**ORIGINAL ARTICLE**

# Curcumin inhibition of bleomycin-induced changes in lung collagen synthesis, deposition and assembly

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## Abstract

**Background** Idiopathic pulmonary fibrosis is characterized by progressive lung tissue remodeling and disproportionate deposition of collagenous proteins with limited therapeutic interventions. The purpose of this study was to determine whether curcumin inhibits bleomycin (BLM)-induced increases in synthesis, degradation and cross-linking of lung collagen in rats.
**Methods and results** Following a single intratracheal instillation of BLM to rats (0.75 U/100 g, sacrificed 3, 5, 7, 14 and 28 days post-BLM), lung collagen synthesis (determined by incorporation of $^3$H-proline) and deposition (determined by lung hydroxyproline content) progressively increased at days 7, 14 and 28 post-BLM injection. Lung lavage fluid hydroxyproline and collagenase levels (a measure of collagen turnover) were increased in BLM rats compared with control groups. In addition, BLM instillation resulted in increased concentrations of collagenase and collagenolytic cathepsin in the lungs. Furthermore, increased cross-linking (as determined by aldehyde content of acid soluble collagen), and decreased susceptibility of fibrotic lung insoluble collagen to denaturing agents occurred in BLM-injured lungs. Significant increases in alveolar macrophage (AM) release of transforming growth factor-β1 (TGF-β1) were noted at various time points (days 3, 5, 7, 14 and 28 post-BLM) during the development and progression of lung fibrosis in rats. Curcumin treatment to BLM rats (300 mg/kg 10 days before and daily thereafter throughout the experimental time period) was associated with marked reductions in lung collagen synthesis and deposition, BALF and lung collagenase activity, BALF hydroxyproline content and lung collagenolytic levels. Additionally, reduced levels of collagen cross-linking and enhanced susceptibility of insoluble lung collagen to denaturing agents were observed in curcumin-treated BLM rats. Finally, curcumin inhibited BLM-induced increases in AM production of TGF-β1.
**Conclusions** Our data demonstrate for the first time that curcumin prevents fibrotic deposits by modulating collagen turnover, assembly and deposition in BLM-instilled rat lungs, and that curcumin treatment protects against BLM activation of macrophages by suppressing the release of TGF-β1.

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## Introduction

In the normal lung, a delicate balance exists between synthesis and degradation of the extracellular matrix (ECM) proteins. However, this dynamic equilibrium is jeopardized during the pathogenesis of idiopathic pulmonary fibrosis (IPF), resulting in an excessive synthesis and abnormal deposition of ECM proteins, particularly collagens, in the lung parenchyma that leads to severe loss of lung function, respiratory failure and death [1–3].

Bleomycin (BLM), a highly efficient antitumor antibiotic used in the treatment of various cancers, causes dose-dependent pulmonary injury, which leads to a progressive destruction of lung parenchyma followed by proliferation

of fibroblasts, and abnormal synthesis and accumulation of extracellular matrix (ECM) components in the alveolar interstitium [4, 5]. Activation of interstitial and subepithelial fibroblasts and myofibroblasts to proliferate and to synthesize collagen apparently follows injury to the alveolar epithelium and endothelium [1, 6]. Biochemical studies demonstrate that there is more collagen in BLM-induced fibrotic lung, and in human interstitial pulmonary fibrosis, than in the normal lung [7–11]. Early increase in type III collagen followed by type I collagen at later stages of fibrosis results in an increased ratio of type I to type III collagen [12]. Mediators of fibroproliferative processes include fibrogenic cytokines such as transforming growth factor-β1 (TGF-β1) that serve to enhance the synthesis and deposition of collagen and other matrix constituents [13]. TGF-β1 exerts its profibrotic effects by inducing the synthesis of ECM, decreasing its degradation, and stimulating the synthesis of integrin matrix receptors [14]. Additionally, connective tissue growth factor (CTGF), platelet derived growth factor (PDGF), and fibroblast growth factor 2 (FGF-2), play major roles in the process of lung fibrogenesis [13–15].

Untreated IPF patients typically have poor quality-of-life linked to progressively disabling dyspnea, with a median survival of around 3–5 years. Current therapies that inhibit abnormal ECM synthesis and deposition and thus progression to end-stage interstitial lung disease are often limited and have proven unsatisfactory. Although clinical interventions with nintedanib and pirfenidone have been appreciated in IPF, these drugs have shown only a modest efficacy on matrix expansion, remodeling and progression of lung destruction [16–18]. Therefore, it is not surprising that modulation of excess collagen deposition has become the center of intense research efforts in pulmonary fibrosis. Continuous efforts are therefore made to find potential novel therapeutic agents for IPF.

Curcumin, diferuloylmethane, a main phenolic compound extracted from turmeric (*Curcuma longa*), has numerous therapeutic properties and its effects in various lung diseases have been reported [19–22]. Our laboratory has previously demonstrated the first protective function of curcumin against BLM-induced lung injury and inflammation collagen content [23]. Studies from other laboratories have also confirmed the protective effect of curcumin on lung collagen deposition in animal models of lung fibrosis [24–27]. However, earlier studies, including our own investigations, on the antifibrotic effect of curcumin were limited to either localization of lung collagen components at the immunohistochemical level or collagen assay; results of detailed collagen metabolism studies were scanty. A detailed understanding of the protective effects of curcumin on BLM-induced changes in collagen metabolism (collagen biosynthesis, extracellular collagen crosslinking and proteinases involved in collagen turnover) during initiation and development of lung fibrosis

is largely unexplored. Therefore, we set to determine the new biochemical insights into the protective function of curcumin on dysregulated lung collagen remodeling in a rat model of BLM-induced pulmonary fibrosis.

## Methods

### Experimental design

Healthy male Wistar rats weighing 325–350 g were obtained from the animal house facilities of Central Leather Research Institute, and allowed to acclimatize for one week before experiments with water and laboratory rat chow ad libitum. A 12-h/12-h light/dark cycle was maintained. All experiments were carried out according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 86–23, revised 1996).The animal ethical committee of CLRI approved the project proposal and experiments were conducted following the rules of the ethical committee.

Rats were divided into four groups. The first group (SAL) consisted of saline-instilled rats, which received a single intratracheal (i.t.) dose of 0.4 ml of sterile physiological saline. The second group (CUR) received 300 mg/kg of curcumin. The third group (BLM) received a single i.t. dose of bleomycin (0.75 U/100 g body weight in sterile physiological saline). The final group (CUR + BLM) received 300 mg/kg of curcumin 10 days before BLM and daily thereafter throughout the experimental period (28-d period). Saline and BLM were instilled to rats through the endotracheal catheter (a non-toxic polyethylene tubing, inner diameter: 1.67 mm; outer diameter: 2.42 mm, length: 70 mm) using a sterile 1-ml syringe under sodium pentobarbital anaesthesia (35 mg/kg) followed by 3 ml of air to distribute the drugs equally. Curcumin, prepared fresh everyday, was suspended in 1% gum acacia and administered by gastric intubation.

### Bronchoalveolar lavage fluid (BALF)

Bronchoalveolar lavage was performed at days 3, 5, 7, 14 and 28 post-BLM administration as described previously. Briefly, the animals were anaesthetized with an intraperitoneal dose of sodium pentobarbital. A polyethylene catheter was placed in the trachea and secured in place. Rats were exsanguinated via the abdominal aorta, and an incision was made in the diaphragm (to allow the lungs to expand during lavage procedures). The lungs were lavaged 5 times with 5 ml/wash of calcium and magnesium-free phosphate-buffered saline, pH 7.4, prewarmed at 37 °C. The BALF was centrifuged at $300 \times g$ for 10 min at 4 °C, and the cell-free supernatant was aliquoted and stored at $-70$ °C.

## Collagen biosynthesis

Lung collagen synthesis was determined following the method of Clark et al. [7] as outlined by [28]. Lung slices were incubated in 5 ml of Dulbecco's modified Eagle's medium containing 10% foetal bovine serum, 50 µg/ml ascorbic acid, 75 µg/ml ferrous sulphate, 200 U/ml penicillin, 200 µg/ml streptomycin and 30 µCi of $^3$H-Proline. Lung slices were equilibrated with 95% $O_2$/5% $CO_2$ and incubated for 4 h at 37 °C in a Controlled Environment Incubator Shaker (New Brunswick Scientific Co. Inc., Edison, New Jersey, USA). At the end of incubation period, lung samples were homogenized with a Polytron homogenizer in 10% trichloroacetic acid (TCA) and washed 4 times with 5% TCA. The TCA soluble fraction was hydrolyzed with 6 N hydrochloric acid (final concentration) and the hydroxyproline content and radioactivity of hydroxyproline were determined [29]. The results were expressed as cpm of $^3$H-hydroxyproline/hour/lung.

## Determination of total hydroxyproline content and the radioactivity of $^3$H-hydroxyproline

The total hydroxyproline content and the radioactivity of $^3$H-hydroxyproline were determined following the procedure of [30]. The samples after acid hydrolysis with 6 N hydrochloric acid were neutralized with potassium hydroxide and made up to known volume. About 2 ml of sample containing 1–10 µg of hydroxyproline was mixed with 1 ml of chloramine-T (0.141 g of chloramine-T mixed with 2 ml of distilled water), 3 ml of peroxide free methyl cellosolve and 5 ml of sodium acetate-citrate buffer, pH 6.0, and allowed to stand for 20 min at room temperature. The oxidation process was stopped by the addition of 0.5 ml of 2 M sodium thiosulphate. After thorough shaking, the reaction mixture was saturated with approximately 2 g sodium chloride. The oxidation product of proline and other interfering substances were removed by extraction with toluene. To convert the oxidation product of hydroxyproline to pyrrole, the aqueous solution was heated in a boiling water bath for 30 min, cooled at room temperature and pyrrole obtained from hydroxyproline was extracted with 6 ml of toluene. One ml of toluene layer containing pyrrole was mixed with 4 ml of Ehrlich's reagent (0.5 g p-dimethylaminobenzaldehyde in 9 ml of absolute ethanol and 1 ml of 12 N hydrochloric acid). The contents were shaken, kept for 30 min at room temperature and the colour developed was read at 557 nm. Aliquots containing 4 ml of the toluene layer were transferred to scintillation vials containing ready gel liquid scintillation cocktail and the radioactivity of $^3$H-hydroxyproline were measured in an automated liquid scintillation system (Beckman LS-1701).

## Estimation of total collagen in lungs

The total collagen content of the lung tissue was determined by the estimation of hydroxyproline, a characteristic imino acid of collagen. Lung tissues were washed with physiological saline, cut into small pieces and defatted with chloroform: methanol (2% v/v). Lung tissue (either dry or wet weight) was hydrolyzed with 6 N HCl at 110 °C for 20 h in sealed tubes. After hydrolysis, the sample was evaporated to dryness; the residue was dissolved in water and made up to a known volume. The hydroxyproline was then estimated by the method of Woessner [30]. The collagen content of lung tissues was then calculated by multiplying the hydroxyproline content by the factor 7.46 [31].

2 ml portions containing 2–10 µg hydroxyproline were placed in test tubes. A series of standards were prepared containing 0–10 µg hydroxyproline in a total volume of 2 ml. Hydroxyproline oxidation was initiated by adding 1 ml of chloramine-T (0.141 g of chloramine-T mixed with 2 ml of distilled water, 3 ml of peroxide free methyl cellosolve and 5 ml of sodium acetate-citrate buffer, pH 6.0) to each tube. The tube contents were vortexed for few minutes and allowed to stand for 20 min at room temperature. The chloramine-T was then destroyed by adding 1 ml of perchloric acid (27 ml of perchloric acid to 100 ml water) to each tube. The contents were mixed and allowed to stand for 5 min. Finally 1 ml of p-dimethylaminobenzaldehyde (PDAB; 20% solution was prepared shortly before use by adding methyl cellosolve to 20 g of PDAB to give a final volume of 100 ml and warmed to 60 °C to facilitate solubilization) solution was added and the mixture was shaken. The tubes were placed in a 60 °C water bath for 20 min, and then cooled in tap water for 5 min. The colour developed was read at 557 nm. Results are expressed as µg/mg dry tissue or mg/g wet tissue.

## Extraction, purification and fractionation of collagen

Extraction, purification and fractionation of collagen were carried out at 4 °C, according to the procedure described by Miller and Rhodes [32].

### Total collagen (TC)

Lung tissues of different experimental groups were washed, weighed and minced in 0.05 M Tris–HCl, pH 7.5. The minced tissues were homogenized in 0.05 M Tris–HCl and a portion of the homogenate was used for the estimation of hydroxyproline content and radioactivity.

### Neutral salt soluble collagen (NSSC)

The remaining portion was extracted twice with 1 M sodium chloride in 0.05 M Tris–HCl pH 7.5 containing 20 mM EDTA and 1 mM phenylmethylsulfonylfluoride at 4 °C in a refrigerated centrifuge. An aliquot of the supernatant was hydrolyzed with an equal volume of 12 N hydrochloric acid, and the collagen content was determined.

### Acid soluble collagen (ASC)

The residue left after sodium chloride extraction was again extracted with 0.5 M acetic acid, pH 3.0, under similar conditions as described above for a total period of 72 h, with daily changes of fresh acid. The supernatants obtained were pooled together and centrifuged at 20,000 rpm for 1 h at 4 °C. An aliquot of the supernatant was subjected to the determination of collagen content.

### Pepsin soluble collagen (PSC)

The residue left after acetic acid extraction was again extracted with 0.5 M acetic acid containing pepsin (1:100 ratio between the weight of the enzyme and dry weight of the tissue to be extracted) under similar conditions as described above for a total period of 72 h, with daily changes of fresh acid. The supernatants obtained were pooled together and centrifuged at 20,000 rpm for 1 h at 4 °C. An aliquot of the supernatant was subjected to the determination of collagen content.

### Insoluble collagen (ISC)

Neutral salt soluble, acid soluble and pepsin soluble collagen were expressed as percentage of total collagen. Insoluble collagen content was calculated by substracting the sum of soluble collagen contents (NSSC + ASC + PSC) from 100.

## Assay of BALF collagenase

Collagenase activity in lung lavage fluid was measured following the method of Christner et al. [33], with modification. Assays were carried out as follows: (1) an aliquot of acetic acid solution of rat skin type 1 collagen was incubated with 0.1 M Tris–HCl (pH 7.5) containing 0.15 M sodium chloride, and 0.1 M EDTA for 60 min at 37 °C. (The amount of lavage fluid added to the assay mixture was 50 µl). The tube was capped and the contents were mixed gently and incubated at 37 °C for 24 h. The reaction tube was placed on ice for 10 min just after incubation, and then centrifuged at 15,000 rpm for 15 min. 50 µl of supernatant including the cleaved collagen was separately hydrolyzed with 6 N hydrochloric acid and assayed for hydroxyproline by the

method of Woessner [30]. Total collagenolytic activity was expressed as µg collagen degraded/hour/ml of lavage fluid.

## Assay of lung collagenase activity

The lung collagenolytic activity was determined according to the method of Woessner [34].

### Preparation of lung samples

Minced lungs from each animal were weighed and frozen. When ready for assay they were thawed, suspended in 5 ml of 0.05 M Tris–HCl buffer (pH7.6) containing 0.25% Triton x-100 and 0.01 M calcium chloride. Each sample was then homogenized in a Polytron homogenizer. After centrifugation at 10,000×g for 15 min, the pellet was resuspended in 5 ml of 0.05 M Tris–HCl (pH 7.6) containing 200 units/ml penicillin and 250 µg/ml streptomycin and 0.15 M sodium chloride. This suspension was used for the assay of lung collagenolytic activity.

### Procedure

The samples to be assayed were divided into two equal portions. The first one served as the blank and contained 0.01 M EDTA and the volume was made up to 10 ml with Tris buffer. The second portion, which served as test contained 0.01 M calcium chloride and made up to 10 ml with the same buffer. To both tubes, 100 µg trypsin was added, incubated at 37 °C for 5 min (to activate latent collagenase) and stopped with 400 µg of soyabean trypsin inhibitor. They were then incubated in a Controlled Environment Incubator Shaker (New Brunswick Scientific Co., Inc. Edison, New Jersey, USA) for 24 h at 37 °C. Soluble hydroxyproline containing peptides were released by collagenase from lung collagen present in the pellets. These products were separated from undigested insoluble collagen by centrifugation of the digests at 4 °C for 30 min at 30,000×g. The resulting supernatants and the pellets were separately hydrolyzed with 6 N hydrochloric acid and assayed for hydroxyproline by the method of Woessner [30]. Total collagenolytic activity was expressed as µg of collagen degraded/mg of collagen.

## Assay of lung collagenolytic cathepsin

The activity of lung collagenolytic cathepsin was assayed by the modified method of Anderson [35] using bovine tendon collagen as the substrate. The bovine tendon collagen was suspended at a concentration of 10 mg/ml in a solution of 0.25% acetic acid in 25% of glycerol (v/v). The collagen was dispersed with the Polytron homogenizer and the resulting homogenate was adjusted to pH 3.5 with acetic acid. Aliquots (0.5 ml) of enzyme were added to 0.5 ml of

collagen suspension in a series of centrifuge tubes. One ml of 0.2 M sodium formate buffer, pH 3.5, containing 10 mM cysteine was added to each tube and mixed thoroughly. The reaction mixture was incubated at 37 °C for 3 h and centrifuged at 3,000×$g$ for 15 min to remove residual collagen. The blank readings were obtained from assay mixture that had been prepared on ice and then centrifuged without prior incubation. Hydroxyproline content in the supernatant was determined by the method of Woessner [30] as described above. The amount of collagen degraded was calculated and enzyme activity was expressed as μM of hydroxyproline liberated/mg protein.

## Estimation of aldehydes in acid soluble collagen

Aldehydes in collagen were measured according to the method of Paz et al. [36]. One ml of 0.2% solution of purified acid soluble collagen in 0.5 M acetic acid was used for the estimation of aldehydes. The solution was heated for 3 min at 100 °C in a screw-capped tube with 0.2 ml of 1% solution of N-methylbenzothiazolone hydrazone (MBTH). To the cooled reaction mixture, 2.5 ml of 0.2% ferric chloride solution was added, reacted for 5 min and then 6.5 ml of acetone was added. The colour developed was read at 670 nm. A reagent blank and a series of standards (2 nmol to 1 micromole/ml of aldehyde) were run simultaneously to give a standard curve.

## Susceptibility of insoluble collagen to denaturing agents

The susceptibility of insoluble collagen to denaturing agents such as urea and potassium cyanide was studied according to the method of Adam et al. [37]. Homogenized insoluble collagen samples of wet weight 900 mg were suspended in 15 ml of 6 M urea and 2 M potassium cyanide separately at room temperature for 24 h with constant stirring. The samples were then centrifuged and the amount of solubilized collagen was calculated by estimating the hydroxyproline content of the supernatant by the method of Woessner [30] as described above.

## Preparation of alveolar macrophage (AM)-conditioned media and determination of rat TGF-β1

AM culture was performed as described before [23] and the concentration of TGF-β1 in AM-conditioned media was determined using a human TGF-β1 ELISA kit (R&D Systems, MN, USA) that detects rat TGF-β1 protein with a sensitivity of 5 pg/ml. The assay detects only the active form of TGF-β1, and samples were activated before measurement

according to the manufacturer's protocol. All assays were done in triplicate wells.

## Statistical analysis

Results are expressed as mean ± standard deviation of six independent experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) for multiple comparisons followed by *post-hoc* Bonferroni test (Graphpad Prism, version 3.01).

## Results

Figure 1 illustrates the time course studies on BLM-induced changes in collagen synthesis (as measured by the incorporation of $^3$H-proline). Compared to control lungs, a dramatic increase in lung collagen synthesis was observed at 7 days post-BLM injection. Collagen synthesis was also elevated at 14 and 28 days after BLM administration. Administration of curcumin markedly reduced BLM-induced increases in lung collagen biosynthesis.

Since a maximal increase in lung collagen was observed at 28 days post-BLM administration, various collagen fractions were isolated and characterized at this time point. Figure 2a demonstrates the concentration of neutral salt soluble and acid soluble collagen in the lungs of BLM-treated rats. Compared with controls, BLM lungs had a decrease in NSSC. In contrast, increased levels of ASC were noted in BLM lungs. BLM-induced changes in collagen fractions were significantly inhibited by curcumin treatment. Concentrations of PSC and TC were increased in BLM-injured lungs (Fig. 2b) as compared with control lungs. However, curcumin inhibited BLM-induced increases in lung PSC and TC.
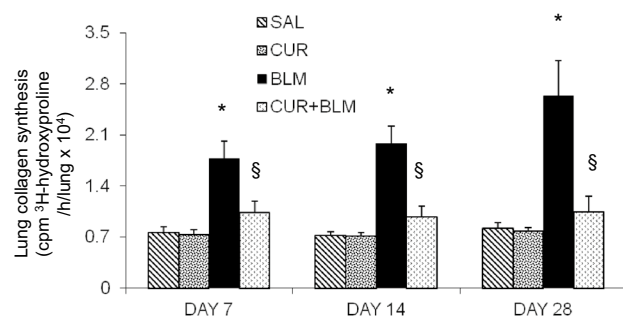


**Fig. 1** Protective effects of curcumin on bleomycin-induced increases in lung collagen synthesis. Results are mean ± SD of six independent observations in each group. Statistical analysis was done using one way analysis of variance with post-hoc Bonferroni test. *Significantly ($p < 0.001$) higher than all groups; §significantly ($p < 0.001$) lower than bleomycin rats
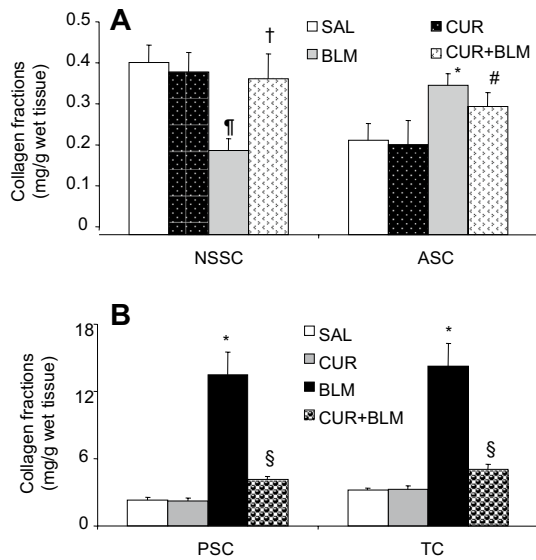
**Fig. 2** Protective effects of curcumin on bleomycin-induced changes in lung collagen fractions: NSSC & ASC (**a**); PSC & TC (**b**). Results are mean ± SD of six rats in each group. Statistical analysis was done using one way analysis of variance with post-hoc Bonferroni test. ¶Significantly ($p < 0.001$) lower than all groups; #significantly ($p < 0.001$) higher than bleomycin rats; *significantly ($p < 0.001$) higher than all groups; §significantly ($p < 0.001$) lower than bleomycin rats. *NSSC* neutral salt soluble collagen; *ASC* acid soluble collagen; *PSC* pepsin soluble collagen; *TC* total collagen

Analysis of BAL fluid revealed a progressive increase in collagenase activity (Fig. 3a) in BLM rats, while collagenase activity was virtually absent in BAL fluid of control rats. BALF collagenase activity was elevated 3 days after BLM and reached its peak at day 7. Thereafter, collagenase level decreased but it was still significantly higher than control values. Similarly, BALF hydroxyproline content was elevated in BLM rats, compared to controls, reaching its peak 7 days after BLM injection (Fig. 3b). Statistically significant ($p < 0.001$) inhibition of BLM-stimulated BALF collagenase and hydroxyproline was found with curcumin treatment.

Levels of lung collagenase (Fig. 4a) and collagenolytic cathepsin (Fig. 4b) increased between 1.5- and 2.5-fold in BLM rats compared with control rats ($p < 0.001$). Both collagenase and collagenolytic cathepsin activities were up-regulated as early as 3 days post-BLM, reached its peak 7 days after BLM, and remained higher until the end of experimental time period. In the fibrotic rats treated with curcumin, levels of lung collagenase and collagenolytic cathepsin were dramatically reduced to near control values.

Levels of aldehyde from acid soluble collagen of BLM-injured lungs increased 3-fold compared with control lungs (Fig. 5a), whereas treatment with curcumin dramatically reduced BLM-induced increases in aldehyde content of acid soluble collagen. The susceptibility of lung insoluble collagen to denaturing agents such as 6 M urea and 2 M KCN were significantly ($p < 0.001$) lower in BLM rats compared
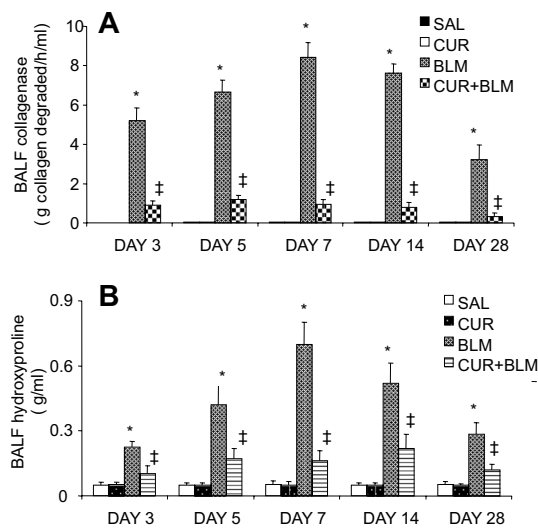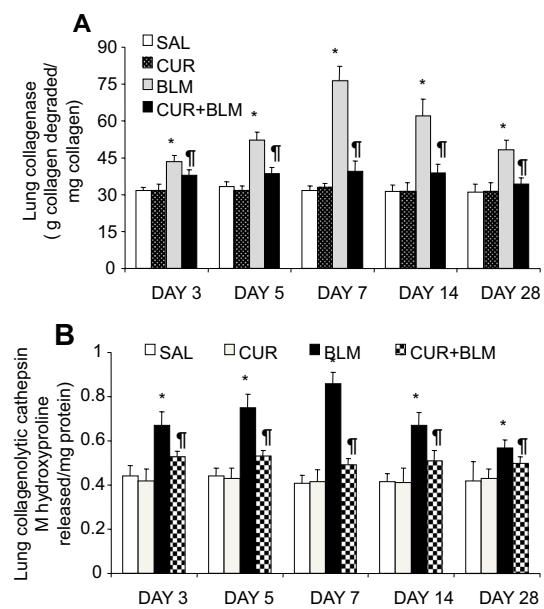


**Fig. 3** Protective effects of curcumin on bleomycin-induced changes in collagenase (**a**) and hydroxyproline (**b**) content in lung lavage fluid. Results are mean ± SD of six rats in each group. Statistical analysis was done using one way analysis of variance with post-hoc Bonferroni test. *Significantly ($p < 0.001$) higher than all groups; ‡significantly ($p < 0.001$) lower than bleomycin rats



**Fig. 4** Protective effects of curcumin on bleomycin-induced changes in lung collagenase (**a**) and collagenolytic cathepsin (**b**). Results are mean ± SD of six rats in each group. Statistical analysis was done using one way analysis of variance with post-hoc Bonferroni test. *Significantly ($p < 0.001$) higher than all groups; #significantly ($p < 0.001$) lower than bleomycin rats
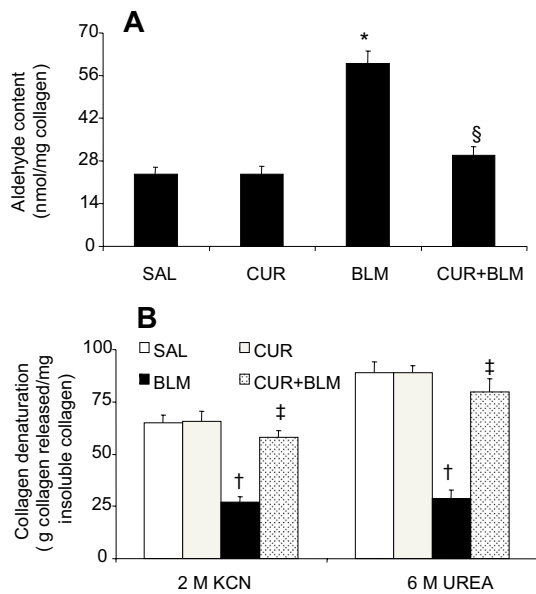
**Fig. 5** Protective effects of curcumin on bleomycin-induced changes in aldehyde content (**a**) and susceptibility of lung collagen to denaturing agents (**b**). Results are mean SD of six rats in each group. Statistical analysis was done using one way analysis of variance with post-hoc Bonferroni test. *significantly ($p < 0.001$) higher than all groups; §Significantly ($p < 0.001$) lower than bleomycin rats; †significantly ($p < 0.001$) lower than all groups; ‡significantly ($p < 0.001$) higher than bleomycin rats
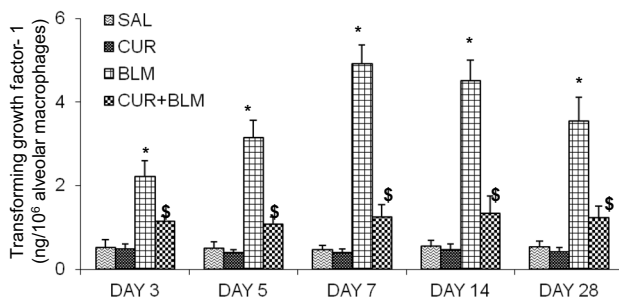


**Fig. 6** Protective effects of curcumin against bleomycin-induced changes in alveolar macrophage release of transforming growth factor-1. Results are mean ± SD of six independent observations in each group. Statistical analysis was done using one way analysis of variance with post-hoc Bonferroni test. *Significantly ($p < 0.001$) higher than all groups; $significantly ($p < 0.001$) lower than bleomycin rats

with control animals (Fig. 5b), but curcumin treatment significantly increased the susceptibility of fibrotic lung insoluble collagen to denaturing agents.

Transforming growth factor-β1 release was measured as an indicator for BLM-induced macrophage activation. AM production of TGF-β1 was significantly ($p < 0.001$) higher in BLM rats: the levels of TGF-β1 increased on days 3 and 5, peaked at day 7, and remained elevated until the end of the

observation time (Fig. 6). It is also interesting to note that AM from BLM rats elaborated greater amounts of TGF-β1 in culture medium without any exogenous stimulus suggesting that BLM has activated ("primed") AM in vivo to release this fibrotic cytokine. Interestingly, curcumin treatment significantly ($p < 0.001$) suppressed BLM-induced AM release of TGF-β1.

## Discussion

Remodeling of the alveolar interstitial matrix is a final common pathway towards lung destruction in pulmonary fibrosis. Increase in lung cellularity and ECM, specifically, collagens, are important histological abnormalities that occur both in animal models and in human pulmonary fibrosis [1, 38, 39]. Increased deposition and remodeling of lung collagen could lead to changes in biochemical, biomechanical and functional properties of the lung tissue in pulmonary fibrosis. Our earlier studies on curcumin inhibition of BLM-induced lung injury led us to postulate whether this protective activity of curcumin extends to modulation of collagen turnover. The present study demonstrates a protective role for curcumin in BLM-induced fibrotic remodeling by regulating lung tissue collagen metabolism, assembly and deposition. In BLM-injured lungs, an increase in collagen synthesis and deposition and a decrease in neutral salt soluble collagen (NSSC) were observed. The NSSC fraction represents the youngest form of collagen that serves as precursor to insoluble collagen [40], and a decrease in NSSC could indicate the involvement of NSSC in the formation of insoluble collagen in pulmonary fibrosis. The data also indicate significant increases in acid soluble collagen (being the more mature fiber-forming protein), and enhanced turnover of insoluble collagen (in this case pepsin soluble collagen), suggesting increased conversion of soluble collagen to insoluble collagen in fibrotic lungs. Interestingly, studies on specific and total activities of soluble and insoluble collagen of sponge-implanted rats in vivo revealed that acid soluble collagen contributes to the formation of insoluble collagen [41].

The observations of the present study reveal increased levels of BALF collagenase and hydroxyproline that ran parallel with the increases in lung collagenase and collagenolytic cathepsin activities in BLM rats. The peak levels of BALF hydroxyproline coincided with the peak increases in myeloperoxidase and collagenase activities in the BALF and lung tissue of BLM rats, indicating that collagen degradation was maximal at the time of intense inflammation. Collagenase, a secretory enzyme, extracellularly cleaves collagens near neutral pH at specific loci in the polypeptide chain across the triple helix. Collagenolytic cathepsins degrade collagens in acidic media at the N-terminal

nonhelical region containing the intramolecular cross-link. In addition, lysosomal proteinases are able to degrade collagen at acid pH (4–7); therefore, the lysosomes may also play a role in the degradation of collagen or of collagen fragments. Newly secreted collagen is also degraded extracellularly before deposition in mature collagen fibers, and this pathway is important in regulating collagen deposition. These enzymes, together with the intracellular degradation of newly synthesized collagen, control the overall rate of collagen degradation in the lung.

The present results are in agreement with the findings of Blaisdell and Giri [42], and that of Bakowska and Adamson [43], who reported increased levels of collagenase in the lungs and BALF, respectively. An increased level of BALF hydroxyproline observed in this study agrees with the findings of Bakowska and Adamson [43]. Increased levels of collagenase have been reported in idiopathic pulmonary fibrosis [44], and in experimental models of lung fibrosis [28] and in patients with adult respiratory distress syndrome [33].

Our observations together with reports from other laboratories indicate that although normal lung contains a small amount of collagenase activity, this activity increases during earlier stages of lung fibrosis. However, this raises a pertinent question as to why an increase in collagen deposition in fibrotic lungs should occur in presence of increased collagenase activity. Increased collagenase activity could be recognized as an essential intervention to reduce excess collagen accumulation; however, this might also make worse the situation further by supplying collagen degradation products that would augment more collagen synthesis and deposition in the fibrotic lung. This is especially the case in idiopathic pulmonary fibrosis [44].

The extent of cross-linking was increased in fibrotic lung collagen, as measured biochemically by the increase in aldehyde content. Increased aldehyde content shows a greater potential for crosslink formation [45, 46]. This crosslink formation is necessary for the stabilization of collagen fibrils and fibers, and for the integrity and elasticity of mature elastin. The degree of cross-linking in collagen is inversely proportional to its solubility [47]. The lower the amount of cross-linking, greater the susceptibility of collagen to denaturing agents. Accordingly, the present study used hydroxyproline release from insoluble collagen as an index of collagen solubility. The data demonstrated a consistent decrease in the release of hydroxyproline from fibrotic lung collagen compared with normal lung collagen, indicating an increase in intermolecular cross-linking of collagen in the fibrotic lung. In this regard, Vater et al. [48] have demonstrated that collagen crosslinks mediated by lysyl oxidase was ten times more resistant to digestion by mammalian collagenase.

Importantly, curcumin administration reduced BLM-induced increases in BALF hydroxyproline and collagenase levels, and lung collagenolytic activities, and also enhanced the susceptibility of insoluble collagen to denaturing agents in BLM-damaged lungs. This may be due to the anti-inflammatory and membrane stabilizing properties of curcumin [19, 49–52], thus preventing leakage and accumulation of collagenolytic activities, which may contribute to protective effects. Importantly, curcumin was reported to lower the secretion of collagenase, elastase and hyaluronidase from rat peritoneal macrophages [53].

Collagen fibers mostly determine the structural stability of lung tissue. Results of the analysis of collagenolytic enzymes on lung structure and function have enabled to characterize the mechanical properties of these fibrous proteins in the lung. Collagens provide structural support and regulate a variety of cellular processes such as cell attachment, migration, proliferation and differentiation. It is well appreciated that fibrotic lung diseases exhibit changes in collagen composition and mechanical properties during disease progression. Abnormal collagen deposition followed by increased collagen crosslinking and ECM remodeling result in an increase in ECM stiffness and scar tissue, traction bronchiectasis, thickened interlobular septae and subpleural honeycombing, compared to normal tissue [54]. Interestingly, curcumin treatment inhibited BLM-induced increases in lung collagen deposition and collagen cross-link formation. The mechanism (s) by which curcumin inhibits BLM-induced increases in lung collagen deposition is not clear. Whether curcumin induces changes in collagen fibril size and morphology in BLM-injured lungs is not known. Furthermore, whether curcumin influences post-translational control of collagen molecules, in addition to transcriptional control of constituent proteins remains to be studied. These are the subjects of our future investigations.

Fibroblast differentiation into myofibroblasts and epithelial-mesenchymal transition (EMT) are key cellular events initiating the development of fibrosis. These cells appear to participate actively in tissue repair, but the production of excess ECM eventually reduces the respiratory function in the lung. We suggest the possibility that curcumin may inhibit the fibrogenic process in bleomycin lungs by affecting cellular processes such as cell proliferation, cell differentiation, production of inflammatory and fibrotic cell mediators [55–59]. Saidi et al. [56] have shown that curcumin inhibited TGF-β1-dependent lung fibroblast differentiation of human lung CCD-19Lu fibroblasts. Furthermore, these authors have reported that the expression of alpha smooth muscle actin (α-SMA), a profibrotic marker, was reduced, and the levels of both soluble and insoluble collagen were reduced to values comparable to those found in undifferentiated fibroblasts. Liu et al. [58] have reported that curcumin displayed anti-fibrosis property by inhibiting TGF-β induced differentiation of mouse lung fibroblasts to myofibroblasts, possibly mediated by the regulation of the expression of

peroxisome proliferator activated receptor γ (PPAR-γ) and platelet derived growth factor R β (PDGFR-β). Thus, curcumin inhibition of cellular processes and fibrogenic mediator/cytokine (in our case TGF-β1) expression opens up the possibility that this would have negative impact on inflammation and development of fibrosis. TGF-β1, a profibrotic cytokine, is involved in the fibrotic response because of its ability to stimulate synthesis of ECM macromolecules, while inhibiting their degradation [13, 60]. Bronchiolar epithelial cells and interstitial fibroblasts express TGF-β1 under normal conditions. Cellular sources of TGF-β1 during tissue injury and inflammation include macrophages and mesenchymal, endothelial and mesothelial cells of the lung [61]. Lung biopsies from fibrotic subjects display increased expression of TGF-β1 [60–62]. Hoyt and Lazo [63] have shown enhanced TGF-β1 mRNA and protein content that occur before the increased expression of collagens in BLM-induced pulmonary fibrosis [63]. Giri et al. [64] have reported the importance of TGF-β1 on bleomycin induced accumulation of lung collagen using a neutralizing antibody to TGF-β1. In the present study, curcumin treatment significantly suppressed AM release of TGF-β1 in BLM rats.

The precise mechanism of suppression of macrophage TGF-β1 secretion in curcumin-treated BLM rats is not known at present, however, it is possible that curcumin may suppress activator protein-1 (AP-1), a transcription factor involved in TGF-β signaling pathway [65, 66], and results in the suppression of TGF-β1 expression. Interestingly, curcumin has been reported to be an inhibitor of AP-1 [67]. Thus, inhibition of TGF-β1 secretion might decrease inflammation and development of fibrosis because of its ability to influence ECM synthesis and deposition. This is supported by the decreased collagen synthesis and deposition in curcumin-treated BLM lungs as described above. We have recently demonstrated that curcumin treatment is associated with a profound inhibition of excess glycoproteins deposition in BLM-induced lung fibrosis [68]. Taken together, the present study demonstrates the significance of curcumin as an important therapeutic compound in not only inhibiting the amount of collagen being synthesized, but also regulating the amount of collagen available for crosslinking in lung fibrosis. Thus, curcumin may be a promising antifibrotic agent to prevent lung ECM remodeling in pulmonary fibrosis.

**Author contribution** Conceived and designed the current manuscript study: PD, VN, MB. Developed study protocols: PD, VN, MB. Data collection: PD, VN, MB. Analyzed and interpreted data: PD, VN, SV, MB. Prepared and edited the manuscript: PD, VN, SV, MB. All authors read and approved the final manuscript.

## Declarations

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