

The inhibitory effect of Yupingfengsan and Siwutang compound formula on inflammation and oxidative stress in COPD rats

Shouzheng Tian, Yang Liu, Xiaomei Zhang, Zhongshan Yang, Jing Peng and Jing Chen*

Faculty of Basic Medical Science, Yunnan University of Chinese Medicine, Kunming, China

Abstract: To investigate the potential roles of the traditional Chinese medicine Yupingfengsan and Siwutang compound formula (YS) in chronic obstructive pulmonary disease (COPD) rats, wistar rats were assigned to control, YS-treated and COPD model groups. The COPD rats model were established by passive smoking and intratracheal instillation of lipopolysaccharide (LPS). Histological changes were detected by hematoxylin/eosin (HE) staining. Protein levels of tumor necrosis factor (TNF)- α , interleukin (IL)-6, transforming growth factor (TGF)- β 1 and phosphorylated-smad2 (p-smad2) were determined by western blot assay. The activities of super oxide dismutase (SOD), glutathion peroxidase (GSH-Px) and the content of malondialdehyde (MDA) in the serum were estimated by biochemical methods. Relative mRNA levels of TNF- α , IL-6 and TGF- β 1 were measured by quantitative real-time polymerase chain reaction (RT-PCR) analysis. The results showed that YS enhanced the above oxidase activity and decreased the yield of MDA, and reduced the levels of TNF- α , IL-6, TGF- β 1 and p-smad2 in YS-treated COPD rats compared with the COPD rats. Our results suggested that YS produced the beneficial effects in COPD rats by antiinflammatory and antioxidative actions. Moreover, our research indicated that YS produced antiinflammatory effects in COPD rats by inhibiting the expression of inflammatory cytokines, possibly through suppressing the TGF- β 1/Smad2 signaling pathway.

Keywords: Antiinflammatory, antioxidative, Yupingfengsan, Siwutang, COPD.

INTRODUCTION

The chronic air-flow obstruction and persistent respiratory symptoms are notable features of COPD (GOLD, 2018). It is the 4th leading place for causes of death in the world, but it is expected to become the 3rd leading cause of death by 2020 (Lozano *et al.*, 2010). With aging issues intensify and continuous exposure to COPD risk factors (cigarette smoke (CS), pollution haze, haze vehicle exhaust and other noxious particles or gas), the burden of this disease will become serious (Mathers and Loncar, 2006). Currently, western medicine treatments of COPD mainly alleviate symptoms (Renard and Drummond, 2015). Corticosteroids and bronchodilators are effective in treating COPD (GOLD, 2018). However, COPD patient refuse to use the above drug because of their side effects (Price *et al.*, 2013). On the contrary, herbal remedies are becoming popular among patients because of their better efficacy and fewer side effects (Wang *et al.*, 2015). For example, a new mixture of herbal medicine i.e., AKL1 has emerged and thought to cure COPD symptoms especially cough related quality of life of COPD patients (Hanif *et al.*, 2018). Yupingfengsan and Siwutang are classical prescriptions of traditional Chinese medicine. Clinically, Yupingfengsan has been widely used to treat respiratory system and immune system diseases, including COPD (Li *et al.*, 2013). Siwutang has been widely used in China and clinically applied to treat blood deficiency and Blood stasis Syndrome for thousands of years. Moreover, Blood stasis has been considered as one of the main pathogenesis of COPD in traditional Chinese

medicine theory. As a result, Yupingfengsan has been widely used to treat COPD in Chinese folk medicine by co-administration with Siwutang. However, as a compound formula, the mechanism of YS for treating COPD remains unclear.

Although the pathogenesis of COPD remains to be further defined, inflammatory response and oxidative stress are clearly associated with the development of COPD (GOLD, 2018). Cigarette smoke consist of reactive oxygen species (ROS) and other hazardous substance that stimulated nonspecific immune cells then mediated the inflammatory response, tissue repair and fibrosis processes of COPD (Oostwoud *et al.*, 2016). ROS and other exogenous irritants activate inflammatory cells and epithelial cells to release ROS, inflammatory mediators and proteases in the respiratory tract (GOLD, 2018; Mannino and Buist, 2007; Barnes, 2010). In turn, inflammatory mediators promote the production of endogenous ROS, proteases over secretion and mucus secretion. Finally, the synergistic effect of inflammation and oxidative stress intensify the development of COPD. Hence, the methods of multi-target intervention, such as traditional Chinese medicine intervention, are regarded to be effective strategies for the treatment, as well as drug research and development of COPD (Matera *et al.*, 2011). This study sought to reveal the potential role of YS in COPD rats.

MATERIALS AND METHODS

Animals

Wistar rats weighing 200 \pm 20g were purchased from

*Corresponding author: e-mail: thaphon@foxmail.com

Chengdu Dashuo Experimental Animal Co., Ltd. (Chengdu, China). Rats were fed at 25°C under a 12 hour day-night cycle with free eating and drinking. All rat-related experiments have been approved by the Animal Ethics Committee of Yunnan University of Traditional Chinese Medicine.

Extract preparation of YS

YS is composed of seven components: Astragali Radix, Atractylodis Macrocephalae Rhizoma, Saposhnikoviae Radix, Angelicae Sinensis Radix, Chuanxiong Rhizoma, Paeoniae Radix Alba and Rehmanniae Radix Preparata (table 1, all the information had been checked with Chinese Pharmacopoeia). Among the seven components, the first three make up Yupingfengsan and the latter four make up Siwutang. All these medicinal herbs were purchased from Yunnan Hongxiang Yixintang Pharmaceutical Co., Ltd. (Kunming, China) and authenticated by Professor Yu Cheng (Yunnan University of Traditional Chinese Medicine, Kunming, China). Voucher specimens of raw medicinal materials were deposited at the Laboratory of Pathogenic Microbiology and Immunology, Yunnan University of Traditional Chinese Medicine (Kunming, China). Extract preparation of YS was according to the following procedures: all medicinal herbs were mixed according to the amount in table 1. The above mixture was decocted three times within boiling water for twenty minutes each time. The water layers were combined and concentrated below 50°C under vacuum to give water extract of YS. The water extract was dissolved at the applied concentration with physiological saline and sanitary filtered by 0.22µm filter before being administered to rats.

Animal grouping

All animals were randomly assigned to control, YS-treated and COPD model groups after being adaptively raised for 7 days. The COPD rat model was established according to the method of Wang Changming *et al.* (Wang *et al.*, 2013). Briefly, 200µg LPS was intratracheal instilled on day 1 and day 14. Continuous fresh CS was administered to the rats for 1 h/day on days 2-13 and 15-42. Self-prepared perspex boxes were used to force rats to smoke according to the literature (Zheng *et al.*, 2009). Cigarettes (Hongtashan filter cigarette; Hongta Group, Yuxi, China) containing 1.1mg nicotine, 11mg tar and 11mg carbonic oxide per cigarette were used for the present study. Simultaneously, rats in normal control group were instilled the same amount of physiological saline solution and inhaled clean air for 6 weeks. $1.26\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ YS was administered intragastrically to the YS-treated group every day from day 15 to day 42, and the equivalent dosage was calculated based on the multiple relation of surface area between humans and rats. Finally, animals were sacrificed and arterial blood as well as lung tissues were harvested immediately for further analysis.

Histological examination

Fresh tissues of left lung were rinsed in normal saline then immersed in 4% formaldehyde solution to complete fixation. After that, the general paraffin section production procedures were performed on these tissues. To be brief, the tissues were dehydrated in an increasing concentration gradient of ethylic alcohol, embedded in paraffin, sectioned to 4µm, dewaxed in dimethylbenzene and rehydration in graded ethanol solution. Finally, the sections were stained with HE and observed under a light microscopy in a blind manner. To estimate the levels of inflammatory cell infiltration, a semi quantitative histological scoring system was performed. Arabic numerals were used to represent different grades in the scoring system: 0 (no cells); 1 (a few cells); 2 (a circle of monolayer cells); 3 (a circle of two to four layers of cells); and 4 (a circle of more than four layers of cells) (Zhou *et al.*, 2016).

Western blot analysis

Lung tissues were smashed in liquid nitrogen then lysed into lysis buffer (Cell Signaling Technology, USA) for ultrasonic homogenation on ice for 10min. The lysates were centrifuged at 12000 rpm for 15 min to get supernatants. Protein concentrations were assessed by a BCA protein analysis kit (Beyotime, China). Total protein was separated using 10% SDS-PAGE then transferred to polyvinylidene fluoride (PVDF) membranes (Millopore, USA). The membranes were blocked with PBS for 1h at room temperature then incubated at 4°C with respective antibodies (anti-p-Smad2 and anti-IL-6, 1:1000 dilution; anti-TGF-β1 and anti-TNF-α, 1:500 dilution; anti-GAPDH, 1:5000 dilution; Bioworld Technology, USA). After washing 5 times for 5 min each time with TBST, the membranes were incubated with IRDyeR680LT secondary antibody (1:20000 dilution; LI-COR, USA) for 1h at room temperature. After that, protein bands were scanned and calculated with Odyssey CLX infrared imaging system (LI-COR, USA). β-actin was used as an internal reference. The ratio of the target band to the internal reference was used as the expression value of the target protein. Relative protein levels of different groups were calculated as the fold change from control group (Guo *et al.*, 2015).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from lung tissues with Trizol Reagent (Tiangen, China). RNA (1µg) was reverse transcribed into cDNA using a reverse transcription kit (Takara, China). Two-step PCR was conducted on the ABI Veriti PCR Thermal Cyclers (Applied Biosystems, Germany) using TaKaRa SYBR Premix Ex TaqII (Takara, China). The optimized PCR procedure was as follows: 95°C for 30s and then 40 cycles were executed, each loop consisted of 95°C for 5s and 62°C for 30s.

Table 1: The composition of YS

English name	Chinese name	Plant species	Plant part	Grams
Astragali Radix	Huangqi	<i>Astragalus membranaceus</i> (Fisch.) Bge.	Root	18
Atractylodis Macrocephalae Rhizoma	Baizhu	<i>Atractylodes macrocephala</i> Koidz.	Rhizoma	6
Saposhnikoviae Radix	Fangfeng	<i>Saposhnikovia divaricata</i> (Turcz.) Schischk.	Root	6
Angelicae Sinensis Radix	Danggui	<i>Angelica sinensis</i> (Oliv.) Diels.	Root	10
Chuanxiong Rhizoma	Chuanxiong	<i>Ligusticum chuanxiong</i> Hort.	Rhizoma	10
Paeoniae Radix Alba	Baishao	<i>Paeonia lactiflora</i> Pall.	Root	10
Rehmanniae Radix Preparata	Shudihuang	<i>Rehmannia glutinosa</i> Libosch.	Root	10

Table 2: The primers used for RT-PCR analysis

Gene	Primer sequence (5' - 3')	Size(bp)
β -actin	F: GGAGATTACTGCCCTGGCTCCTA R: GACTCATCGTACTCCTGCTTGCTG	124
TNF- α	F: GTCGTAGCAAACCACCAAGC R: GAAGAGAACCTGGGAGTAGATAAGG	147
IL-6	F: AGTTGCCTTCTTGGGACTGA R: ACTGGTCTGTTGTGGGTGGT	102
TGF- β 1	F: ATTCCTGGCGTTACCTTGG R: AGCCCTGTATTCCGTCTCCT	120

F: forward; R: reverse.

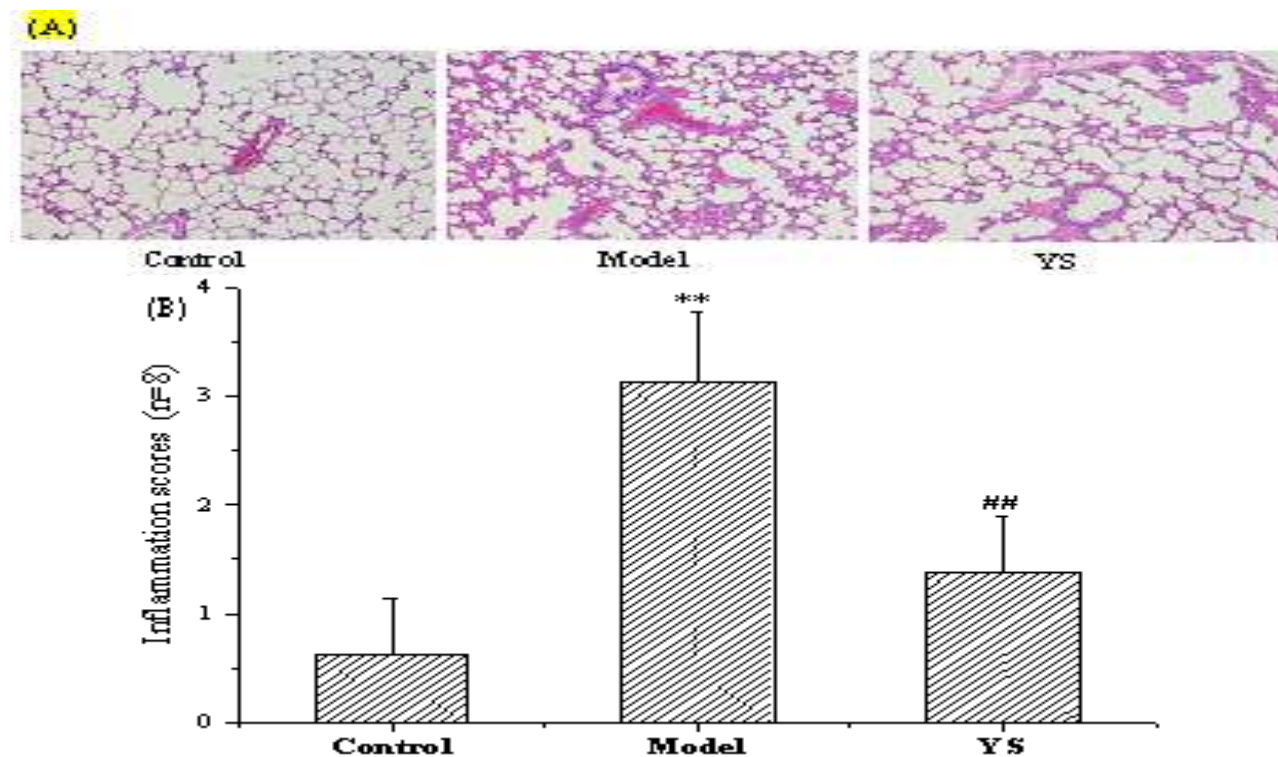


Fig. 1: The effects of YS on pulmonary pathological changes in CS and LPS-induced COPD rats. Rats were intragastrically treated with $1.26\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ YS from day 15 until week 6. (A) Lung tissues were sliced and stained by HE, magnification $\times 100$. (B) The level of inflammatory cell infiltration was measured by a histological scoring system. Data are expressed as the means \pm SDs. Compared with the control group: *** $P < 0.01$; Compared with the model group: ## $P < 0.01$.

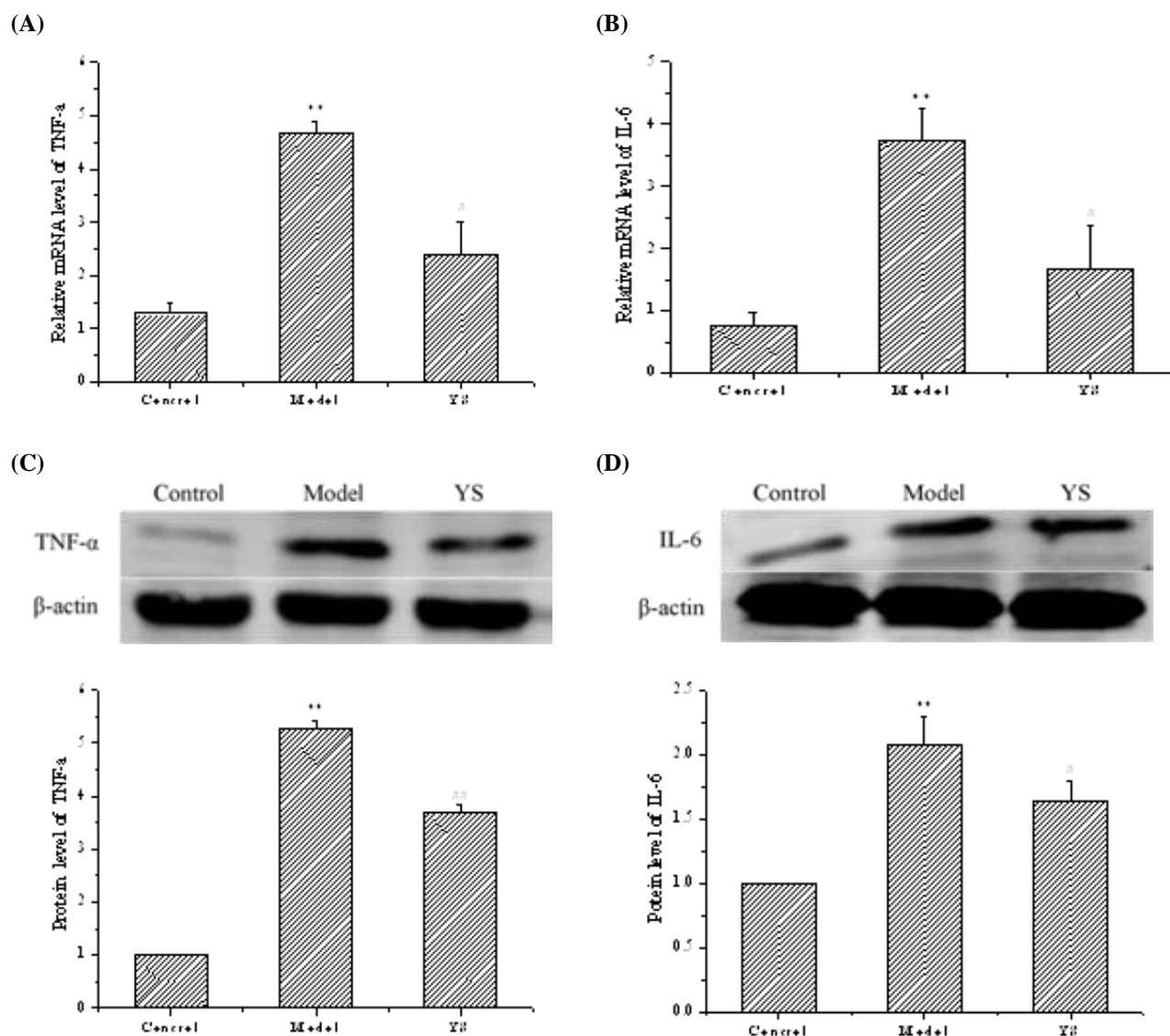


Fig. 2: The effects of YS on inflammatory cytokines in CS and LPS-induced COPD rats. After rats were intragastrically treated with $1.26\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ YS from day 15 until week 6, both mRNA and protein levels of TNF- α and IL-6 in lung tissues were measured by using RT-PCR and western blot assay, respectively. (A) Relative mRNA level of TNF- α . (B) Relative mRNA level of IL-6. (C) Protein level of TNF- α . (D) Protein level of IL-6. Data are expressed as the means \pm SDs. Compared with the control group: ** $P < 0.01$; Compared with the model group: # $P < 0.05$ and ## $P < 0.01$.

The primers used for PCR were shown in table 2. After getting the cycle threshold (Ct) values, the Ct values of target genes subtracted that of the internal reference genes β -actin to obtain the ΔCt value, ΔCt values of normal group subtracted that of different groups to obtain $\Delta\Delta\text{Ct}$, and relative mRNA levels of target genes connected with internal reference genes were represented as $2^{-(\Delta\Delta\text{Ct})}$ (Xi *et al.*, 2016).

Antioxidant detection

The levels of SOD, GSH-Px and MDA in serum were evaluated by using relevant analytical kits (Nanjing

Jiancheng Bioengineering Institute, Nanjing, China).

STATISTICAL ANALYSIS

All the analyses were carried out using SPSS 19. Data from the experiments were expressed as means \pm standard deviations (SDs). The differences among different treatment groups were analyzed by using one-way analysis of variance (ANOVA) with Student-Newman-Keuls test. $P < 0.05$ were considered as statistically significant.

RESULTS

Effect of YS on lung histological changes in COPD rats

The results from pulmonary pathological examination revealed that there were no apparent pathological changes in control group rats. However, the samples of the model rats were mainly featured with evident mucosal epithelial cells injury, inflammatory cells infiltration, mucus hyper secretion, alveolar septum thickens and fusion of pulmonary alveoli. On the contrary, the pathological changes in the YS-treated rats indicated a weakened degree compared to those in the model group. The histological scoring system showed an increased value in model rats compared with the control group. Nevertheless, rats treated with YS remarkably reduced scores compared with model group. Histological changes in different experimental groups indicated that YS signally improved the lung pathological injury in COPD rats (fig. 1).

Effect of YS on inflammatory cytokines in COPD rats

As showed in fig. 2, both TNF- α and IL-6 in COPD rats were significantly increased, regardless of mRNA levels and protein levels, compared with the normal control group ($p < 0.01$). On the contrary, both mRNA and protein levels of TNF- α and IL-6 in YS-treated rats were decreased in different degrees compared to those in COPD rats (fig. 2). These data indicated that YS reduced yields of TNF- α and IL-6, further alleviated inflammatory injury in COPD rats.

Effect of YS on TGF- β 1/Smad2 signaling pathway in COPD rats

As revealed in fig. 3A and 3B, both mRNA and protein levels of TGF- β 1 in model rats were higher than that in normal control group rats ($p < 0.01$). However, the TGF- β 1 mRNA levels in YS-treated rats were significantly reduced compared with that in model rats ($p < 0.05$), and protein index exhibited similar trend with the mRNA levels ($p < 0.01$). To further determine whether the TGF- β 1/Smad2 signaling plays a role in COPD rats, the p-Smad2 protein levels were tested. The results revealed that the phosphorylation levels of Smad2 in model rats were higher than that in the control group ($p < 0.01$), while the levels were reduced in YS-treated rats compared with that in the model rats ($p < 0.05$), as shown in fig. 3C. Based on the above analysis, it was obvious that YS played an important role in inhibiting TGF- β 1/Smad2 signaling pathway in COPD rats.

Effect of YS on antioxidant activity in COPD rats

In order to assess the effects of YS on antioxidant activity in COPD rats, the levels of SOD, GSH-Px and MDA in serum of the different experimental groups were measured. fig. 4A and 4B showed that enzyme activities of SOD and GSH-Px in model groups were reduced compared with that in the normal rats ($p < 0.01$). Conversely, YS improved enzyme activities of SOD and GSH-Px compared with

that in COPD rats ($p < 0.05$). fig. 4C showed the opposite trends compared with fig. 4A and 4B, suggested that YS played an important role in keeping a certain low levels of MDA in serum of COPD rats. The results illuminated that YS could improve antioxidant capacities of COPD rats.

DISCUSSION

Although many problems need to be clarified, inflammatory response and oxidant/antioxidant imbalance play crucial roles in the pathogenesis of COPD (GOLD, 2018). In our current research, we revealed anti-inflammatory and antioxidant function of YS in COPD rats. And for all we know, it was the first evidence of YS ameliorated inflammation and oxidative stress in the COPD rats.

Cytokines as crucial inflammatory mediators, many have been involved in patients with COPD, including TNF- α , IL-6 and TGF- β 1 (Barnes, 2016). Airway epithelial cells and many other immunity-associated cells in lung tissues can be activated by CS and other respiratory irritants to yield inflammatory cytokines TNF- α and IL-6, which are increasingly being reported as inflammatory markers of COPD patients. Dong *et al* found in 2014 that the productions of TNF- α were elevated in COPD patients compared to the normal individuals (Dong *et al.*, 2014). Queiroz *et al* reported that the levels of IL-6 in serum were higher in patients with COPD than healthy controls (Queiroz *et al.*, 2016). In addition, the levels of TNF- α and IL-6 in COPD animals shown the same trends compared with that in patients (Kim *et al.*, 2017; Yang *et al.*, 2016). In our current research, the results of TNF- α and IL-6 were consistent with the above literatures which suggested that the COPD animal model was established successfully. Nevertheless, the reduced yields were observed in YS-treated rats compared with the COPD groups. From the above analysis, we know that YS could suppress the expression of some inflammatory cytokines in COPD rats.

TGF- β 1 is a key factor associated with chronic inflammation of the respiratory tract. The major reasons involve that TGF- β 1 promotes fibrosis, induces collagen deposition and prevents them from being degraded (Massague, 1990). In addition, TGF- β 1 also has a chemotactic effect on macrophages and neutrophil to promote development of inflammation (Luo *et al.*, 2008). The results above prompted us to pay attention to the expression of TGF- β 1 in COPD rats, so TGF- β 1 was measured. As described in the results section, both mRNA and protein levels of TGF- β 1 in COPD rats were elevated compared with that in normal rats. However, the TGF- β 1 mRNA levels in YS-treated rats were significantly reduced compared with the model group and protein index exhibited similar trend with the mRNA levels. TGF- β 1 produces biological effects through binding its receptor

complex, activated TGF- β receptor phosphorylates Smad2 and Smad3, which forms a complex with Smad4, then the

complex was transferred into the nucleus to regulate many inflammatory genes (Gagliardo *et al.*, 2013).

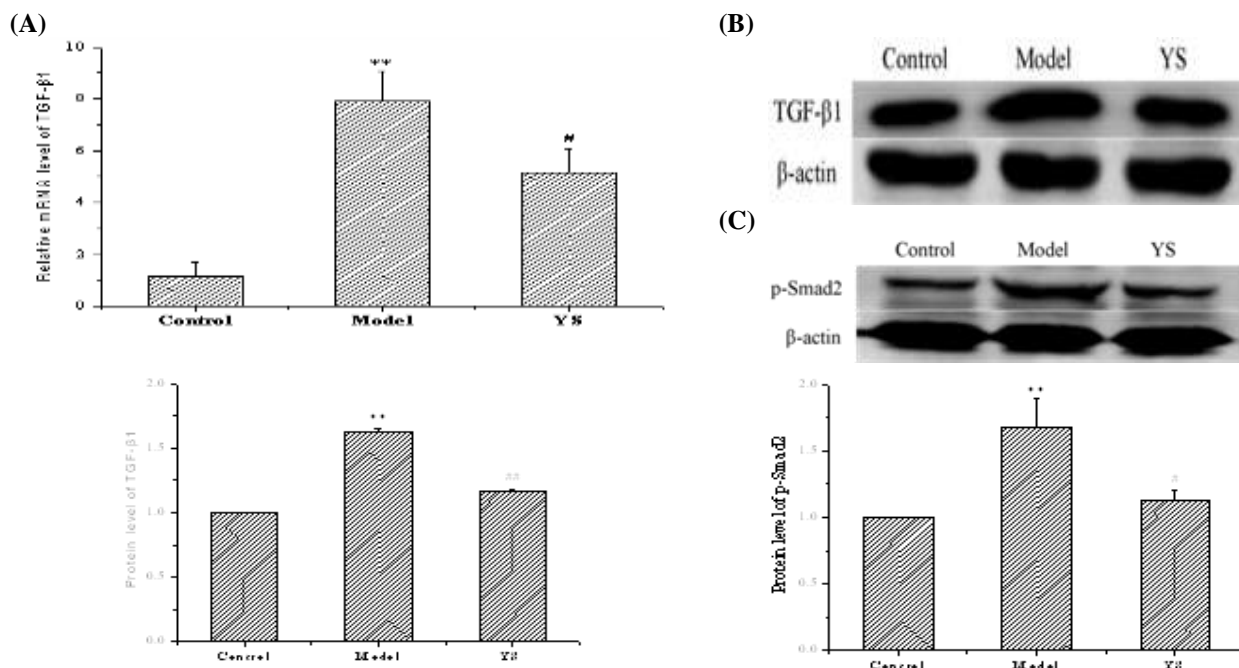


Fig. 3: The effects of YS on TGF- β 1/Smad2 Signaling in CS and LPS-induced COPD rats. After rats were intragastrically treated with $1.26\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ YS from day 15 until week 6, relative mRNA level of TGF- β 1 (A) were measured by using RT-PCR and protein levels of TGF- β 1 (B) and p-Smad2 (C) were measured by using western blot assay. Data are expressed as the means \pm SDs. Compared with the control group: ** P <0.01; Compared with the model group: # P <0.05 and ## P <0.01.

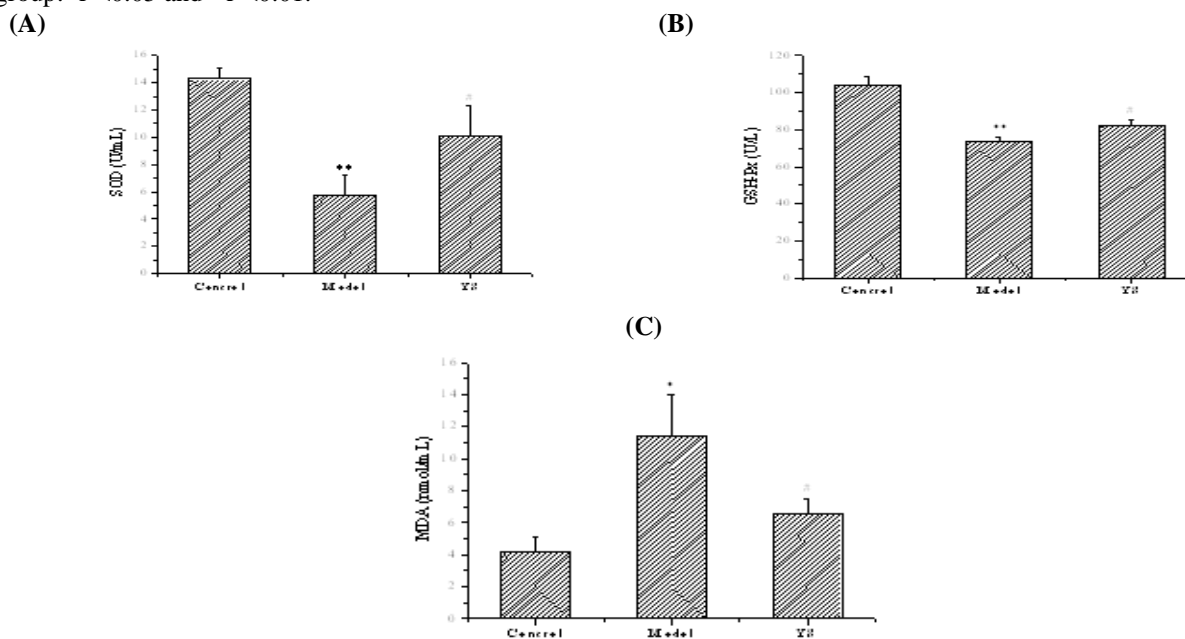


Fig. 4: The effects of YS on antioxidant activity in CS and LPS-induced COPD rats. After rats were intragastrically treated with $1.26\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ YS from day 15 until week 6, the levels of SOD (A), GSH-Px (B) and MDA (C) in serum of the different experimental groups were measured by using relevant analytical kits. Data are expressed as the means \pm SDs. Compared with the control group: * P <0.05 and ** P <0.01; Compared with the model group: # P <0.05.

Recent studies had shown that the over expression of TGF- β 1/Smad2 signaling had been involved in variety of inflammation in patients or animals (Queiroz *et al.*, 2016; Rosenbloom *et al.*, 2017). To further determine whether the TGF- β 1/Smad2 signaling pathway plays a role in COPD rats, the p-Smad2 protein levels were tested. These data revealed that the Smad2 phosphorylation levels in model rats were higher than that in the controls. However, the levels were tilted downwards in YS-treated rats compared to the model group. Based on the above analysis, it was obviously that YS played an important role in inhibiting TGF- β 1/Smad2 signaling pathway in COPD rats, which may play a role in YS inhibiting inflammation in COPD rats. As is well-known, oxidative stress is associated with the development of COPD. Tobacco smoke contains many harmful substances, including oxygen free radicals and nitrogen free radicals, which are the major factors induced antioxidative system imbalances and tissue damage in COPD patients (Wrobel *et al.*, 2012).

Oxidative stress triggers COPD by multiple mechanisms including mucous hypersecretion, epithelium injury, neutrophil infiltration and evoked inflammation (Bajpai *et al.*, 2017). Many molecular markers included in the oxidative stress process are connected to COPD (Singh *et al.*, 2017). As is well-known, in order to maintain the redox balance, a perfect antioxidant defensive enzyme system was founded in the human body, SOD, GSH-Px and other endogenous antioxidants act on COPD by scavenging ROS and RNS, and thus preventing tissues and cells from oxidative stress. According to the literatures, SOD and GSH-Px had been reported that there is a reduced yield in COPD patients and animals (Cai *et al.*, 2017; Vibhuti *et al.*, 2007). Membrane lipid is sensitive to ROS and RNS, which can cause changes in membrane mobility and thereby result in dysfunction of the cells. Malondialdehyde (MDA), the peroxidation product of membrane lipid, is a major indicator of oxidative stress. Previous studies had shown that the higher serum concentrations of MDA were found in patients and animals with COPD (Lin *et al.*, 2017; Dhakal *et al.*, 2015). The same results, associated with SOD, GSH-Px and MDA, were revealed in this study in COPD rats, and these data further demonstrated that the COPD rat model was established successfully. However, after rats were intragastrically treated with 1.26g•kg⁻¹•day⁻¹ YS from day 15 until week 6, the increased activities of SOD and GSH-Px were observed, while the concentrations of MDA were decreased compared with the COPD groups. These results indicated that YS may play a protective role by alleviating oxidative stress in COPD rats.

CONCLUSION

In conclusion, we reported the first evidence to show the positive effects of YS on CS and LPS-induced COPD rats through suppression inflammatory response and

attenuation oxidative stress. Moreover, our research indicated that YS produced anti-inflammatory effects in COPD rats by inhibiting the expression of inflammatory cytokines, possibly through suppressing the TGF- β 1/Smad2 signaling pathway.

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