, antitumor, and neuroprotective effects and a modulatory effect on the immune system[4].

Table 1. Hematology of mice of different groups 8 weeks post-treatment.

Groups	Hb (g/dL) (Mean±SE)	TLC (x10³/µl) (Mean±SE)	DLC	
			Neutrophil	Mononuclear Cells
SHAM	10.92±0.29^{c}	10.18±0.12^{a}	33.46±1.35^{a}	66.46±1.41^{a}
BLEO	6.90±0.18^{b}	10.40±1.34^{a}	37.78±0.79^{a}	62.22±0.88^{a}
BLEO/BAIC/0.1	7.65±0.62^{b}	9.92±1.11^{a}	35.52±1.11^{a}	64.48±1.47^{a}
BLEO/BAIC/1.0	8.10±0.27^{bc}	8.39±0.34^{a}	37.67±1.09^{a}	62.33±1.01^{a}
BLEO/BAIC/10	9.95±0.25^{bc}	8.22±0.28^{a}	34.92±1.03^{a}	65.08±0.96^{a}

The values (Mean±SE) in a column having different superscript differ significantly from each other at 5% level of significance. The values (Mean±SE) in a column having same superscript do not differ significantly from each other at 5% level of significance.

MATERIALS AND METHODS

Animals and Treatments

The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana. Male albino mice (n=30, 4-6 weeks age), were obtained from Disease free small animal house, Central Research Institute, Kasauli, Himachal Pradesh and housed in the small animal house of GADVASU. All mice received humane care. After the acclimatization period of 7 days, the animals were weighed again and, 30 male albino mice were randomly divided into five experimental groups (6 animals each) and named as per the challenge and treatment: SHAM/PBS (saline-only/control group), BLM (bleomycin @ 1 mg/kg bwt treated group), BLM/BAIC/0.1 (bleomycin @ 1 mg/kg bwt + baicalein (0.1 mg/kg bwt), BLM/BAIC/1 (bleomycin @ 1 mg/kg bwt + baicalein (1 mg/kg bwt) and BLM/BAIC/10 (bleomycin @ 1 mg/kg bwt + baicalein (10 mg/kg bwt). Mice received a single intratracheal instillation of saline containing bleomycin sulfate @ 1 mg/kg bwt (Sigma, USA) in a volume of 50 µl in all groups, except vehicle group. Baicalein (Sigma, USA) in three different doses (0.1, 1.0, 10 mg/kg bwt) was given intraperitoneally daily for 8 weeks. All the mice were sacrificed after 8 weeks by ketamine and xylazine overdose.

Collection of Blood and Bronchoalveolar lavage (BAL) fluid

The blood was withdrawn by cardiac puncture after sacrificing the animal and it was collected in EDTA vials for the hematological parameter estimations *viz.* hemoglobin concentration (Hb) and total leucocyte count (TLC). The collection of bronchoalveolar lavage was done three times through a tracheal cannula which was attached to 1 ml syringe with 0.5 mL of PBS in each animal from the left lung. Bronchoalveolar lavage fluid (BALF) was processed to get cell pellets and supernatants as described earlier[5]. TLC and DLC were performed of the cell pellets of BALF with a hemocytometer and the smears were stained with Leishman stain. The supernatant of BALF was collected and stored at -80°C for estimation of other parameters like protein concentration, TNF-α and IL-6.

Table 2. Lung W/D weight ratio and Protein concentration in bronchoalveolar lavage fluid (BALF) in different groups of mice 8 weeks post treatment.

Groups	Lung W/D Weight Ratio (Mean±SE)	Protein Concentration in BALF (µg/ml) (Mean±SE)
SHAM	3.04±0.17^{ab}	406.65±9.08^{ab}
BLEO	6.89±0.62^{d}	1114.60±112.58^{f}
BLEO/BAIC/0.1	4.89±0.46^{c}	925.95±45.55^{e}
BLEO/BAIC/1.0	4.08±0.45^{bc}	910.75±36.88^{e}
BLEO/BAIC/10	3.73±0.22^{abc}	730.43±17.79^{d}

The values (Mean±SE) in a column having different superscript differ significantly from each other at 5% level of significance. The values (Mean±SE) in a column having same superscript do not differ significantly from each other at 5% level of significance.

Wet to Dry Lung Weight Ratio

The similar lobe of the right lung from each animal was collected after sacrificing and was weighed immediately after its excision, which was termed as wet weight. After weighing the lung lobes were dried at 60^{O}C for 72 h, and weighed again to get the dry weight. This wet dry (W/D) lung weight ratio was calculated as an indicator of pulmonary edema.

Estimation of protein in bronchoalveolar lavage fluid

Commercially available BCA Protein Assay Kit (Thermo Scientific, USA) was used to estimate protein in BALF as per manufacturer's instruction.

Histopathological studies

After the BAL fluid collection, the tissue samples from the lungs were collected and were fixed in 10% neutral buffered formalin and were further processed and embedded in paraffin. Five μm thick sections were stained with hematoxylin and eosin. To ascertain fibrosis and collagen deposition Masson Trichrome and Picro-sirius red staining were also done respectively. The slides were viewed and photomicrographs were taken by microscope attached with camera (BX 61, Olympus Corporation, Japan). Histopathologically, the lung injury was assessed experimentally by blind experts and was graded semi-quantitatively using modified Ashcroft's scoring method[10] with a score range of 0-8 score. For Picro-sirius red staining, the slides were stained with picro-sirius red stain (0.1% Sirius red in aqueous saturated picric acid) for 1 hour followed by washing with acidified water (0.5% glacial acetic acid), dehydration and mounting with DPX. Collagen was red in colour while non-collagen components were orange. The images were analyzed using Image J (Fiji) software (http://fiji.sc). The intensity of the picrosirius red positive area was expressed as percentage area (μm^2).

Estimation of Lipid peroxidation and Superoxide dismutase in lung homogenates

The lung tissue (10 mg) was homogenised in 1 ml of ice-cold phosphate buffered saline (pH 7.4), using a tissue homogenizer with a teflon pestle at 4^{O}C. The resultant tissue homogenates were used for measurements of Total protein, Lipid peroxidation (LPO) and Superoxide dismutase (SOD) activity. Total proteins were estimated using commercially available BCA Protein Assay Kit (Thermo Scientific, USA) as per manufacturer's protocol. LPO was calculated and denoted in terms of MDA (malondialdehyde) production, by the thiobarbituric acid (TBA) method as described with slight modifications. Briefly, 1 ml of tissue homogenate were incubated at 37^{O}C for 2 hours after which 1 ml of 10% w/v trichloroacetic acid was added to each sample. The mixture was mixed thoroughly and centrifuged at 2000 rpm for 10 min. To 1 ml of supernatant after centrifugation, an equal volume of 0.67% w/v TBA was added and kept in boiling water bath for 10 min. The samples were cooled down and then diluted with 1 ml of distilled water. The absorbance was read at 535 nm. The amount of LPO was expressed in nanomoles of MDA formed per gram of wet tissue. SOD activities were estimated as per the method described[6]. It basically involves generation of superoxide by autoxidation of pyrogallol and the inhibition of superoxide-dependent reduction of the tetrazolium dye, 3-(4-5 dimethyl thiazol 2-yl) 2, 5 diphenyl tetrazolium bromide (MTT), to its formazan, which was measured at 570 nm.

Table 3. MPO activity in different groups of mice at 8 weeks post treatment.

Groups	MPO (mμ/ml)
SHAM	54.30±1.72^{a}
BLEO	164.50±8.71^{cde}
BLEO/BAIC/0.1	145.42±3.96^{bcd}
BLEO/BAIC/1.0	137.31±11.80^{bcd}
BLEO/BAIC/10	103.74±18.47^{b}

The values (Mean±SE) in a column having different superscript differ significantly from each other at 5% level of significance. The values (Mean±SE) in a column having same superscript do not differ significantly from each other at 5% level of significance.

Estimation of Myeloperoxidase (MPO) activity

Myeloperoxidase Colorimetric Activity Assay Kit (SIGMA ALDRICH, St. Louis, USA) was used to measure myeloperoxidase (MPO) activity in the lung homogenates using as per the manufacturers instructions. The absorbance was measure at 412 nm using microplate reader.

Estimation of cytokines by ELISA

Mouse IL-6 and Mouse TNF-α ELISA Kit (Krishgen Biosystem) and Mouse TGF-β ELISA kit (YH Bioresearch Laboratory) were used to measure Interleukin-6 (IL-6), Tumor necrotic factor-alpha (TNF-α) in BALF and transforming growth factor-β in lung homogenatesas

Table 4. LPO and SOD level in lung homogenates of different groups of mice at 1 and 8 weeks post treatment.

Groups	LPO (nM/g) (Mean±SE)	SOD (U) (Mean±SE)
SHAM	1.05±0.25^{a}	4.75±0.15^{g}
BLEO	2.00±0.00^{cde}	2.81±0.32^{bcd}
BLEO/BAIC/0.1	1.51±0.13^{abc}	2.89±0.14^{cd}
BLEO/BAIC/1.0	1.71±0.33^{cd}	3.10±0.26^{de}
BLEO/BAIC/10	1.15±0.13^{ab}	3.94±0.15^{f}

The values (Mean±SE) in a column having different superscript differ significantly from each other at 5% level of significance. The values (Mean±SE) in a column having same superscript do not differ significantly from each other at 5% level of significance.

per the manufacturer instructions.

Estimation of collagen by hydroxyproline Assay

Hydroxyproline (HYP) assay was undertaken to estimate the collagen content in the lung homogenates.Briefly, 100 µl of lung homogenate were hydrolyzed with equal amount of concentrated HCl in a pressure-tight, teflon capped vial at 120^{O}C for 3 hours followed by clarification with activated charcoal. 10 µl of each hydrolyzed sample were then transferred to a 96-well plate and the liquid portion of the sample was evaporated to dryness under vacuum. Hydroxyproline standard (1 mg/ml) was used to prepare the standard curve. Chloramine T reagent (100 µl) was added to each sample and standard and the incubated at room temperature for 5 min. After this DMAB reagent was added (100µl) and the plate was incubated at 60^{O}C for 90 min. The absorbance of each sample was read at 560 nm using a microplate reader.

Table 5. TNF-α and IL-6 level in BALF of different groups of mice at 1 and 8 weeks post treatment.

Groups	TNF-α (Mean±SE)	IL-6 (Mean±SE)
BLEO	612.02±18.56^{g}	154.58±12.29^{d}
BLEO/BAIC/0.1	562.24±25.42^{f}	140.71±13.68^{cd}
BLEO/BAIC/1.0	458.77±13.11^{e}	124.82±8.58^{bc}
BLEO/BAIC/10	281.74±7.85^{d}	72.00±4.43^{a}

The values (Mean±SE) in a column having different superscript differ significantly from each other at 5% level of significance. The values (Mean±SE) in a column having same superscript do not differ significantly from each other at 5% level of significance.

Immunohistochemistry (IHC)

Immunohistochemical analysis for epithelial and mesenchymal markers (E-cadherin and alpha-smooth muscle actin) was performed. Briefly, 5-µm paraffin sections on poly-L-lysine coated slides were rehydrated. After antigen retrieval (heat induced) and blocking of endogenous peroxidase in tissue slides, the slides were incubated with primary antibodies to mouse monoclonal alpha-smooth muscle actin (α-SMA) (Abcam, UK) and to rabbit polyclonal E-cadherin (Gentex, USA) which was followed by incubation with secondary antibody (ABC, Universal, Vector). The colour was developed using diaminobenzidine (DAB) substrate and counter stained with hematoxylin. In negative control, tissue sections were processed without application of primary antibody. Semi-quantitative immunohistochemical analysis was performed using scoring pattern[7] as per Lomas *et al* (2012) with a score range of 0-5 where 0 (0 Positives staining cells (%), no expression), 1 (<1 %, Negligible expression), 2 (1 to 10%, Scanty expression), 3 (10 to 33%, Low-moderate expression), 4 (33 to 66%, Moderate expression) and 5 (>66, Extensive expression).

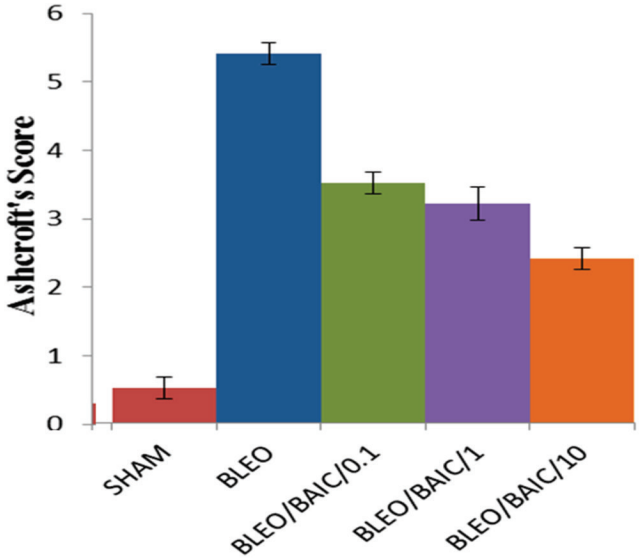

Fig. 1. Ashcroft's scoring scale for assessment of histological changes of different groups of mice 8 weeks post treatment.

Ultrastructural examination of lung tissue

After sacrificing the animals, lung tissues were removed and dissected at size of 1 mm^{3} and were fixed in Karnovsky's fixative for 6 hours at 40^{O}C. The tissues were processed for transmission electron microscopy as described earlier[8]. After several washings, the 1 mm^{3} lung tissues were post fixed in 1% osmium tetraoxide for 1 hour at 40^{O}C followed by dehydration at various grade of acetone (30-100% acetone) for 30 min each at 40^{O}C and the clearing was done with 2 changes of toluene for 30 min at room temperature. The tissues were further processed and embedded in pure epoxy resin to make blocks. Ultrathin sections (70 nm) were mounted on the copper grid of 300 meshe size and stained with uranyl acetate and lead citrate. The sections were visualized with Tecnai 200Kv transmission electron microscope (Tecnai, Fei Electron Optics) at Elecron Microscopy acility at All India Institute of Medical Sciences (AIIMS), New Delhi.

Statistical Methods

Data generated from various experiments were presented as Mean ± SE. All the grouped data was evaluated using SPSS/10.0 software. One-way analysis of variance (ANOVA) was used to detect differences among groups and the means were compared by Duncan's and LSD post hoc test and a value of $P \leq 0.05$ was taken as significant.

RESULTS

Effect of baicalein on hematology in bleomycin (BLM) induced lung injury

In the present study, mean values of hemoglobin at 8 weeks in different groups was lowered in BLM group (6.90±0.18 g/dL), the Hb value was improved by baicalein

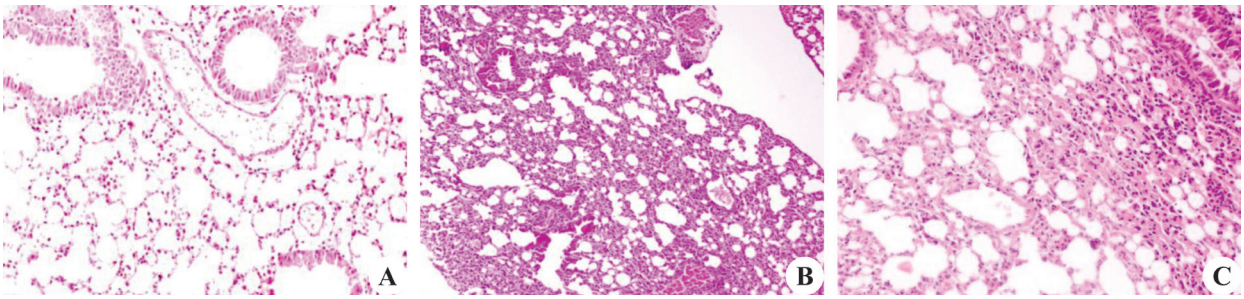

Fig. 2A. SHAM: Lung: Showing normal histology with normal bronchi and alveolar epithelium (H&E x100). **B.** BLEO/8 week post treatment: Lung: Showing severely damaged lung architecture with inflammatory cells infiltration, fibrosis, collagen deposition and decreased alveolar spaces with edematous changes (H&E x100). **C.** BLEO/BAIC/10/8 week post treatment: Lung: Moderate fibrin deposition with reduction in inflammatory cells infiltration, edema with improved lung architecture compare to BLEO/8 week (H&E x100).

in BLM/BAIC/0.1 group (7.65±0.62 g/dL), BLM/BAIC/1.0 group (8.10±0.27 g/dL), BLM/BAIC/10 group (9.95±0.25 g/dL) with inclined dose rate and maximum effect was seen at highest dose rate where, the mean value of the group was comparable to SHAM group (10.92±0.29 g/dL) demonstrating the preventive effect of baicalein @ 10 mg/kg dose rate (Table 1).

Similar trend was seen in the pattern of TLC in the animals, where the TLC decreased by baicalein treatment in different groups *viz.* SHAM, BLM, BLM/BAIC/0.1, BLM/BAIC/1.0 and BLM/BAIC/10 i.e. (10.18±0.12×10^3/µl), (10.40±1.34×10^3/µl), (9.92±1.11×10^3/µl), (8.39±0.34×10^3/µl) and (8.22± 0.28×10^3/µl) which signalled that baicalein had a protective effect (Table 1).

Baicalein attenuated bleomycin induced pulmonary edema and microvascular permeability

The wet/dry (W/D) lung weight ratio was calculated as an indicator of pulmonary edema. In the present study the BLM group (6.89±0.62) showed increased mean

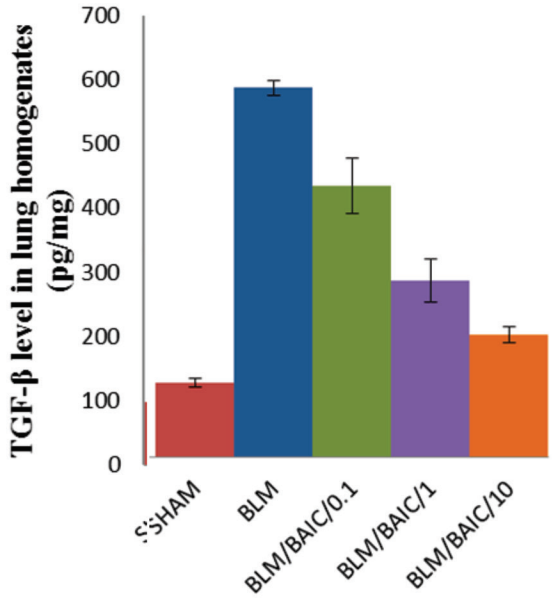

Fig. 3. TGF-β levels in lung homogenates of different groups of mice at 8 weeks post treatment.

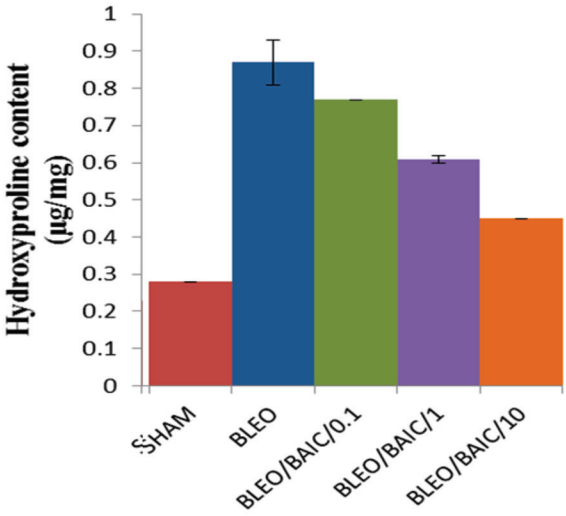

Fig. 4. Mean values for hydroxyproline content in lung homogenates of different groups of mice at 1 and 8 weeks post treatment.

wet/dry (W/D) lung weight ratio, while SHAM group (3.04±0.17) and BLM/BAIC/10 group (3.73±0.22) showed comparable values. The animals revealed dose dependent fall in the mean wet/dry (W/D) lung weight ratio in the BLM/BAIC/0.1 (4.89±0.46) and BLM/BAIC/1.0 (4.08±0.45) groups, which further supported that baicalein @ 10 mg/kg attenuated bleomycin induced lung edema. Mean values of wet-to-dry lung weight ratio are presented in Table 2.

The total protein concentration in Bronchio-alveolar Lavage fluid (BALF) was used as a measure to ascertain the microvascular permeability in the present study. The total protein concentration in BALF of BLM group (1114.60±112.58 µg/ml) was significantly ($p<0.05$) higher as compared to that of SHAM (406.65±9.08 µg/ml). But baicalein treatment BLM/BAIC/10 group (730.43±17.79 µg/ml) showed significant decline in total protein concentration as compared to that of BLM group. The improvement was seen in BLM/BAIC/0.1 and BLM/BAIC/1.0 groups also but maximum improvement was seen in highest dose group. Hence, this showed that BAIC at maximum dose was able to maintain vascular

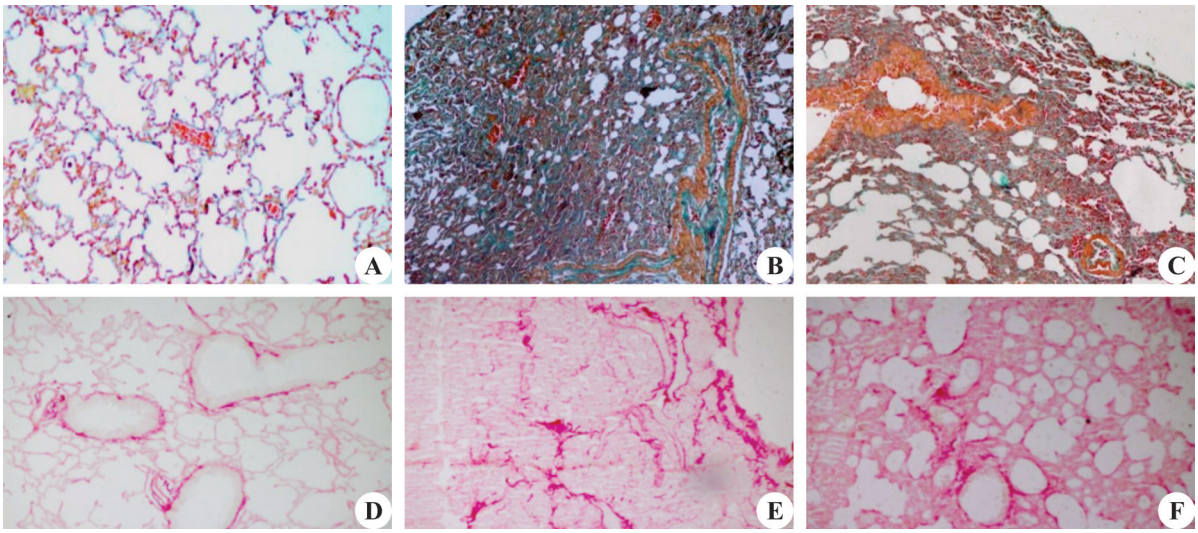

Fig. 5A. SHAM: Lung: Showing normal histology with normal alveolar architecture (Masson's trichrome x100). **B.** BLEO/8 week post treatment: Lung: Massive deposition of collagen fibers (green color stained areas) with thickening of alveolar septa and in interstitial areas depicting pulmonary fibrosis (Masson's trichrome x200). **C.** BLEO/BAIC10/8 week post treatment: Lung: Moderate pulmonary fibrosis with decreased deposition of collagen in baicalein treated group (Masson's trichrome x200). **D.** SHAM: Lung: Normal architecture of bronchioles and alveoli (Picro-Sirius red x100). **E.** BLEO/8 week post treatment: Lung: Massive deposition of collagen fibers (red color stained areas) with thickening of alveolar septa and in interstitial areas depicting pulmonary fibrosis (Picro-Sirius red x100). **F.** BLEO/BAIC/10/8 week post treatment: Lung: Moderate pulmonary fibrosis with decreased deposition of collagen in interstitial area (Picro-Sirius red x100).

permeability in lungs. The mean protein concentration values in BALF for different groups are presented in Table 2.

Baicalein attenuated pulmonary inflammatory cells infiltration

Meanvalues of TLC were measured in BALF of all the groups and it was observed that there was massive increase in the Total Leukocyte Count of BALF in BLM (4.25±0.52×10^3/µl) group compared to SHAM (0.60±0.05×10^3/µl) group. TLC value of BLM/BAIC/0.1 (3.32±0.40×10^3/µl) and BLM/BAIC/1.0 (2.45±0.28×10^3/µl) groups were comparable to each other but significant drop in TLC was seen in BLM/BAIC/10 group (1.65±0.08×10^3/µl). This proved the anti-inflammatory action of baicalein. Mean value of TLC alteration are presented in Fig. 3a. Moreover, the percentage of mononuclear cells in mice showed increase in the percentage of mononuclear cells in BALF which were 64.67±2.27 in BLM group, 61.40±2.20 in BLM/BAIC/0.1 group, 57.60±1.93 in BLM/BAIC/1.0 group and percentage was subsidized to 54.67±1.30 in BLM/BAIC/10 group. This further showed that baicalein @ 10 mg/kg bwt was able to make balance in DLC of BALF.

Myeloperoxidase (MPO) activity was measured as an indicator of inflammatory cells accumulation especially macrophages in the chronic stage. Mean values of MPO activity in lung homogenates of mice in SHAM group (54.30±1.72 mu/ml) and BLEO/BAIC/10 (103.74±18.47 mu/ml) showed comparable difference but the difference was significant from mean values of BLM (164.50±8.71 mu/ml), BLM/BAIC/0.1 (145.42±3.96 mu/ml) and BLM/BAIC/1.0 (137.31±11.80 mu/ml) groups (Table 3). These all findings suggested that baicalein was able to limit the infiltration of inflammatory cells mainly macrophages in lungs.

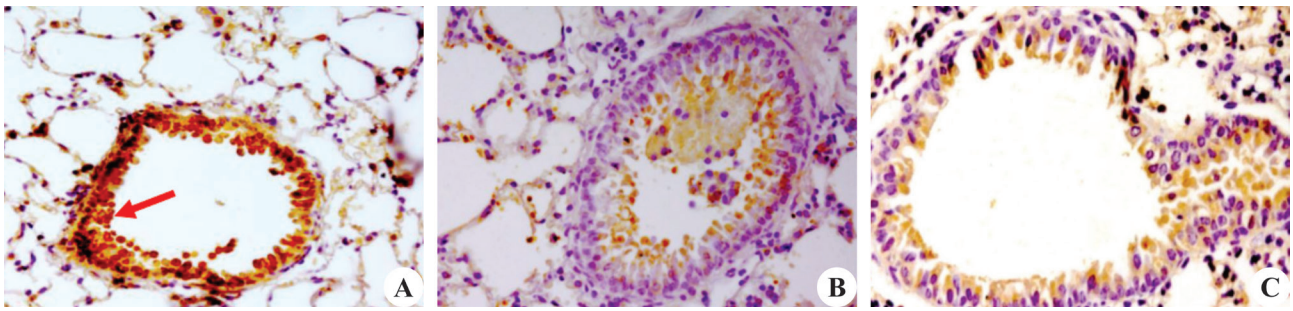

Fig. 6A. SHAM/1 week post treatment: Lung: Bronchial and alveolar epithelial cells distinctly expressing E-cadherin (Arrow) (IHC x200). **B.** BLEO/8 week post treatment: Lung: Expression of E-cadherin was seen only on the tip of bronchial epithelial cells (IHC x200). **C.** BLEO/BAIC/10/8 week post treatment: Lung: Restoration of E-cadherin expression in the bronchial epithelial cells (IHC x200).

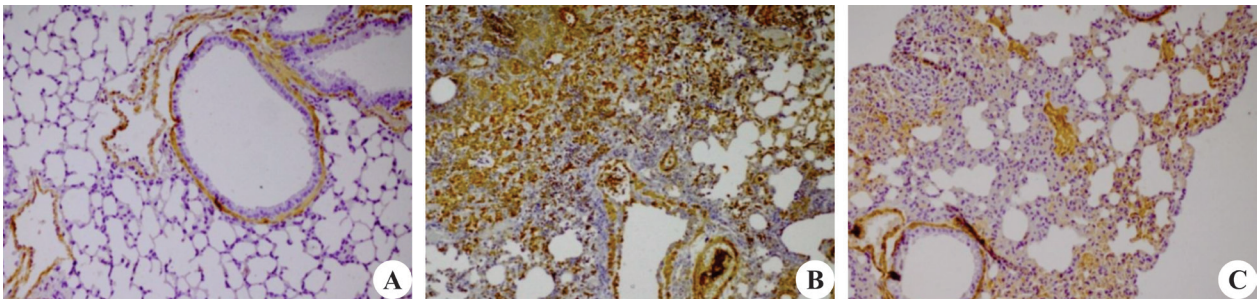

Fig. 7A. SHAM: Lung: Alpha-smooth muscle actin (α-SMA) normally expressed in the bronchial as well as vascular smooth muscles (IHC x100). **B.** BLEO/8 week post treatment: Lung: Increased immunolocalization of α-SMA in the myofibroblasts present in the alveolar interstitial area (IHC x100). **C.** BLEO/BAIC/10/8 week post treatment: Lung: BAIC treatment restored the expression of α-SMA in vascular endothelial area and decreased expression of α-SMA in the alveolar interstitial area (IHC x100).

Baicalein attenuated bleomycin induced oxidative stress

The lung malondialdehyde (MDA) level of the mice in BLM group rose to 2.00±0.00 nM MDA/g while it was significantly decreased in BLM/BAIC/10 group (1.15±0.13 nM MDA/g) which was comparable to control (SHAM) group (1.05±0.25 nM MDA/g). Baicalein also lowered the level of MDA in BLM/BAIC/0.1 (1.51±0.13 nM MDA/g) and BLM/BAIC/1.0 (1.71±0.33 nM MDA/g) groups (Table 4). This specifies that baicalein reduces the increased level of Lipid peroxidation (LPO) by bleomycin.

Further, the Superoxide Dismutase (SOD) level also decreased significantly in BLM group (2.81±0.32 U) in contrast to (SHAM) group (4.75±0.15 U). But it was raised in animals treated with baicalein in a dose dependent manner BLM/BAIC/0.1 (2.89±0.14 U), BLM/BAIC/1.0 (3.10±0.26 U) and BLM/BAIC/10 (3.94±0.15 U). Thus, it hence showed that baicalein reversed the bleomycin-induced decrease in the superoxide dismutase activity (Table 4).

Baicalein attenuated bleomycin induced inflammatory cytokines production

Interleukin-6 (IL-6) and Tumor necrotic factor-alpha (TNF-α) level were estimated in BALF. The level of IL-6 was significantly increased in BLM (154.58±12.29 pg/mg) group as compared to SHAM (71.34±2.34 pg/mg). In contrast to this, BLM/BAIC/10 (72.00±4.43 pg/mg) showed marked reduction in the IL-6 level (Table 5). Further, TNF-α in BLM (612.02±18.56 pg/mg) was higher than SHAM (58.44±4.43 pg/mg) while BLM/BAIC/10 (281.74±7.85 pg/mg) showed reduction in the level of TNF-α level (Table 5).

Baicalein attenuated bleomycin induced histopathological changes

Pulmonary morphological changes as assessed by Ashcroft's scoring scale showed reduction of the score in BAIC treated group when compared to that of BLM group (Fig. 1). Significant and consistent lesions in lung sections of different groups were categorized according to Ashcroft's (a numerical fibrotic scale) lung injury assessment scale from 0 to 8. The animals sacrificed showed infiltration of inflammatory cells mainly mononuclear cells along with edematous changes, deposition of collagen fibers, fibrosis in the sub-pleural areas in BLM group which was reduced by baicalein treatment (Fig. 2A-C).

Baicalein attenuated bleomycin induced pulmonary fibrosis

To determine the effect of baicalein on bleomycin induced pulmonary fibrosis, TGF-β level and hydroxyproline assays in lung homogenates along with Masson's trichrome and Picro Sirius red staining were performed. As TGF-β1 plays most important role in the development of pulmonary fibrosis, we determined the effect of BAIC on the levels and expression of TGF-β1. In the present study, the level of TGF-β was increased in BLM (573.75±12.31 pg/mg) group as compared to SHAM (114.97±6.82 pg/mg). BAIC treatment resulted in the reduction in the expression and levels of TGF-β in lung (Fig. 3). Further, the collagen content in the lung homogenates was examined by a hydroxyproline assay which showed increase in collagen content in lungs. Collagen content of BLM group (0.87±0.06 μg/mg) was raised which was decreased by baicalein in BLM/BAIC/10 group (0.45±0.00 μg/mg) and was comparable to SHAM

Table 6. Score range of E-cadherin and alpha-smooth muscle actin expression in different groups of mice 8 weeks post treatment.

Groups	Score of E-Cadherin expression (Mean±SE)	Score of alpha-smooth muscle actin expression (Mean±SE)
SHAM	4.66±0.21^{c}	1.83±0.16^{a}
BLEO	2.16±0.16^{a}	4.66±0.21^{cd}
BLEO/BAIC/10	3.66±0.21^{b}	2.16±0.16^{b}

The values (Mean±SE) in a column having different superscript differ significantly from each other at 5% level of significance. The values (Mean±SE) in a column having same superscript do not differ significantly from each other at 5% level of significance.

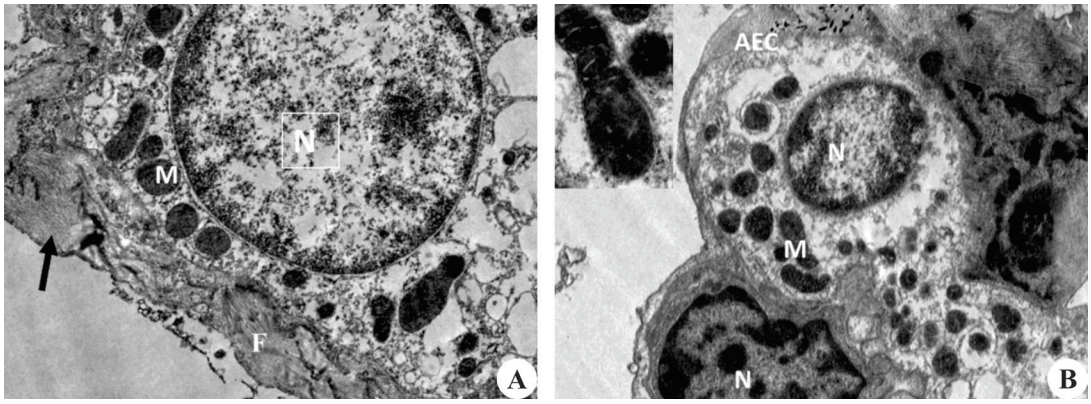

Fig. 8A. BLEO/8 week post treatment: Lung: Type 2 alveolar epithelial cells with swollen mitochondria and surrounded by deposition of collagen fibers (arrow) (Bar = 1μm) (N = nucleus, F = collagen fibers, M = mitochondria). **B.** BLEO/BAIC/10/8 week post treatment: Lung: Type 2 alveolar epithelial cells showing restoration of mitochondrial damage with fine cristae in mitochondria (inset) (Bar = 1μm) (N = nucleus, AEC = alveolar epithelial cell, M = mitochondria).

group (0.28±0.00 μg/mg). Collagen deposition was also decelerated by baicalein in BLM/BAIC/0.1 (0.77±0.00 μg/mg) and BLM/BAIC/1.0 (0.61±0.01 μg/mg) groups. Hence, this signifies that baicalein can control collagen deposition in lungs (Fig. 4).

Moreover, by Masson's trichrome and Picro Sirius red staining, there was a decreased fibrosis in baicalein treated groups. Score of picrosirius red stained section in % area was higher in BLM (11.29±1.38 μm^2) as compared to that of SHAM (0.41±0.06 μm^2) group. However, this was reduced in BLM/BAIC/10 group (6.44±1.66 μm^2) (Fig. 5A-F). The decrease was more pronounced in BLM/BAIC/10 group suggesting the antifibrotic role of baicalein in dose dependent manner.

Baicalein restored bleomycin induced changes in the expression of e-cadherin and alpha-smooth muscle actin

In the present study, to study the impressions of epithelial and mesenchymal markers, immunohistochemistry was done using primary antibodies against epithelial (e-cadherin) and mesenchymal marker (alpha-smooth muscle actin). There was reduced expression of epithelial marker e-cadherin in BLM group when compared to that of SHAM. In SHAM group the expression of E-Cadherin was observed in the cytoplasm of the bronchial and alveolar epithelial cells with a score of (4.66±0.21) whereas it was seen mainly on the tip of bronchial epithelial cells in BLM (2.16±0.16). However, the treatment with BAIC treatment restored the expression of e-cadherin in the epithelial cells (3.66±0.21) (Fig. 6A-C). Alpha-smooth muscle actin (α-SMA) was normally expressed in the bronchial as well as vascular smooth muscles as observed in SHAM mice (1.83±0.16). However there was expression of α-SMA in the alveolar interstitial areas in BLM mice (4.66±0.21) which was further reduced by baicalein treatment (2.16±0.16) (Fig. 7A-C). The scoring pattern of both immunohistochemical markers are given in Table 6.

Baicalein restored bleomycin induced ultrastructural changes

Transmission electron microscopy was performed to determine the ultra-morphological changes in bronchial and alveolar epithelial cells. The alveolar epithelial cells showed degeneration and complete loss of mitochondrial cristae along with presence of collagen fibers deposited around the alveolar epithelial cells, severe degeneration of alveolar epithelial cells was observed with loss of mitochondria as vacuolation in BLM group (Fig. 8A). The effects were ameliorated in BLM/BAIC/10 mg group where alveolar epithelial cells showed restoration of mitochondrial damage. The mitochondria showed fine cristae (Fig. 8B).

DISCUSSION

Pulmonary fibrosis (PF) is a chronic lung disorder marked by damage to alveolar epithelial cells, inflammation, fibroblast proliferation, and extracellular matrix deposition, leading to scar formation. Key areas affected include the alveolar walls and surrounding connective tissue. This process results in decreased lung elasticity and reduced alveolar surface area, impairing gas exchange and pulmonary function. In research, a single intratracheal injection of bleomycin in mice is used to model lung injury that leads to pulmonary fibrosis[9].

Baicalein (BAIC), a bioflavonoid, has various pharmacological effects, including antioxidant, anti-inflammatory, and neuroprotective properties. It works by scavenging Reactive Oxygen Species and improving antioxidant status, thus modulating the immune system[9]. However, the effectiveness of BAIC against BLM-induced chronic lung injury and its impact on Epithelial Mesenchymal Transition (EMT) is not well understood. This study aims to investigate whether BAIC treatment can improve pulmonary fibrosis and EMT following BLM exposure.

In many organs including lung, EMT is involved in pathogenesis of fibrosis. Chronic lung inflammation is always accompanied with EMT, collagen deposition, and lung fibrosis[10]. Thus in the present study focus was given to bleomycin induced chronic lung injury and subsequent EMT. And it has been observed that BAIC treatment showed amelioration in the chronic lung injury and pulmonary fibrosis caused by BLM.

The significant decrease in hemoglobin, in the present study might be because heme proteins are liable to release iron when peroxides are present. Organic peroxides and hydrogen peroxide can be produced during the bleomycin reaction leading to iron release from haemeproteins resulting in decrease in hemoglobin concentration[11]. The hemoglobin level was improved in group BLEO/BAIC/10 and was comparable to control because of baicalein has a strong antioxidant activity which quench the peroxides present and might prevent the release of iron from the heme proteins[13], thereby improving the Hb level.

Bleomycin leads to altered lung fluid balance leading to increased permeability in the endothelium leading to pulmonary edema which is a pathophysiological hallmark of lung injury.

Increased pulmonary capillary permeability leads to edema by allowing more fluid and protein to enter the lung interstitium[14]. This type of pulmonary edema, characterized by high protein content, arises from less restricted plasma proteins moving across the capillary membrane. The severity of alveolar flooding during lung injury depends on factors like interstitial edema extent, alveolar epithelial injury, and the epithelium's ability to clear edema fluid[15].

Because of the increase in micro vascular permeability in lung injury, concomitant increases in lung micro vascular hydrostatic pressure (as might occur with aggressive volume resuscitation) will lead to even greater formation of pulmonary edema. The injury is triggered by the release of inflammatory cells, proinflammatory cytokines and ROS which ultimately damage the endothelial cells. In the present study, the damage to the endothelial cells might have led to permanent influx of protein and edematous fluid into the lung alveoli leading to increase in protein content and wet to dry lung ratios. In the present study baicalein in BLM/BAIC/10 group @ 10 mg bw I.P considerably reduces the pulmonary edema due to its anti-inflammatory, antioxidant activity[16]. Mechanistically, baicalein treatment appears to mitigate lung damage by suppressing accumulation of inflammatory cell, decreasing IL-6, TNFα and inactivating NFκB pathway which results in inactivation of pro-inflammatory genes, with release of the cytokines[17].

The TLC in BALF was increased due to increase in the number of mononuclear cells at 8 weeks as during any chronic inflammation the MNCs predominate[18]. In addition, the alveolar inflammatory cells have been regarded as a major source of the release of pro-inflammatory cytokines and chemokines, promoting inflammatory cell accumulation in tissues, as well as reactive oxygen species (ROS) formation[19]. Accordingly, the immune cell-derived inflammatory mediators play a critical role in the pathogenesis of pulmonary fibrosis[20]. The number of inflammatory cells in the BLM/BAIC/10 group decreased and showed that baicalein ameliorated the effect of bleomycin induced lung injury due to its anti-inflammatory effect[21]. These changes might be due to the fact that baicalein leads to impairment of reactive oxygen intermediates production, through scavenging reactive oxygen intermediates by antagonizing ligand-initiated Ca^{2+} influx by baicalein that accounts for the inhibition of Mac-1-dependent leukocyte adhesion that confers the anti-inflammatory activity of baicalein[22], hence, reduced leukocyte infiltration which ultimately lead to reduced MPO activity post treatment of BLM. Importantly, the increased amount of pro-inflammatory cytokines (IL-6 and TNF-α) in BALF of mice challenged by BLM was remarkably inhibited by BAIC. These findings suggest that the protective effect of BAIC in chronic lung injury may be, at least in part, attributed to the suppression of inflammatory cell sequestration and infiltration into lungs, in turn attenuating pro-inflammatory cytokine release.

Bleomycin binds to iron (Fe^{2+}), undergoes redox cycling and catalyzes the formation of ROS that plays a major role in the progression of pulmonary fibrosis by targeting DNA, protein and lipids with ultimate progression of lipid peroxidation[23]. In this study the decreased LPO production observed in BAIC treated group, might also be due to the iron chelating activity of BAIC and has been shown to possess relatively potent metal chelating properties[24]. SOD is a ubiquitous enzyme that catalyzes the dismutation of superoxide into oxygen and hydroperoxides thereby protecting the cells from detrimental superoxide anion[25]. A notable descend in the activities of SOD was observed in bleomycin-induced animals, which might be due to increased LPO and overproduction of ROS[26]. The activity of SOD was maintained to near normal values upon treatment with Baicalein. This may be due to the direct action of BAIC on superoxide, hydroxyl and alkoxyl radical coupled with its ability to attenuate LPO, which in turn reduces free radical generation and oxidative stress during BLM induced chronic lung injury.

Histopathological examination of the lungs showed infiltration of inflammatory cells along with edematous changes, deposition of collagen fibres, fibrosis in the sub-pleural areas in BLM group which was reduced by

baicalein treatment. The pulmonary fibrosis caused due to bleomycin was restored backed by BAIC due to the antifibrotic effect of BAIC as observed in the present study depicted by reduction in the deposition of collagen as shown by Masson's trichrome, picro-sirius red stained lung sections as well as hydroxyproline content in the lung. In BLM induced chronic lung injury TGF-β plays the most important role in pulmonary fibrosis. TGF-β, a potent fibrogenic cytokine is elevated after bleomycin administration in the epithelial cells, endothelial cells, alveolar macrophages and interstitial fibroblasts and thereby initiates inflammatory response, apoptosis of epithelial cell and proliferation of fibroblast along with collagen deposition and EMT[27]. It has been postulated that the initial activation of TGF-β is due to the initial inflammatory response and generation of ROS[28]. In this study, baicalein had reduced the expression of TGF-β along with the reduction of collagen deposition in interstitial regions which might be due to inhibition of the increased expression of TGF-β1 and p-Smad-2/3 in bleomycin-treated mice[29].

Epithelial-mesenchymal transition (EMT) is a physiological process in which epithelial cells acquire the motile and invasive characteristics of mesenchymal cells[30]. E-cadherin, a cell adhesion molecule, normally expressed by epithelial cells is repressed during EMT. During EMT, these cells leave the epithelial layer and migrate through the lining basement membrane followed by accumulation in the tissue interstitium where they eventually loss the epithelial markers and gain a mesenchymal phenotype[31]. In the present study, the expression of e-cadherin was suppressed in BLM mice which were restored with baicalein treatment. Further in the present study, BLM administered mice showed increased TGF-β with decreased expression of E-cadherin. This may be due to the fact that TGF-β represses E-cadherin production in epithelial cells[32].

Further, alpha smooth muscle actin (α-SMA), a contractile protein and actin isoform, are expressed mainly in smooth-muscle cells of blood vessels and plays an important role in fibrogenesis[33]. In the present study, besides vascular and bronchiolar smooth muscles, α-SMA expression was observed in the alveolar interstitial areas in BLM administered mice which was restored with baicalein treatment. α-SMA positive myofibroblasts have been demonstrated in type 2 EMT associated with tissue regeneration and organ fibrosis. This occurs due to destabilize interactions between epithelial cells and/or cell to extracellular matrix. Further, stressed and injured epithelium can give rise to myofibroblasts and thereby contribute to fibrogenesis[34]. Thus, it indicates that EMT plays an important role in bleomycin induced pulmonary fibrosis.

Ultrastructural studies showed injured and apoptotic epithelial cells in BLEO treated lung which was restored with baicalein treatment. Evidently, baicalein treatment is associated with the restoration of mitochondrial functions with mitochondrial ultrastructural changes in bronchial epithelia either due to 15-LOX inhibition, or by indirect mechanisms such as the reduction lipid peroxidation. Baicalein may limit apoptosis, possibly via the inhibition of both the extrinsic and intrinsic pathways of apoptosis, including the inhibition of TNF-α production and modulation of pro- and anti-apoptotic signaling elements[35].

CONCLUSION

In conclusion, baicalein was effective in attenuating the BLM induced Pulmonary fibrosis through suppression of oxidative stress, inflammation, histological as well as ultrastructural damages and EMT especially during the chronic stage injury. This study will provide an additional knowledge on the ameliorative effect of baicalein during the later stages of lung injury where the EMT starts and this may help in the therapeutic or management of clinical chronic lung injury associated pulmonary fibrosis.

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