Drug Design, Development and Therapy

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ORIGINAL RESEARCH

RETRACTED ARTICLE: Casticin Improves Respiratory Dysfunction and Attenuates Oxidative Stress and Inflammation via Inhibition of NF-κB in a Chronic Obstructive Pulmonary Disease Model of Chronic Cigarette Smoke-Exposed Rats

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Objective: The present study was conducted to elucidate the protective effect of Casticin against chronic obstructive pulmonary disease (COPD) in rats.

Methods: The COPD in rats was induced by the controlled cigarette smoke, and CST (10, 20, and 30 mg/kg) was injected into the cigarette-smoke exposed rats. Blood was taken from the abdominal vein and centrifuged (1500 g, 4°C, 15min); plasma was collected and used for the determination of various biochemical parameters.

Results: The results of the study suggested that CST significantly improved the lung functions of the rats in a dose dependent manner. It also causes a reduction of white blood cells, neutrophils, and macrophages in BALF of rats. The plasma level of leptin and C-reactive protein together with pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were also significantly restored to near to normal in CST-treated group. In Western blot analysis, CST causes significant inhibition of the NF-κB and iNOS pathway.

Conclusion: Our study demonstrated that the CST protects lungs against COPD via improving lung functions and inhibition of oxidative stress and inflammation.

Keywords: COPD, Casticin, smoke, inflammation, oxidative stress, NF-κB

Introduction

Chronic obstructive pulmonary disease (COPD) is a devastating illness affecting approximately 300 million people each year across the world.[1] It is characterized by chronic airway inflammation which causes irreversible hindrance to normal breathing in the affected individuals with concurrent episodes of breathlessness, if not treated well in time.[2] The difficulty in breathing was triggered by the amalgamation of emphysema and chronic bronchitis. According to an estimate, currently, it accounts for the death of more than 3 million people, which will be responsible for approximately 8 million deaths each year by 2030 making it the third leading cause of death worldwide.[3–5] Studies have shown that inflammation in COPD greatly contributes to important comorbidities, such as lung cancer.[6] Unfortunately, on the other hand, there is no effective treatment that can effectively manage COPD and its complications. Consequently, the current therapeutic modalities against COPD provides only symptomatic relief.[7]

Various studies have shown the critical role of nuclear factor-kappa B (NF-κB) in the airway inflammation of COPD patients which regulates the expression of various inflammatory genes in airway cells.[8–10] In COPD individuals, the bronchial biopsies and inflammatory cells showed aberrant activation of NF-κB. It has been found that NF-κB activation is an oxidative stress-driven mechanism due to an imbalance between oxidant and antioxidant in the airways. It is also activated by numerous inflammatory mediators, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α or via induction of toll-like receptors (TLRs) due to provocation by bacterial or viral-organisms. Initially, after activation, the NF-κB translocates to the nucleus from cytoplasm after dissociating from IκBα, the negative regulator of NF-κB which is deteriorated during the process. It involves the phosphorylation of IκBα catalyzed by IκB kinase (IKK). It promotes the release of cytokines such as interleukin-6 (IL-6), IL-8, and TNF, thus encouraging translocation of immune cells to inflamed tissues in the airway of COPD patients.-[10–16]

Despite extensive research on COPD, still, the etiology of the disease is not completely understood. Still, the therapeutic intervention against COPD is mainly relying on anti-inflammatory agents or bronchodilators. However these treatments are not much effective in delaying COPD progression. Therefore, a potent NF-κB inhibitor combined with and anti-inflammatory property would the purpose against COPD.

Casticin (CST) belongs to a poly-methoxyflavone (Molecular weight: 374.34) obtained from the *Vitex sp.* (Family: Verbenaceae).[17] It displayed a wide range of pharmacological properties such as, anti-inflammatory,[18] anti-cancer,[19–21] ulcerative colitis,[22] antioxidant.[23] However, recently study has been conducted to elucidate the effect of CST against COPD but it does not provide any possible mechanism behind this pharmacological effect.[24] Therefore, in the present study, we intended to analyze the effect of CST in experimentally induced COPD and to elucidate the possible mechanisms underlying its effect.

Materials and Methods

Chemicals

The chemicals were purchased from Sigma Aldrich (USA) unless otherwise stated. Casticin (CST, ≥98%) was obtained from Sigma Aldrich, USA.

Animals

The animals (Male Wistar rats) were obtained from the institutional animal house and housed in polypropylene cages with an ad-libitum supply of food and water. The rats were provided with alternate dark and light cycle with strict hygienic conditions. The animal experiments were approved by the Animal Ethical Board of the Shenzhen Institute of Respiratory Disease, Shenzhen People's 90 Hospital (The First Affiliated Hospital of Southern University of Science and Technology, The Second Clinical Medical College of Jinan University), and were performed as per the relevant national guidelines proposed by NIH, USA.

Experimental Induction of COPD by Cigarette Smoke Exposure

After obtaining rats from the animal house, they were randomly grouped into five groups needed for different treatments. The rats except for the control (exposed to free air) were exposed to cigarette smoke twice a day around 9 A.M and 3 P.M for continuous twelve weeks using special smoking apparatus. The three groups were administered with graded dose of CST (10, 20, and 30 mg/kg). The one group serves as disease control with no-treatment.

Drug Treatment

The CST after dissolving in the vehicle (5% D-mannitol) was injected to the cigarette-smoke exposed rats in the concentration as indicated above by sub-cutaneous injection b.i.d before the exposure of cigarette smoke for the experimental period. The sham and disease control group received the vehicle with no-treatment.

Respiratory Function Analysis

On the 77th day, before that administration of CST, the tidal volume and peak expiratory flow-rate were recorded with a whole-body plethysmograph with a respiratory function analysis system (Biosystem XA, USA) when the rats were awake. On the 85th day, the rats were anesthetized and a cannula was inserted into the trachea to obtain functional residual capacity and forced expiratory volume at 100 ms using a forced maneuvers system (Bio-System for Maneuvers, Data Sciences International).

Analysis of Reactive Protein in Plasma

After recording respiratory function, the blood was taken from the abdominal vein and centrifuged (1500×g, 4°C, 15min), plasma was collected and stored at 80°C. Plasma levels of leptin as an index of fat mass and C-reactive

protein as an index of systemic inflammation were determined with high sensitivity ELISA assay kit and a mouse high sensitivity C-reactive protein enzyme-linked immunosorbent assay, respectively.

Analysis of Cells in Bronchoalveolar Lavage Fluid (BALF)

The BALF samples were centrifuged at 800 × g for 10 min at 4°C and the supernatants were obtained as pellets in 1 mL of saline after harvesting from the lungs of the rat. Turk's solution was added to the bronchoalveolar lavage fluid pellet suspension, and the number of white blood cells per 1 μL was counted with a hemocytometer. Moreover, the numbers of neutrophils, monocytes/macrophages, white blood cells were counted under a microscope after staining with Wright–Giemsa solution.

Histopathology Analysis

Tissue samples collected from the right upper lobe were fixed in 4% paraformaldehyde, embedded in paraffin and paraffin sections (5 μm) were cut. Paraffin sections were then stained with hematoxylin-eosin (HE) and visualized using a light microscope.

Biochemical Determination

The lung tissue homogenate were lysed with 0.05 M Tris. HCl (Beijing Biotopped Science & Technology Co., Ltd. Beijing, China) extraction buffer on ice. The cell lysates were centrifuged at 4°C 12,000 x g for 10 min. The resulting cell lysates were used to assess the SOD, and GSH-Px activity, and MDA content. The MDA level was based on thiobarbituric acid (TBA) reactivity. In brief, 50 μL of homogenate or an adequate volume of MDA working standard solution was introduced into 10 mL glass tubes containing 1 mL of distilled water. After addition of 1 mL of the solution containing 29 mmol/L TBA in acetic acid (pH of the reaction mixture, 2.4–2.6) and mixing, the samples were placed in a water bath and heated for 1 h at 95–100 0 C. After the samples cooled, 25 μL of 5 mol/L HCl was added (final pH 1.6–1.7), and the reaction mixture was extracted by agitation for 5 min with 3.5 mL of n-butanol. We separated the butanol phase by centrifugation at 1500 x g for 10 min, and measured the fluorescence of the butanol extract with a Perkin-Elmer fluorometer (Model LS50B, Perkin-Elmer, UK) at wavelengths of 525 nm for excitation and 547 nm for emission. The SOD estimation was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with 2 -(4-iodophenyl)-3(4-nitrophenol)-5- phenyltetrazolium chloride to form a red formazan dye. The SOD activity is then measured by considering the degree of inhibition of this reaction. The GSH activity was measured based on the fact that GSH-Px catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH the oxidized glutathione is immediately converted into the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm is measured.

Enzyme-Linked Immunosorbent Assay (ELISA)

Inflammatory mediators such as TNF-α, IL-1b, and IL-6 were measured by solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Code RPN 2751, RPN 2718 and RPN 2708, Amersham Pharmacia Biosciences, Piscataway, NJ, USA) without prior extraction or purification according to the manufactures' instructions. Results were calculated by using nonlinear regression of a four parameter logistic model.

Western Blot Assay

Lungs were homogenized in WB and IP lysis buffer (WB007, Xitang, China) and incubated for 30 min at 4°C. Proteins were extracted, respectively, according to instructions of a protein extraction kit (Xitang, China). Cell debris was removed by micro-centrifugation, and supernatants were quickly frozen. The protein concentration was determined by Bradford assay (BSA) method. Proteins from the sub-cellular fractions were mixed with 2× SDS sample buffer (100 mmol L^{-1} Tris-HCl (pH 6.8), 4% SDS (w/v), 20% glycerol, 200mmol L^{-1} DTT, and 0.1% bromophenol blue (w/v) and boiled in a water bath for 5 min, before separation on 8% polyacrylamide gels. After quantification by the BSA method, protein samples were separated by 10% SDS PAGE for 4 h and subsequently transferred to nitrocellulose membranes. The filter was then blocked in tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% dried milk powder (wt/vol) for 2 h at room temperature. The nitrocellulose filters were incubated with primary antibody (1:1000; Abcam, USA) for overnight at 4°C. After washes with TBST, the filters were incubated with IgG HRP (1:10,000) (Abcam, USA) for 1 h at room temperature and further washed for 30min with TBST. Immunoreactive proteins were visualized using the enhanced chemiluminescence Western blotting detection system (Millipore, Billerica, MA, USA) and photographed with Image Quant LAS 4000 (GE, USA) and were analyzed by Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

All data are recorded as mean ± SEM of three independent experiments. Data were statistically analyzed by one-way analysis using statistical software GraphPad Prism 5.0 (California, USA). The P-value < 0.05 was considered as statistically significant.

Results

Effect of CST on Lung Function of Rats

As shown in Figure 1, as compared with the sham, the tidal volume peak expiratory flow volume and forced expiratory volume was found to be reduced significantly. Moreover, functional residual volume together with lung capacity (a biomarker of lung emphysema) found elevated in the model group which is exposed to cigarette smoke as compared to the sham group. On the other hand, these levels were found to restore near to normal in CST-treated group in a dose-dependent manner. These results indicated that CST attenuated the exacerbations after cigarette smoke in rats.

Effect of CST on Cells in BALF

To further examine the protective effect of CST against COPD, the level of white blood cells, neutrophils and macrophages were quantified with or without CST treatment in different animal groups. As shown in Figure 2, the level of white blood cells, neutrophils, and macrophages were found significantly higher in cigarette exposed (model) rats in comparison to sham. The CST-treated rats showed a significant reduction in the elevated level of these biomarkers in a dose-dependent manner as compared to the model group.

Effect of CST on the Level of Plasma Leptin and C-Reactive Protein

As shown in Figure 3 the plasma leptin level was found to be reduced together with an increase in plasma C-reactive protein in the model group as compared to sham. However, the level of plasma C-reactive protein was approximately restored near to normal, with a considerable increase in plasma leptin levels in CST-treated groups.

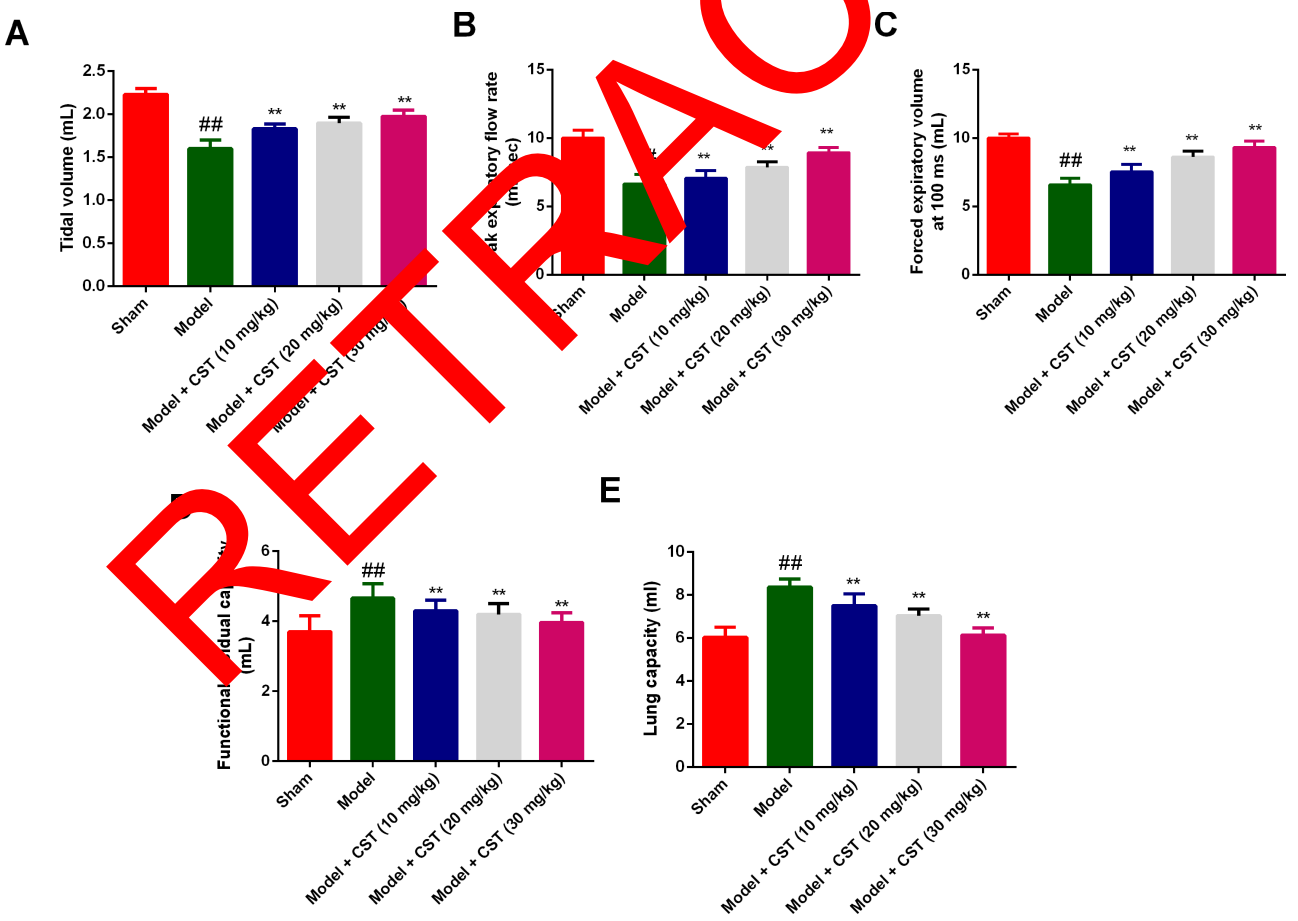

Figure 1 Effect on lung respiratory function of rats. (**A**) tidal volume, (**B**) peak expiratory volume, (**C**) forced expiratory volume, (**D**) functional residual capacity, (**E**) lung capacity. Data are expressed as mean ± SEM. ##P < 0.05 vs sham; **P < 0.05 vs model, one-way analysis of variance followed by a Tukey's post hoc test.

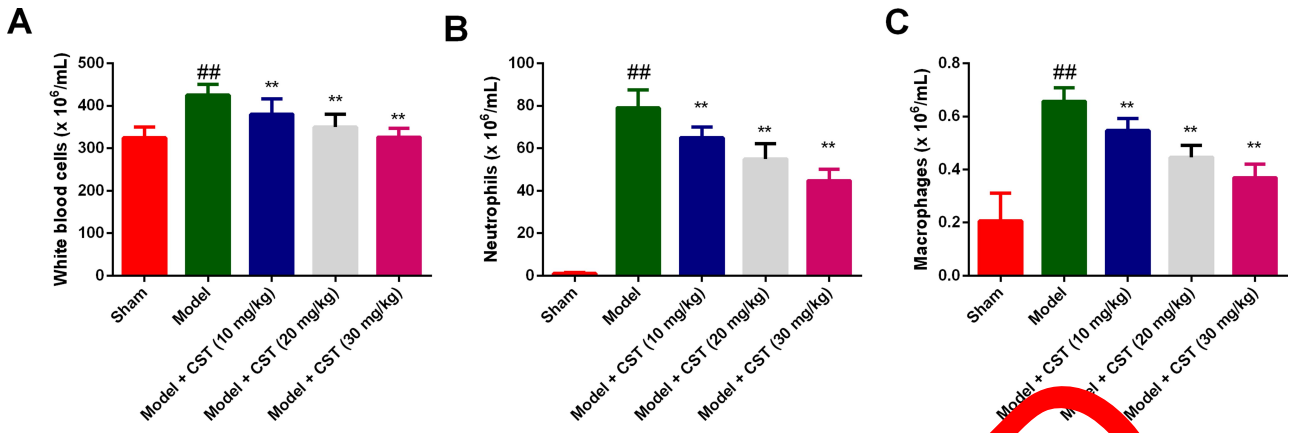

Figure 2 Effect of CST on plasma level of (**A**) white blood cells (**B**) neutrophils, and (**C**) macrophages. Data are expressed as mean ± SEM. ##P < 0.05 vs sham; **P < 0.05 vs model, one-way analysis of variance followed by a Tukey's post hoc test.

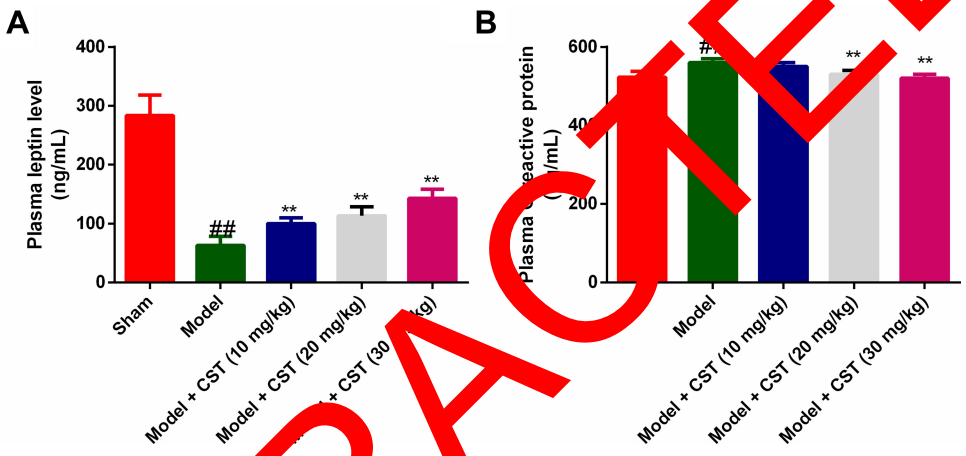

Figure 3 Effect of CST on the plasma (**A**) leptin level and (**B**) C-reactive protein level. Data are expressed as mean ± SEM. ##P < 0.05 vs sham; **P < 0.05 vs model, one-way analysis of variance followed by a Tukey's post hoc test.

Effect of CST Oxidative Stress Biomarker

The effect of CST was studied on the level of oxidative stress mediators in rats after exposure to cigarette smoke and results are presented in Figure 4. The level of GSH and SOD was found reduced in the model group together with an increase in MDA as compared to sham. On the contrary, CST causes a reduction of MDA and increases the level of GSH and SOD in a dose-dependent manner. The prominent activity was achieved in the case of a 30 mg/kg treated group.

Effect of CST on Pro-Inflammatory Cytokines

Inflammation is another hallmark of COPD, thus the effect of CST was quantified on the level of various pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6. As shown in Figure 5, the level of these cytokines were found significantly higher in the model group as compared to sham. However, the CST significantly decreased the level of TNF-α, IL-1β, and IL-6 in a dose-dependent manner.

Effect on Histopathology

On close examination of histopathology of lung tissues, the model group showed enlarged alveolar spaces together with degradation of alveoli which got fused into bullae as compared to sham. The model group also showed signs of emphysema which was found absent in the sham group. All the observed anomalies were significantly ameliorated by the treatment in CST-treated group (Figure 6).

Effect of CST on NF-κB Downstream Mediators

In the next instance, the effect of CST was analyzed on the levels of various downstream g mediators of the NF-κB

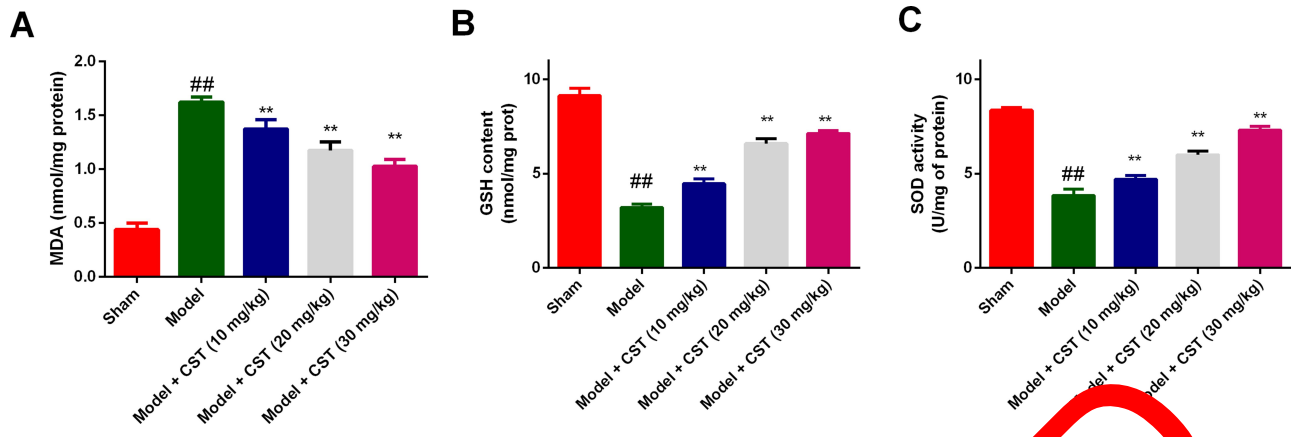

Figure 4 Effect of CST on oxidative stress indices. (**A**) MDA, (**B**) GSH, and (**C**) SOD. Data are expressed as mean ± SEM. ##P < 0.05 vs sham; **P < 0.05 vs model, one-way analysis of variance followed by a Tukey's post hoc test.

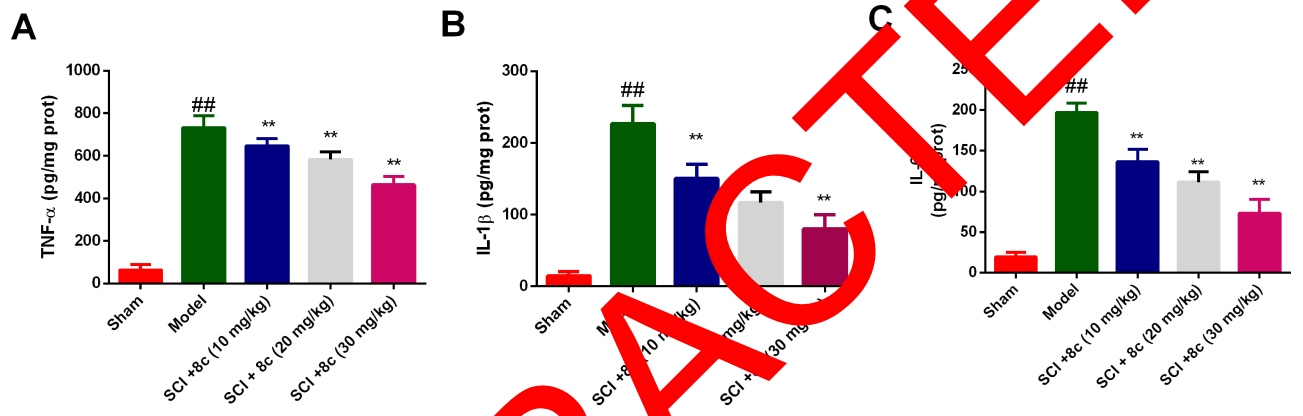

Figure 5 Effect of CST on pro-inflammatory cytokines. (**A**) TNF- α (**B**) IL-1β, and (**C**) IL-6. Data are expressed as mean ± SEM. ##P < 0.05 vs sham; **P < 0.05 vs model, one-way analysis of variance followed by a Tukey's post hoc test.

signaling pathway. As shown in Figure 7A, the level of TLR-4 (Figure 7B) together with phosphorylated NF-κB (Figure 7C) and IκBα (Figure 7D) was found significantly increased in the model group as compared to sham. CST significantly reduced the level of these mediators in a dose-dependent manner.

Discussion

COPD is a devastating illness that leads to impaired breathing function due to emphysema and chronic bronchitis. It affected more than millions of individuals across the globe. The increase in pollution of the atmosphere further worsens the situation which requires immediate attention to deal with COPD.[25,26] As per the recent estimates, the developing nations are overburdened with COPD due to rapid industrialization and lack of adequate diagnostic and treatment facilities. More than 90% of COPD-related deaths are observed alone in low and middle-income countries.[27,28] Thus, novel agents that can provide relief or protect against COPD are urgently needed. Our study successfully demonstrated the protective effect of CST in the experimental COPD model in rats. We found that CST causes inhibition of oxidative stress and inflammation a possible mechanism to protect lungs against cigarette smoke exposure. It has been well established that, rats exposed to cigarette smoke for 12 weeks showed COPD like illness such as, lung emphysema, which was caused by respiratory dysfunction and alveolar neutrophil infiltration. The dysfunction of the lung's ability to breathe is the characteristic hallmark of COPD. In COPD, the bronchial tubes of the lungs become inflamed and narrowed. When the person breathes out, they tend to collapse and become clogged with mucus and prevents air movement across the lungs. Therefore, initially, we intend to investigate the effect of CST on the lung ability and breathing capacity via various

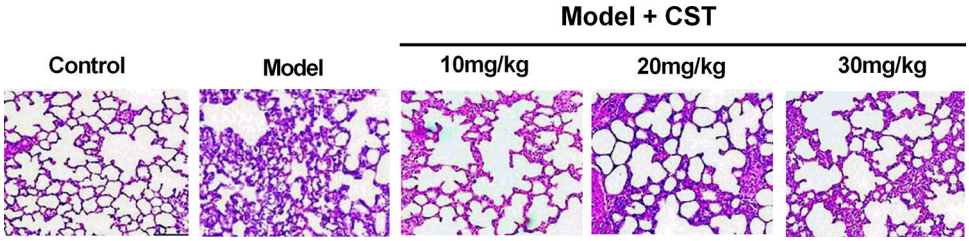

Figure 6 Effect of CST on the histopathology of lung tissues of rats.

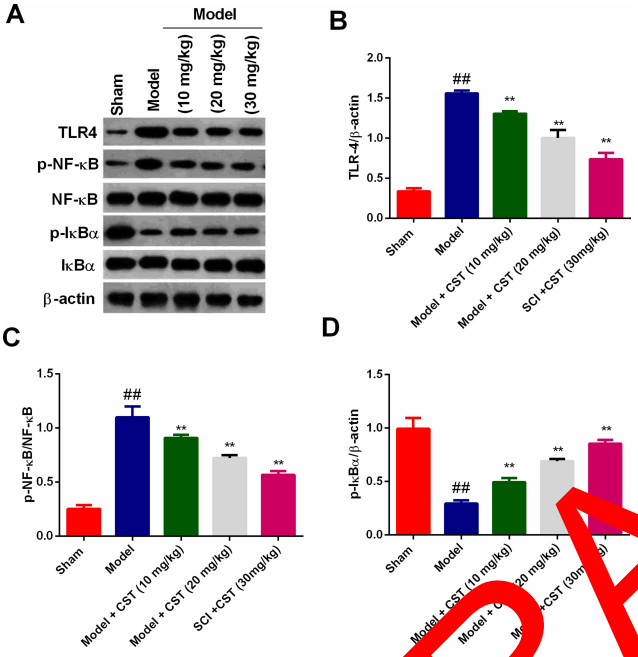

Figure 7 Effect of CST on (**A**) NF-κB downstre[am] signal[ing] m[edi]ators by western blot analysis, quantitative bar graph of (**B**) TLR[4], (**C**) p-NF-κ[Bα] and (**D**) p-IκBα. Data are expressed as mean ± SEM. ##P < 0.05 [vs] Sham; **P < 0.05 [vs] model, one-way analysis of variance followed by a Tuke[y] post [hoc] test.

parameters.[29–31] The results suggested that CST improved tidal volume, forced respiratory volume, and forced expiratory volume in 1 s which indicates that CST causes a reduction in airway blockage and improves breathing ability. Studies have shown that patients affected by COPD have higher level of WBCs, macrophages, and neutrophils.[32–34] In the present study, CST causes a dose-dependent reduction of these indices. Systemic inflammation is another feature of COPD, which often needs to be taken into account. As shown in the study, CST causes a reduction of plasma leptin level and plasma reactive protein–C, which is considered as an important index of systemic inflammation.[35–37] Numerous studies have shown the importance of oxidative stress and damage due to oxidative stress in the pathogenesis of COPD. It has been found that increased oxidative stress is directly linked to oxidants from the environmental exposure, such as pollutants in the air, cigarette smoking together with increased concentration of reactive oxygen species (ROS) and reactive nitrogen species (RNS) from the leukocytes and macrophages responsible for the generation of the inflammatory cascade in lungs of COPD patients.[38–40] These ROS and RNS promote oxidative damage to DNA, lipids, carbohydrates, and proteins that contribute to the progression and advancement of COPD. In the lungs, they also recruit resident cells, particularly epithelial cells and alveolar macrophages, to produce chemotactic molecules. These molecules further stimulate other inflammatory cells such as, neutrophils, monocytes, and lymphocytes into the lung, which in turn propagates oxidative stress in the lung.[41–44] In the present study, CST causes a significant reduction in the oxidative stress as evidenced by an increase of SOD and GSH levels together with a reduction in MDA level. Various studies have shown the highly interrelated connection between oxidative stress and inflammation in COPD.[45–49] The oxidative stress is believed to initiate and augment inflammation in COPD subjects.[10,50] In our study, we have demonstrated that CST reduces systemic inflammation by reducing serum levels of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α. These observations were further supported by histopathological analysis of lung tissue, where CST showed to prevent damage to alveoli of the lung and restore the normal architecture of lung tissues. The above results were found in line with an earlier study where CST showed a protective effect against COPD.[24] The effect of CST was further quantified on the downstream mediators of the NF-κB signaling pathway which believed as a key mediator to promote inflammation in lung tissues of COPD patients.[9,51,52] NF-κB suggested to have a key role in regulating the expression of inflammatory genes in airway cells and found aberrantly overexpressed in animal models of COPD and COPD affected individuals. Our study has demonstrated that CST causes inhibition of

TLR4 activation in Western blot analysis together with inhibition of phosphorylation of NF-κB and IκBα.

Conclusion

Collectively, our results showed that, Casticin improves lung capacity and attenuates oxidative stress and inflammatory response. It also causes inhibition of downstream mediators of NF-κB signalling pathway as a possible mechanism for amelioration of airway and systemic inflammation.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competi rests.

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