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Research Paper

Effects of *Schisandra chinensis* extracts on cough and pulmonary inflammation in a cough hypersensitivity guinea pig model induced by cigarette smoke exposure

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ABSTRACT

Schisandra chinensis (*S. chinensis*) is a traditional Chinese medicine commonly used in prescription medications for the treatment of chronic cough. However, the material basis of *S. chinensis* in relieving cough has not been completely elucidated yet. This study established a guinea pig model of cough hypersensitivity induced by 14 days of cigarette smoke (CS) exposure, to evaluate the antitussive, antioxidant, and anti-inflammatory effects of three *S. chinensis* extracts. And then the function of four lignans in reducing expression of TRPV1 and TRPA1 was examined using A549 cells induced by cigarette smoke extract (CSE). The results demonstrated that both ethanol extract (EE) and ethanol–water extract (EWE) of *S. chinensis*, but not water extract (WE), significantly reduced the cough frequency enhanced by 0.4 M citric acid solution in these cough hypersensitivity guinea pigs. Meanwhile, pretreatment with EE and EWE both significantly attenuated the CS-induced increase in infiltration of pulmonary neutrophils and total inflammatory cells, as well as pulmonary MDA, TNF-α, and IL-8, while remarkably increased activities of pulmonary SOD and GSH. According to H&E and immunofluorescence staining assays, airway epithelium hyperplasia, smooth muscle thickening, inflammatory cells infiltration, as well as expression of TRPV1 and TRPA1, were significantly attenuated in animals pretreatment with 1 g/kg EE. Moreover, four lignans of EE, including schizandrin, schisantherin A, deoxyschizandrin and γ-schisandrin, significantly inhibited CSE-induced expression of TRPV1, TRPA1 and NOS3, as well as NO release in A549 cells. In conclusion, *S. chinensis* reduces cough frequency and pulmonary inflammation in the CS-induced cough hypersensitivity guinea pigs. Lignans may be the active components.

1. Introduction

Cough hypersensitivity syndrome (CHS) is a new clinical phenotype of chronic cough, for which there is still no clinically effective drugs in the current treatment strategies (Chung, 2011). The pathogenesis of CHS has not yet been completely elucidated. Groneberg et al. (2004) showed that there was an increase in transient receptor potential vanillic-1 (TRPV1) containing subepithelial sensory nerves within the bronchial wall of chronic cough patients. Moreover, the existing results showed that cigarette smoke (CS) is an important cause of CHS, which may mediate expression and activation of transient receptor potential ankyrin-1 (TRPA1) (Benich and Carek, 2011; Shapiro et al., 2013). Previous study has described an exacerbated cough model mimicing to CHS by exposing the guinea pigs to CS for 10 days. It was demonstrated that the cough enhanced by citric acid was significantly increased after 10 days of CS exposure, which was significantly inhibited by codeine, DNK333 (a selective NK1/NK2 antagonist), terbutaline, or atropine (Lewis et al., 2007). Moreover, the CS

Abbreviations: BALF, Bronchoalveolar lavage fluid; CHS, Cough hypersensitivity syndrome; CS, Cigarette smoke; DMEM, Dulbecco's modified eagle medium; EE, Ethanol extract; Eos, Eosinophil; EWE, Ethanol–water extract; GSH-Px, Glutathione peroxidase; H&E, Hematoxylin–eosin; HPLC, High performance liquid chromatography; IL-8, Interleukin 8; IgG, Immunoglobulin G; Lym, Lymphocyte; Mac, Macrophage; MDA, Malondialdehyde; Neu, Neutrophil; NK1, Neurokinin-1 receptor; NK2, Neurokinin-2 receptor; NO, Nitric oxide; PBS, Phosphate buffered saline; PCR, Polymerase chain reaction; PEG, Polyethylene glycol; Penh, Enhanced pause; RIPA, Radio Immunoprecipitation Assay; RNA, Ribonucleic Acid; SOD, Superoxide Orgotein Dismutase; TNF-α, Tumor necrosis factor α; TRPV1, Transient receptor potential vanillic-1; TRPA1, Transient receptor potential ankyrin-1; WE, Water extract

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exposure-evoked cough has been fully justified in massive researches based on clinical data, CS-exposed rodent models, and *in vitro* models (O'Malley et al., 2014; Itoh et al., 2014; Nie et al., 2012; Lee et al., 2013; Jin et al., 2003). Previous studies revealed that CS exposure induces oxidative stress, which increases airway inflammatory cells influx, lipid peroxidation and pro-inflammatory cytokines release such as TNF-α and IL-8 (Nie et al., 2012). The contents of TNF-α and IL-8 within the airways always increase in nonasthmatic patients with chronic dry cough (Jatakanon et al., 1999). Moreover, SOD and GSH, the main pulmonary antioxidant enzymes, were found to be decreased in human bronchial epithelial cells after CS-exposure (Hoffmann et al., 2013). Thus, the CS-exposure model in rodents might be useful for investigating the anti-oxidant, anti-inflammatory and antitussive effects of novel ingredients which might have potential therapeutic effects to CHS.

Schisandra chinensis (*S. chinensis*), officially listed as a sedative and tonic drug in the Chinese Pharmacopoeia, has been commonly used in prescription medications of traditional Chinese medicine for the treatment of chronic cough (Kim et al., 2014). *S. chinensis* was also considered as an adaptogen in cases of physical exhaustion for many years in Russian states, and it has been proved to be indispensable in the prescription medications for treating chronic cough (Panossian and Wikman, 2008). Schisandra lignans such as schisandrin, schisantherin A, deoxyshisandrin and γ-schisandrin, are the major constituents of *S. chinensis* and more than 40 of them have been isolated by now (Cheng et al., 2014). Besides, volatile oils, organic acids, citral, sterols, vitamins, and carbohydrates were also reported to be extracted from *S. chinensis* (Shen et al., 2010). However, the effects and related material basis of *S. chinensis* on cough and chronic pulmonary inflammation have not been fully confirmed.

Pharmacological studies on animals showed that extracts of *S. chinensis* significantly inhibited carrageenan-induced mice paw edema and lipopolysaccharide -induced releases of NO and prostaglandin E2 (PGE2). Extracts of *S. chinensis* can also reduce expressions of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in a RAW 264.7 macrophage cell line (Guo et al., 2008; Hu et al., 2014). Moreover, *S. chinensis* had strong antioxidant, hepatoprotective and anti-inflammatory effects in an acute liver injury mice model damaged by CCl_4. Pretreatment with extracts of *S. chinensis* significantly attenuated acute liver injury through improving hepatic activities of SOD, GSH and CAT, as well as normalizing levels of Serum glutamic pyruvic transaminase (SGPT), Serum glutamic-oxaloacetic transaminase (SGOT) and P450 (Zhu et al., 1999). Furthermore, numerous clinical trials have demonstrated the efficiency of *S. chinensis* in asthenia, neuralgic and psychiatric disorders, pneumonia, chronic sinusitis and neuritis.

Therefore, this study sets up a chronic cough hypersensitivity guinea pigs model induced by CS exposure, to evaluate the effects of *S. chinensis* extracts against cough and chronic pulmonary inflammation. The effects were evaluated by cough frequency, enhanced pause (Penh), inflammatory cells infiltration, release of cytokines and chemokines, and the expression of TRPV1 and TRPA1. By analyzing the correlation between efficacy and composition in the three extracts of *S. chinensis*, we hypothesized the lignans would be active ingredients, and then the A549 cells induced by cigarette smoke extract (CSE) were used to verify the effects of lignans on expression of TRPV1, TRPA1 and NOS3, as well as the release of NO.

2. Materials and methods

2.1. Materials and reagents

The commercially available filtered cigarettes (trade name: Hongmei, from the Guangdong Tobacco Industrial Co., Ltd. China) containing 12 mg tar and 1.2 mg nicotine per cigarette were used in this study. Codeine tablets (30 mg per tablet, made by China National Pharmaceutical Industry Co. Ltd.) were provided by The First Affiliated Hospital of Guangzhou Medical University for research use. Antibodies against β-actin, antibodies against TRPV1 and TRPA1, antibody of NOS3 were all purchased from Santa Cruz (Biotechnology Inc., CA, USA). TITC-rabbit Anti-goat IgG and Texas red donkey anti-rabbit IgG were purchased from EarthOx. TNF-α and IL-8 assay kits were purchased from Nanjing Jiancheng Insititute of Bioengineering, (Nanjing, China), BCA assay kit (Beyotime, Shanghai, China), Trizol kit (Invitrogen, Carlsbad CA, USA), SuperScript™ II reverse transcriptase reagents and the qSYBR Green PCR Kit (Takara, Dalian, China) were used for biological detection.

The fruits of *S. chinensis* were collected in Anshan County, Liaoning Province, China in November 2013. The plant was identified by Dr. S. Zhong and a voucher specimen (No. 20131129S) was deposited at the State Key Laboratory of Respiratory Diseases. The fruits were dried and grounded to powder before using. Standards of schizandrin, schisantherin A, deoxyschizandrin and γ-schisandrin (purity $>99\%$) were purchased from National Institute for Food and Drug Control (NIFDC, Beijing, China). CH_3CN was HPLC grade. Deionized water was prepared using a Millipore water purification system. All other analytical grade reagents were purchased from Guangzhou chemical reagent factory.

2.2. Instruments

The HPLC system was equipped with an Agilent Series 1200 liquid chromatography, with G1379B vacuum degasser, G1312A binary pump, G1329A autosampler, G1316A column oven and G1315B Diode array detector, connected to Agilent ChemStation software (Agilent, Waldbronn, Germany). The smoking exposure apparatus used in the present study was previously reported (Nie et al., 2012). The citric acid-induced cough measurement was done with a Buxco system as our research team's report (Chen et al., 2013). TU-1901 UV–vis spectrophotometer (Purkinje General Instrument Co., Ltd., Beijing, China), Nikon fluorescence microscope (Eclipse E600), and laser scanning confocal microscope (Leica TCS SP; Bucks, UK) were used for histological evaluations.

2.3. Animals

Hartely strain guinea pigs (male, 300 ± 50 g) were purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China). Animals were kept 4–5 per cage and acclimatized at a constant room temperature of 23 ± 3 °C and humidity of $55 \pm 15\%$ with a 12:12 h light–dark cycle. Food and water were available *ad libitum*. The animal study was performed according to the international rules considering animal experiments and the internationally accepted ethical principles for laboratory animal use and care.

2.4. Cells

A549 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured with DMEM (Gibco BRL, Gland Island, NY) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad CA, USA), 100 mg/mL streptomycin (Invitrogen, Carlsbad CA, USA), and 100 U/mL penicillin (Invitrogen, Carlsbad CA, USA) in a humidified atmosphere of 5% CO_2 at 37 °C.