

Original Article

Tetrastigma hemsleyanum (Sanyeqing) extracts reduce inflammation and oxidative stress in a chronic obstructive pulmonary disease rat model

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Abstract: Patients with chronic obstructive pulmonary disease (COPD) have poor prognosis. This study aims to investigate the protective effects of Sanyeqing and its potential mechanism in a rat model with COPD. Total of 45 rats were randomly divided into three groups: normal control group, COPD group and Sanyeqing group. COPD model was established by cigarette smoking and intratracheal instillation of lipopolysaccharide. All rats were sacrificed after 4-week Sanyeqing treatment. Right middle lobes of lung tissues were harvested for histopathological examination. Peripheral blood from carotid artery and bronchoalveolar lavage fluid (BALF) were also collected. Levels of IL-8 and CRP in serum and BALF were analyzed by ELISA. Local malondialdehyde (MDA) and total superoxide dismutase (SOD) were examined in both pulmonary tissue homogenate and BALF. The expression of nuclear factor-erythroid 2-related factor 2 (Nrf2) in lung tissues was evaluated by quantitative RT-PCR and Western blotting. Sanyeqing treatment appeared to reduce COPD induced inflammatory response by decreasing cell inflammation and the levels of IL-8 and CRP in both serum and BALF ($P<0.05$). Sanyeqing treatment also reduced local MDA levels as well as increased SOD levels ($P<0.01$). Moreover, the expression of Nrf2 in lung tissues was improved by Sanyeqing at both the mRNA level and the protein level. These results suggest that Sanyeqing, at least in an animal model, can attenuate COPD through its antiinflammation and antioxidant activity, which may provide a novel approach in the treatment of COPD.

Keywords: Tetrastigma hemsleyanum (Sanyeqing), chronic obstructive pulmonary disease, inflammation, oxidative stress

Introduction

Chronic obstructive pulmonary disease (COPD) is a slowly progressive, poorly reversible disease characterized by an abnormal inflammatory response and oxidative stress in the lung [1]. The incidence of COPD in general populations is increasing, along with its great burden on public health [2, 3]. Therefore, the development of new longer lasting, targeted therapeutic strategies is a matter of great urgency. At present, clinical trials and experimental studies have shown that certain Chinese medicines can effectively treat COPD, by improving pul-

monary function, respiratory muscle fatigue, immunity, and lung blood flow [4, 5].

Tetrastigma hemsleyanum (Sanyeqing), an herbal medicine belongs to the grape family Vitaceae, is widely distributed in the Southern China. In Traditional Chinese Medicine, its extracts from both leaves and roots are widely used for the treatment of lots of diseases, such as high fever, infantile febrile convulsion, pneumonia, asthma and tumors, *et al.* In recent years, modern pharmacological studies have proven that Sanyeqing has a variety of biological activities including immunoregulation, anti-inflam-

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matory, antioxidant and antiproliferation [6-10]. However, to our knowledge, little is known about its effect on COPD.

Therefore, in this study, we aimed to investigate the protective effect of Sanyeqing and its potential mechanism in rats with chronic obstructive pulmonary disease.

Methods

Animals and treatments

All procedures were approved by the animal care committee of Medical College of Jishou University. A total of 45 healthy male SD rats, weighting from 255 to 302 g (mean 274 ± 29 g) were obtained from the experimental animal center of technology services in Changsha, China. Sufficient food and water were available ad libitum for all rats. The animals were maintained on a 12-hour dark/light cycle with ambient temperature of $25 \pm 1^\circ\text{C}$ and relative humidity $50 \pm 10\%$ throughout the experimental period.

After two weeks' acclimatization of laboratory conditions, rats were randomly divided into three groups: (1) normal control group (n=15); (2) COPD group (n=15); and (3) Sanyeqing group (n=15). The latter two groups were performed on a rat model of COPD, which was established by cigarette smoking and intratracheal instillation of lipopolysaccharide (LPS, 200 μg , Sigma-Aldrich, USA) as previously described [11]. For cigarette smoking, rats were exposed to smoke from 16 cigarettes for twice a day (each 30 min) for 28 days. Rats in Sanyeqing group were fed extract of *Tetrastigma hemsleyanum* [7] by gavage device before smoking every day (dose 1.0 g/kg, plus saline to 2 ml), lasted 28 days.

Samples collection

At the end of the 4-week Sanyeqing treatment, all rats were sacrificed under pentobarbital anesthesia (30 mg/kg, intraperitoneal injection). Right middle lobe of lung tissues, peripheral blood from carotid artery and bronchoalveolar lavage fluid (BALF) were collected for further analysis. Collected lung tissues were fixed in formalin and embedded in paraffin for histological analysis. An aliquot of the lung tissue was homogenized for biochemical analysis.

For BALF collection, after anesthesia and thoracotomy, right side of the main bronchus was tied. The left lung was then lavaged three times with 3 ml saline solution at 37°C , and the BALF was collected. All BALF samples were immediately centrifuged at 1200 rpm for 10 min at 4°C . The cell free supernatant were obtained and stored at -20°C for further analysis.

Histological study

Hematoxylin and eosin (HE) staining was performed for evaluation of structural changes. Four consecutive 5 μm sections of lung tissue were collected for each slide, and five slides were made from each sample. The sections were routinely stained with HE and observed under a Leica photograph microscope (Leica Microscope Ltd., Wetzlar, Germany).

Determination of levels of inflammation

Levels of CRP and IL-8 in serum and BALF were measured with an ELISA method using commercially available kits (R&D Systems, Minneapolis, Minnesota, USA). Operation procure was performed according to the manufacturer's instruction. Furthermore, white blood cell count and neutrophil in BALF were determined under the optical microscope. Briefly, after centrifuge, the cell deposit of BALF was collected and added 0.8 ml Hank's buffer, smeared by device, dyed by Wright-Giemsa, and then counted at least 300 cells for cell counting and classification.

Determination of levels of oxidative stress

Superoxide dismutase (SOD) and malondialdehyde (MDA) in both BALF and pulmonary tissue homogenate were measured as described previously [12]. Briefly, MDA was reacted with thiobarbituric acid by incubating for 1 h at $95-100^\circ\text{C}$. Following the reaction, fluorescence intensity was measured in the n-butanol phase with a fluorescence spectrophotometry (Hitachi, Model F-4010, Japan), and by comparing with a standard solution of 1, 1, 3, 3 tetramethoxypropane, results were expressed in terms of nmol/ml. SOD activity was measured by reduction of nitrobluetetrazolium (NBT) and xanthine-xanthine oxidase system. Enzyme activity leading to 50% inhibition was accepted as one unit. Results were expressed as U/ml.

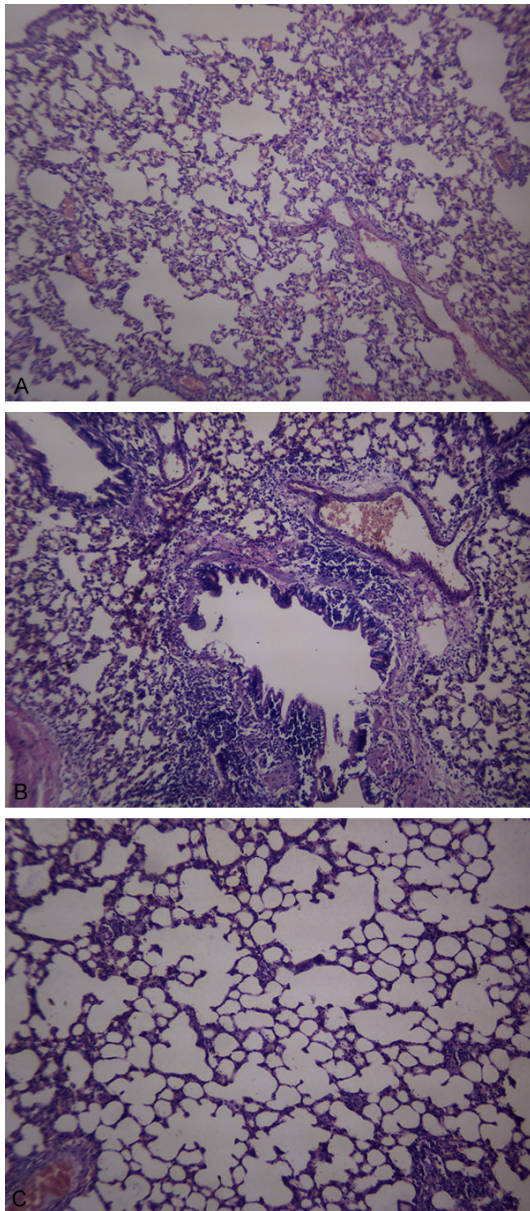


Figure 1. Micrographs of lung histopathology. Histological changes of lung segment stained with HE after Sanyeqing treatment. A. Normal control group: No abnormalities were seen; B. COPD group: pulmonary tissue showed a severe inflammatory response with visible increases in inflammatory cells. Alveolar diameter was significantly higher. C. Sanyeqing group: inflammation lessened after sanyeqing therapy. The number of inflammatory cells decreased. (Magnification, $\times 100$).

Determination of nuclear factor erythroid 2-related factor 2 (Nrf2) levels

Since nuclear factor-erythroid 2-related factor 2 (Nrf2) is a central transcription factor that regulates antioxidant and anti-inflammatory

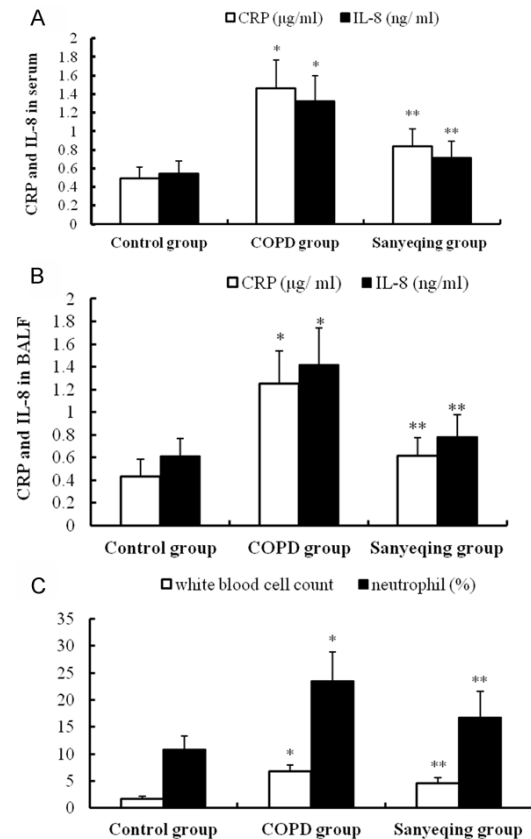


Figure 2. Effects of Sanyeqing on inflammation in COPD rats. ELISA was performed to detect levels of CRP and IL-8 in both serum (A) and BALF (B) White blood cell count and neutrophil in BALF were determined under the optical microscope (C) * $P < 0.05$, vs Control group; ** $P < 0.05$, vs COPD group.

genes [13], we further evaluated both mRNA and protein levels of Nrf2 in lung tissues using quantitative RT-PCR and Western blotting respectively.

For mRNA evaluation, RNA from lung tissues was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA with random primers using the M-MLV enzyme (Invitrogen). Quantitative realtime PCR was performed using specific primers and SYBR Green master mix on a 7900HT Fast-Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Each sample was run in triplicate. The relative mRNA abundance of each gene was normalised to the expression level of the house-keeping gene β -actin.

For protein detection, lung tissues were mechanically homogenised in ice-cold lysis buffer and centrifuged at 14,000 g for 10 min at 4°C.

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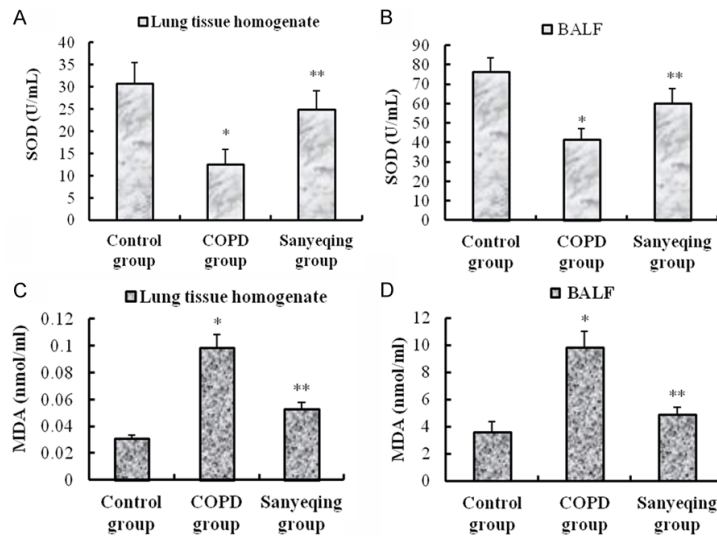


Figure 3. Effects of Sanyeqing on oxidative stress in COPD rats. SOD and MDA in both BALF and pulmonary tissue homogenate were measured as described in methods. * $P<0.01$, vs Control group; ** $P<0.01$, vs COPD group.

The resulting supernatant fraction was separated by SDS-PAGE, and immunoblotted with antibodies against Nrf2 (1:500), and β -actin (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunocomplexes were detected using the chemiluminescence method (ECL-plus kit, Amersham Biosciences UK Limited, UK). Three independent experiments were performed for both human and rat samples.

Statistics

Statistical analyses were performed by using SPSS software (Version 13.0). Quantitative variables are presented as the mean \pm standard deviation. Data with a normal distribution were analyzed by one-way ANOVA followed by least significant difference test. $P<0.05$ was considered to be statistically significant.

Results

Sanyeqing improve pulmonary injury in COPD Rats

Representative histopathological photographs of sections from all three groups are shown in **Figure 1**. Compared to controls, COPD rats showed a severe inflammatory response with visible increases in inflammatory cells. Alveolar diameter was significantly higher in the

COPD group. These COPD-related phenomena were relieved with sanyeqing treatment. Furthermore, sanyeqing treatment alleviated the inflammatory response, as shown by a significant decrease in the number of inflammatory cells present in lung tissues.

Sanyeqing alleviate inflammation in COPD rats

Compared to the normal control group, serum levels of CRP and IL-8 were significantly higher in the COPD group ($P<0.05$), while those in the sanyeqing group were significantly lower than those in the COPD group ($P<0.05$, **Figure 2A**). Similar to the results obtained from serum, BALF levels of CRP and IL-8 were also significantly increased in COPD rats ($P<0.05$), while sanyeqing treatment reverse above changes ($P<0.05$, **Figure 2B**).

We further counted total number of white blood cell and neutrophil in BALF. As shown in **Figure 2C**, both white blood cell and neutrophil in COPD group were significantly higher than those in normal control group ($P<0.01$), while sanyeqing treatment decreased above cell numbers in COPD conditions ($P<0.01$). Taken together, these findings indicate that sanyeqing can alleviate both system and local inflammation in COPD rats.

Sanyeqing attenuate local oxidative stress in COPD rats

To further determine the effect of Sanyeqing on oxidative stress in COPD rats, SOD and MDA contents in both pulmonary tissue homogenate and BALF were performed. As shown in **Figure 3A**, levels of SOD in the COPD group were significantly lower than those in the control group ($P<0.01$). While after Sanyeqing treatment, although the SOD activities were still lower than those in normal group, they were significantly higher than those in COPD group (**Figure 3B**). Contrary to the antioxidant enzyme SOD, MDA as one of the major oxidative damage marker, its levels were significantly increased in COPD rats ($P<0.01$), while sanyeqing treatment reduced MDA levels ($P<0.01$, **Figure 3C** and **3D**).

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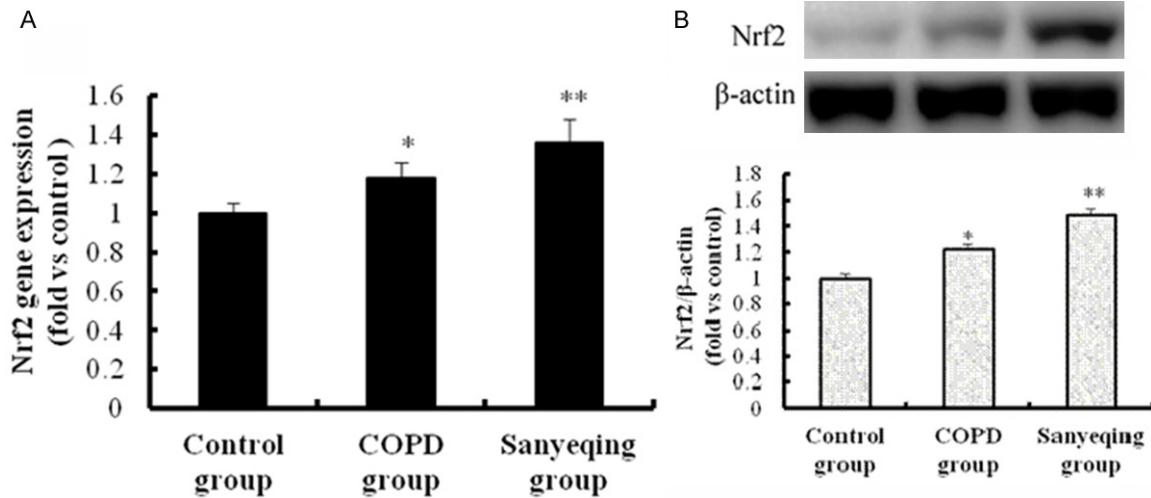


Figure 4. Sanyeqing improve Nrf2 expression in COPD rats. Both mRNA and protein expression of Nrf2 in lung tissues were measured as described in methods. * $P < 0.05$, vs Control group; ** $P < 0.05$, vs COPD group.

These findings indicate that sanyeqing can alleviate increased oxidative stress induced in COPD condition.

Sanyeqing improve Nrf2 expression in COPD rats

To further investigate underline mechanism of the antiinflammatory and anti-oxidative effects of Sanyeqing, the effects of Sanyeqing on Nrf2 expression were detected. As shown in **Figure 4**, both mRNA (**Figure 4A**) and protein (**Figure 4B**) expression of Nrf2 in lung tissues were upregulated in COPD rats. These increases in Nrf2 expression were augmented by Sanyeqing.

Discussion

The current study demonstrated that treatment of Sanyeqing showed a protective effect in a rat model with COPD, accompanied by inhibition of inflammation and oxidative stress. Sanyeqing protected against COPD through activating Nrf2 signaling pathway.

Chronic obstructive pulmonary disease is a common disease of respiratory system, which is in the research focus of all over the world. The pathogenesis of COPD is complicated with inflammation, oxidative stress and imbalances between protease and antiprotease activity, as well as tissue destruction and reconstruction [14]. Although great progress has been made in the basic and clinical research, patients suffered from COPD still have to face poor progno-

sis. Sanyeqing is a Chinese herb and extensively used to treat patients with variety of diseases [6-10]. However, few studies have yet explored its protective effect on COPD. In this study, using a rat model of COPD established by cigarette smoking and LPS [11], our results showed that treatment with sanyeqing significantly improved lung tissue pathology, which may be potentially related to its antiinflammation and antioxidative capacity.

As a chronic and systemic inflammatory disease, the inflammatory reaction in the lungs may be either the cause or the result of the systemic effects associated with COPD [15]. Previous studies have shown that members of inflammatory cells such as neutrophils, lymphocytes, and alveolar macrophages were aggregated in blood, sputum, BALF, and bronchial mucosa in COPD patients [16]. Neutrophils have an important role in the pathogenesis of airway inflammation in COPD. Activated neutrophils cause lung destruction through the release of oxygen radicals and proteolytic enzymes [17]. In addition, neutrophils can release cytokines and chemokines, which can potentiate inflammation and trigger an immune response [18]. Among these cytokines and chemokines, IL-8 is considered to be a major driver of neutrophilia. As a neutrophil attractant, IL-8 is usually overexpressed in COPD patients, which can induce local pathological damage, aggravate airway inflammation as well as reflect the severity of airway inflammation [19]. In this study, our results showed that total number of

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white blood cells and neutrophil percentage in BALF were significantly decreased after sanyeqing treatment. Meanwhile, both serum and BALF levels of IL-8 and CRP, another well-known marker of inflammation, were also reduced by sanyeqing treatment in COPD rats. Taken together, these findings indicate that sanyeqing have an antiinflammation effect in both whole body and local lung tissues under COPD conditions.

Oxidative stress, defined as loss of the balance between production of reactive oxygen species and antioxidant defenses, is considered to cause oxidative damage to lipids, proteins, and DNA. Many experiments have shown that COPD patients suffer from oxidative stress [20]. Meanwhile, oxidative stress is also proven to play an important role in the pathogenesis of COPD [21]. To determine the local oxidant/antioxidant levels, we further measured the MDA and SOD levels in both pulmonary tissue homogenate and BALF from all three groups. Our results showed that Sanyeqing treatment significantly reduced MDA levels and improved SOD activities in both pulmonary tissue homogenate and BALF under COPD conditions. Since both MDA (the product of peroxidation of lipids) and SOD (an oxygen radical scavenger) are often used to evaluate the extent of oxidative stress, our results provided new evidence that Sanyeqing can significantly decrease COPD induced oxidative stress in lung.

Nuclear factor-erythroid 2-related factor 2 (Nrf2), also known as NFE2L2, is ubiquitously expressed at high concentrations in various human organs including the lungs. Recently, studies have indicated that Nrf2 is a key factor in the regulation of antioxidant and anti-inflammatory genes, and plays a crucial role in the pathogenesis of COPD [13]. Previous studies showed that activation of Nrf2 had protective effects against cigarette smoking induced lung inflammation [22]. Moreover, Nrf2 activation in macrophages reduced oxidative stress [23]. Therefore, to further investigate the potential underline mechanism of Sanyeqing on antiinflammation and antioxidative activity, we compared Nrf2 expression in COPD rats with or without Sanyeqing treatment. Our results showed that both mRNA and protein expression of Nrf2 in lung tissues were upregulated in COPD rats, and to a greater extent after Sanyeqing treatment. These results suggest that

the protective effect of Sanyeqing on COPD rats may be achieved via activating Nrf2.

Taken together, our studies provide evidence that Sanyeqing, at least in an animal model, can attenuate COPD. The protective effect of Sanyeqing is potentially related to its antiinflammation and antioxidative capacities via Nrf2 activation, which may provide a novel approach in the treatment of COPD. Certainly, further studies are needed to clarify the cellular and molecular mechanisms of Sanyeqing, as well as its safety and therapeutic potency in COPD patients.

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Disclosure of conflict of interest

None.

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