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Andrographolide ameliorates silica induced pulmonary fibrosis

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ABSTRACT

The purpose of this study was to investigate the protective effect of andrographolide in silica-induced pulmonary fibrosis (PF) in mice and its underlying mechanisms. Male Swiss albino mice were divided into five groups: Normal control group, disease control group (1.5 mg silica/60 μL/mice) via oropharyngeal route, low dose (LD) group received silica + andrographolide (3 mg/kg), high dose (HD) group received silica + andrographolide (10 mg/kg), andrographolide per se group received 10 mg/kg andrographolide. Various bronchoalveolar lavage fluid (BALF) and biochemical parameters, inflammatory cytokines, histology and protein expression studies were carried out. Andrographolide significantly reduced total protein concentration, albumin, accumulation of inflammatory cells and lactate dehydrogenase (LDH) level in BALF. We found that andrographolide intervention led to decreased levels of the inflammatory cells including neutrophils, macrophages and lymphocytes in the BALF of the treated animals. In addition, andrographolide significantly reduced nitrite ($p < 0.01$ at HD), malondialdehyde ($p < 0.01$ at HD) and upregulated glutathione ($p < 0.01$ at HD) in silica challenged animals. Andrographolide showed anti-fibrotic activity by reducing collagen deposition and inflammation in lung. Histopathology revealed that andrographolide decreased irregular cellular nodules, inflammatory infiltration and fibrosis. Andrographolide intervention significantly reduced the expression of N-cadherin, α-SMA and vimentin (mesenchymal markers) and upregulated the expression of E-cadherin (an epithelial marker). Hence, andrographolide elicits its anti-pulmonary fibrotic effect by halting the progression of epithelial-to-mesenchymal transition (EMT) via affecting fibroblasts. We, to the best of our knowledge prove for the first time that andrographolide possesses potent antifibrotic activity by targeting inflammatory cells and EMT associated fibroblasts.

1. Introduction

Pulmonary fibrosis (PF) is a chronic, progressive lung disease with increasing global prevalence. It kills thousands of people every year worldwide. The incidence and prevalence of PF are not clearly known. In the USA prevalence ranged from 14 to 27.9 and incidence ranged from 6.8 to 8.8 cases per 100,000 population while in Europe the prevalence ranged from 1.25 to 23.4 and incidence ranged from 0.22 to 7.4 per 100,000 population [1]. Further, prevalence of PF is found to be higher in males as compare to females [2]. The risk factors responsible for PF include infection, dusts, genetic, drugs, cigarette smoking, radiation, autoimmune reactions, allergic responses and tissue injury [3]. Clinically, it is characterized by inflammation, loss of alveolar architecture through apoptosis of cells, proliferation of fibroblasts and excessive deposition of extracellular matrix (ECM) [4]. Occupational inhalation of crystalline silica (silicon dioxide) is one of the leading causes of PF. Silica is a widely used industrial raw material and is used for multiple applications. The pathology of silica induced PF includes the phagocytosis of insoluble crystalline particles of silica by macrophages. This event is distinct from bleomycin induced PF which is largely mediated by reactive oxygen species (ROS) induced oxidative stress. The ingested silica particles are then enzymatically hydrolyzed into smaller particles and are released which trigger the inflammatory reaction and initiates fibrogenic response [5, 6]. Mounting evidence indicate that after inhalation or instillation of silica particles, alveolar macrophages (AMs) are activated which release chemokines and inflammatory cytokines, cause stimulation of ROS, fibroblasts proliferation and differentiation into expansion of activated mesenchymal cells (myofibroblasts) by epithelial-to-mesenchymal transition (EMT), collagen and extracellular matrix deposition and ultimately fibrosis (silicosis) [7–9]. AM's participate in regulation of inflammatory response and pulmonary pathologies [4, 10]. There have been many theories to

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explain the pathogenesis of silicosis including the role of transforming growth factor (TGF-β) signaling pathway, inflammatory response, oxidative and nitrosative stress etc. but none explains the pathogenesis clearly [11, 12].

The EMT pathway in fibrotic condition is identified by upregulation of α-smooth muscle actin (α-SMA) and downregulation of E-cadherin and regulation of several growth factor and cytokines [13, 14]. The US-food and drug administration (FDA) approved nintedanib (Ofev) and pirfenidone (Esbriet) for the treatment of PF. However, lung transplantation is the last option for PF [15, 16]. The side effect related limitations of current strategies require investigations of novel molecules for potential benefit against this deadly disease.

Natural products present an attractive option for treating various acute and chronic inflammatory and fibrotic diseases [17–19]. Andrographolide is a natural compound and labdane diterpenoid lactone. It is isolated from traditional Chinese medicine *Andrographis paniculata* Nees (Acanthaceae) and was first reported in the Indian Medical Gazette in 1951 [20]. Andrographolide is used as folk medicine in China and is currently used for treatment of rheumatoid arthritis, upper respiratory tract infection, asthma, laryngitis and sore throat [21, 22]. In India it is used for the treatment of dysentery, viral infections, fever and diarrhea. Other studies have reported that it is particularly efficient at regulating immune responses [23, 24]. In the present study, we hypothesized that andrographolide owing to its well-known anti-inflammatory and antioxidant effect, may prove beneficial in amelioration of silica induced PF. We chose a noninvasive, highly homogeneous and reproducible model of occupational PF produced by oropharyngeal instillation of crystalline silica. We evaluated the protective effects of andrographolide by evaluation of various bronchoalveolar lavage (BAL) fluid parameters including the levels of inflammatory cells like macrophages, neutrophils and lymphocytes; and oxidative and nitrosative measurement. In addition, we studied the role of this potential natural product against silica induced inflammation and histological damage. Further, the molecular mechanisms were studied by Western blotting and immunohistochemistry. We focused on the effect of andrographolide on the EMT associated fibroblasts and evaluated the expression of various epithelial and mesenchymal transition associated proteins. Our results demonstrate the potential benefits of andrographolide against oropharyngeally administered silica induced occupational PF model.

2. Materials & method

2.1. Drugs and chemicals

Andrographolide (98% pure, characterized by NMR spectroscopy, Supplementary Fig. S1), Bovine serum albumin (BSA), Bradford reagent, Crystalline silica (Min-U-Sil-5, average particle size 1.5–2 μm), Chloramine-T, Direct red 80,5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), Ehrlich reagent, Formaldehyde, Glacial acetic acid, Griess reagent, Hydrochloric acid (HCl), Paraffin, Potassium chloride, Reduced glutathione (GSH), Sodium dodecyl sulphate (SDS), Sodium hydroxide, Sodium nitrite, *trans*-L-Hydroxyproline, 2-Thiobarbituric acid (TBA), Tris-HCl, Xylene, were purchased from Sigma Aldrich, USA. Enzyme linked immunosorbent assay (ELISA) kits for IL-1β, IL-6, TNF-α and TGF-β were purchased from Invitrogen, Thermo Scientific, USA. Primary antibodies against β-actin, E-cadherin, N-cadherin, COX-2, CTGF, α-SMA and HRP conjugated anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnologies, Santa Cruz, USA. Primary antibody for vimentin, p-smad 2/3 and HRP conjugated anti-rabbit secondary antibody was procured from Cell Signaling Technology, USA. The immunohistochemistry kit was procured from PathnSitu, India. The biochemical kits for total protein, albumin content and lactate dehydrogenase (LDH) were purchased from Accurex Biomedical Pvt. Ltd., India.

2.2. Experimental animals

The study was carried out on Male Swiss albino mice (weight 25-30 g) of age range 6–8 weeks purchased from National Institute of Nutrition (NIN), Hyderabad, India. Animals were housed (3 animals per cage) in a temperature controlled environment at 23 ± 2 °C, relative humidity of 55 ± 15 with 12 h light/dark cycle [25]. Animals were acclimatized at least one week prior initiating experiment. All procedures of the study were approved by Institutional Animal Ethics Committee (Approval No: NIP/10/2017/PC/260), NIPER-Hyderabad, India. All the experiments were conducted in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

2.3. Induction of pulmonary fibrosis

The required amount of silica was weighed and heated (180 °C for 2 h) to remove endotoxins. The crystalline particles were suspended in normal sterile saline (1.5 mg/60 μL) and the resulted suspension was sonicated for 15 min with the help of probe sonicator (Sonics, USA) to ensure uniform suspension. The suspension was vigorously mixed by a vortex shaker (Stuart, UK), before instillation in every mouse. The animals were lightly anesthetized with isoflurane and kept on a 60° inclined board with the help of rubber bands. The tongue of mice was pulled and held with the help of blunt forceps. Pulmonary fibrosis was induced by single dose (1.5 mg/60 μL/mice) of crystalline silica via oropharyngeal route using a micropipette [26]. The intraperitoneal (i.p.) dosing of drug was started 4 h after silica administration to avoid any stress.

2.4. Experimental design

The animals were divided into five groups (n = 10): Group 1: normal control (NC), received daily i.p. sterile 0.9% saline; Group 2: disease control (DC), received oropharyngeal silica (1.5 mg/mice, only once on first day); Group 3: low dose (LD), received oropharyngeal silica (1.5 mg/mice, only once on first day) + daily andrographolide (i.p., 3 mg/kg); Group 4: high dose (HD), received oropharyngeal silica (1.5 mg/mice, only once on first day) + andrographolide (i.p., 10 mg/kg); Group 5: per se group received high dose of andrographolide alone (i.p., 10 mg/kg). Andrographolide doses were continued for 21 days once daily. The dosage of andrographolide was selected based upon previous studies [27–29]. The animals from all the groups were sacrificed on day 21. A scheme of the experimental design is shown in Fig. 1.

2.5. Bronchoalveolar lavage fluid (BALF) collection

Mice were anesthetized by inhalation with the help of isoflurane; a small incision was made on the trachea and BAL fluid was collected by cannula. Briefly, cannula was inserted into trachea with sufficient amount of ice-cold PBS (1.5 mL each time) to collect BALF. The procedure was repeated thrice, and 70–80% recovery of collected BALF was observed. After collection of BALF, it was used for total cell count and differential cell count using a hematology system (ADVIA 2120i, Siemens, Germany). Later, remaining BAL fluid was centrifuged at 4000 rpm for 10 min at 4 °C, and supernatant was used for determination of total protein, albumin content and LDH.

2.6. Lung-body weight index and lungs morphology

The animal body weights were taken every third day and, lungs were collected, washed and weighed after sacrifice of animals. Lung morphology was observed in all the groups and images were captured using a digital camera. The lung weights were normalized with body weight to obtain organ index (Lung weight/Body weight) and results were expressed in mg/g.