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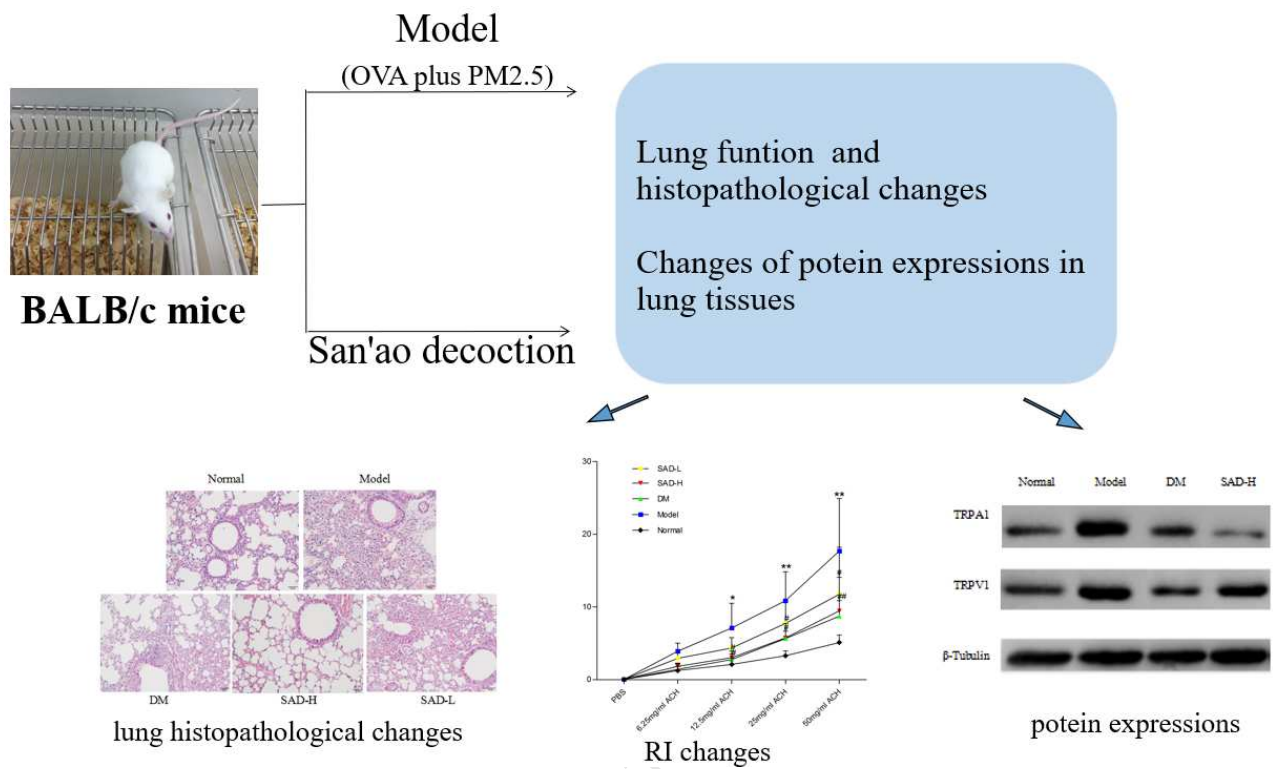
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Effect of San'ao Decoction on Aggravated Asthma Mice

Model Induced by PM2.5 and TRPA1/TRPV1 Expressions

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Abstract

Objective: San'ao decoction (SAD), a traditional Chinese prescription, is well-known in asthma treatment. In the current study, the protective role of SAD and its mechanism in aggravated asthma mice model via regulation of TRP channel were evaluated and explored.

Methods: UPLC-QTOF-MS was used for analyzing the chemicals in SAD. The major chemical components in SAD were separated and detected under an optimized chromatographic and MS condition. 75 BALB/c mice were randomly divided into five groups: normal group, model group, dexamethasone group ($0.75\text{mg}\cdot\text{kg}^{-1}$), SAD-high dose group ($1.8\text{ g}\cdot\text{kg}^{-1}$) and SAD-low dose group ($0.9\text{ g}\cdot\text{kg}^{-1}$). A 42 days aggravated asthmatic model was established in mice induced by ovalbumin (OVA) plus PM2.5 ($1.6\text{mg}\cdot\text{kg}^{-1}$). After treated with corresponding medicine, peripheral blood and bronchoalveolar lavage fluid (BALF) from each group were assessed, airway responsiveness was determined, histopathological changes in lungs were detected, relevant cytokines and neurokines levels were measured, TRPA1 and TRPV1 mRNA and protein expressions in lung tissues were examined as well.

Results: 21 signal peaks of the chemicals in SAD were identified with the method of UPLC-QTOF-MS. SAD, especially SAD-high dose exerted significant effects on OVA plus PM2.5 mice model in relieving lung injury score ($P<0.05$), reducing eosinophil (EOS) count in blood ($P<0.05$) and inflammatory cells ratio in BALF ($P<0.05$, $P<0.01$), decreasing RI value ($P<0.05$) while increasing Cdyn value ($P<0.05$), reducing IL-13, PGD_2 and NGF levels in BALF ($P<0.01$), as well as down-regulating TRPA1 and TRPV1 mRNA and protein expressions in lung tissues ($P<0.05$, $P<0.01$).

Conclusion: SAD could improve pulmonary functions, relieve lung injury, as well as reduce IL-13, PGD_2 and NGF levels of OVA plus PM2.5 aggravated asthma model in mice. The effect and mechanism of SAD might be related to the inhibition of TRPA1 and TRPV1 channels.

Keywords: San'ao decoction; asthma; PM2.5; TRPA1; TRPV1

Abbreviations: SAD, San'ao decoction; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; DM, dexamethasone; ACH, acetyl choline; EOS, eosinophil; RI, resistance; Cdyn, dynamic lung compliance; IL-13, interleukin-13; SP, substance P; PGD₂, prostaglandin D₂; NGF, nerve growth factor

1. Introduction

Asthma is a chronic obstructive airway disease with recurrent attacks of cough, dyspnea, chest tightness and wheezing (Sharifi et al. 2018). It is characterized by progressive airway remodeling, airflow obstruction and bronchial hyperresponsiveness (Carr and Kraft, 2017). Asthma affected 235 million people and caused 383,000 deaths worldwide estimated by World Health Organization (2017), as well as brought huge health and economic burden to the patients and society (Accordini et al. 2013).

The inhalation of environmental exposure can stimulate allergic reactions and lead to high susceptibility of asthma (Meldrum et al. 2017). As one of the environmental pollutants, the increasing concentration of particulate matter 2.5 (PM_{2.5}) may lead to asthma incidence rising continually (Gorai et al. 2016; Mirabelli et al. 2016). The polycyclic aromatic compounds of PM_{2.5} are the organic constituents with high toxicity and could cause bronchial mucosal injury, alveolar epithelial tissue hyperplasia and chronic inflammation (He et al. 2015; Yang et al. 2016).

San'ao Decoction (SAD) was first recorded in *<Taiping huimin heji jufang • Volume Two>* of Song dynasty. It consisted of Herba Ephedrae (Herbaceous stem of Ephedra sinica Stapf.), Semen Armeniacae amarum (Dry seed of Prunus armeniaca L.) and Radix Glycyrrhizae (Radix and Rhizoma of Glycyrrhiza uralensis Fisch.), which were all recorded in Pharmacopoeia of the People's Republic of China (2015 edition) issued by Chinese Pharmacopoeia Commission (2015). With the function of dispersing lung and dispelling cold, nowadays SAD is well known and commonly applied to cough and asthma treatment on clinic (Fan et al. 2015).

In our early studies, SAD was proved to play the significant roles in resisting airway allergic inflammation, reducing airway responsiveness, protecting bronchial epithelial cells, etc. Also, seven identified bio-active compounds in SAD including glycyrrhizic acid, formononetin, amygdalin, etc. were analyzed by using a HPLC-DAD method as confirmed previously (Shu et al. 2012). The complex effect and mechanism of SAD mainly rested in the following ways: reducing airway inflammatory reaction and eotaxin-3 expressions induced by IL-4 (Li et al. 2010), decreasing airway hyperresponsiveness in the LPS-enhanced asthma model through regulating Th1/Th2 balance, and playing anti-asthmatic effect through regulating Treg/Th17 level (Gu et al. 2011). Besides, formononetin extracted from SAD was confirmed to take part in the inflammation control and immune adjustment through activating PPAR γ (Zhou et al. 2009).

Recently, TRP (Transient receptor potential ion channel protein) channels were confirmed to have the effects of regulating respiratory symptoms, and TRPV1 was demonstrated to play parts in controlling sensory nerve function as well as modulating the functions of CD4⁺ T cells in allergic asthma (McAlexander and Taylor, 2011;

Baker et al. 2016). Our previous studies also affirmed that asthma exacerbation was found in the OVA plus PM2.5 mice model characterized by airway hyperresponsiveness, severe lung tissue injury, increased TRPA1 protein expression, etc., indicating that TRP channel might play a role in the process of asthma (Liu et al. 2017).

Due to the therapeutic effects of SAD in treating asthma on clinic, as well as the increasingly serious environmental pollution, in this study the aggravated asthma model was duplicated in mice induced by OVA plus PM2.5 to explore the effect and mechanism of SAD in treating asthma exacerbation. The effects of SAD were observed through airway responsiveness, pulmonary histopathology, inflammatory cell ratio in bronchoalveolar lavage fluid (BALF), as well as the level of cytokines and neurokines in BALF. Also, the mechanism of SAD was further elaborated by comparing the TRPA1 and TRPV1 protein expressions in different groups.

2. Material and methods

2.1 Drugs and reagents

In this study, the ratio of *Herba Ephedrae*, *Semen Armeniacae Amarum* and *Radix Glycyrrhizae* in SAD was 2:3:1, as early acquired from the orthogonal test (Wang et al. 2012). All crude dry herbs were purchased from Chinese herbal medicine market of Bozhou, Anhui (Batch No. 20150227). The botanical identities of the herbs were identified by Prof. Dekang Wu of Medicinal Plant Department, Nanjing University of Chinese Medicine.

OVA was purchased from Shanghai Bo'ao Biotech Co., Ltd. (Batch No.150623); dexamethasone (DM) was purchased from Shanghai Sinepharm Co., Ltd.(Batch No. 015140402); acetyl choline (ACH) was purchased from Sinopharm Chemical Reagent Co., Ltd (Batch No. 20141024); Wright's stain was purchased from Nanjing KeyGEN Biotech Co., Ltd. (Batch No. 20150717); IL-13, SP, PGD₂ and NGF assay kits were purchased from Shanghai Yunhan Biotech Co., Ltd, China; anti-TRPA1 antibodies and anti-TRPV1 antibodies were purchased from Abcam Inc., UK; β -Tubulin antibodies were purchased from CST Inc., USA; PrimeScriptTM RT reagent Kit with gDNA Eraser (No. RR047A) was purchased from TaKaRa Bio Inc., Japan. FastStart Universal SYBR Green Master(ROX) (No. 04913850001) was purchased from Roche Co., Ltd., Germany.

2.2 Apparatus

Ultrasonic nebulizer was purchased from Jiangsu Yuwell Medical Equipment and Supply Co., Ltd.; Buxco FinePointeTM RC system was purchased from Buxco Inc., USA; Enzyme-linked immune detector was purchased from Bio-Tek Instruments Inc., USA; ABI7500 RT PCR upgrade was purchased from Invitrogen Co., Ltd; Vertical electrophoresis apparatus was purchased from Bio-Rad Laboratories Inc., USA; Gel documentation system was purchased from Bio-Rad Laboratories Inc., USA. Upright Fluorescence Microscope BX53 was purchased from Olympus Co., Ltd, Japan.

2.3 Sample preparation

SAD was prepared for high dose and low dose, with the concentration ratio of 2:1. In the study, high dose of SAD ($1.8\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) for mice is equivalent to the

application to patients ($0.2\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) on clinic. Herbal samples in SAD were weighted accurately and mixed according to the ratio. The mixed herbs were soaked into 10 times of water for 1h and boiled 30min to get the extracted solution, then boiled another 30min with 5 times of water for the extracted solution. The two times extracted solutions were mixed together and centrifuged for 5min ($3000\text{r}\cdot\text{min}^{-1}$). The supernatant was concentrated to each milliliter containing 1~2g of crude herbs, which was regarded as the decoction sample.

2.4 UPLC-MS/MS conditions

SAD was dissolved in methanol solution (50%). The mixture was then centrifuged to get the supernatant. Afterwards, SAD solution was made into the concentration of 150mg/ml. UHPLC was performed on a Waters ACQUITY UPLC system (Milford MA), consisting of a binary solvent delivery system, an autosampler and PDA detector system. An ACQUITY UPLC BEH C18 column (2.1 mm $\times$ 100 mm i.d., 1.7 μm ; Waters) was used for all analyses, respectively maintaining at 30°C and 10°C. The mobile phase was composed of 0.1% formic acid (v/v) (A) and acetonitrile (B). The flow rate was 0.4 ml/min with 30 min of gradient elution: 0-1 min, 5-5% B; 1-10 min, 5-20% B; 10-25 min, 20-50% B; 25-27 min, 50-95% B; 27-29 min, 95% B; 29-30 min, 95-5% B.

The MS analysis was performed on a Waters ACQUITY Synapt Q-TOF mass spectrometer connected to the Waters ACQUITY UPLC system via an electrospray ionization (ESI) interface. High purity nitrogen was chosen to be the nebulizer and auxiliary gas while argon as the collision gas. The Q-TOF MS was operated in the negative ion mode with a capillary voltage of 3 kV, a sampling cone voltage of 35V, a desolvation gas flow of 600L/h, a collision energy of 6 eV, an ion source temperature of 120°C, and full scan spectra from 100 to 1500 Da. The LockSpray was used to maintain MS accuracy. The $[\text{M}-\text{H}]^{-}$ and $[\text{M}+\text{H}]^{+}$ ions of leucine-enkephalin at m/z 554.2615 and 556.2771 were used as the lock mass in respectively negative and positive ESI modes, with a concentration of 50 pg/ml and a infusion flow rate of 10 $\mu\text{L}/\text{min}$.

2.5 Animals

A total of 75 female Balb/c mice (8-12 weeks old, $20\pm 2\text{g}$) were purchased from the Comparative Medicine Centre of Yangzhou University (Yangzhou, Jiangsu, China), with the certification No. SCXK (Su) 2012-0004. The mice were acclimated to laboratory conditions (Temp: $24\pm 1^{\circ}\text{C}$, relative humidity: 50%~70%) with free access to tap water for one week before experiments commenced. All animal procedures were approved by the Ethical Committee for the Experimental Use of Animals at Nanjing University of Chinese Medicine (Nanjing, Jiangsu, China) and conformed to the Guide for the Ethical Care and Use of Laboratory Animals (National Academy of Sciences, 2011)

2.6 Model establishment and drug treatment

75 mice were randomly divided into 5 groups with 15 mice each: normal group, model group, SAD high dose group ($1.8\text{g}\cdot\text{kg}^{-1}$, SAD-H), SAD low dose group ($0.9\text{g}\cdot\text{kg}^{-1}$, SAD-L) and dexamethasone group ($0.75\text{mg}\cdot\text{kg}^{-1}$, DM). A 42 days aggravated asthma model induced by OVA plus PM2.5 ($1.6\text{mg}\cdot\text{kg}^{-1}$) was established as (Liu et al.

2017) previously described. In brief, mice in all groups except the normal group were given two injections subcutaneously and intraperitoneally of 0.5mL sensitizing solution containing 25 μ g OVA and 2mg aluminium hydroxide gel on day 1 and day 8 respectively. Then these groups were challenged with OVA (5%) for 20 min daily from the 15th day through the 28th day and every other day from day 30 to day 42. Mice in normal group received NS (40 μ l), while mice in other groups received PM2.5 solution (1.6mg·kg⁻¹, 40 μ l) through intratracheal injection on day 29, 33, 37 and 41 respectively. Mice in the three medication groups were administrated intragastrically 30 min before OVA atomization with SAD high dose (1.8 g·kg⁻¹) in SAD-H group, SAD low dose (0.9 g·kg⁻¹) in SAD-L group and dexamethasone (0.75mg·kg⁻¹) in DM group once a day from day 29 through day 42, while mice in normal and model groups were given equal volume of NS. 24h after the last challenge, all mice were anaesthetized with 4% chloral hydrate (0.1ml/10g) to test pulmonary functions, as well as to collect blood, lung tissues and BALF for further research.

2.7 Eosinophil (EOS) count in peripheral blood

After anaesthetized with 4% chloral hydrate (0.1ml/10g), peripheral blood was withdrawn from mice orbit. 20 μ l peripheral blood was mixed with 0.38 ml of EOS count solution (Nanjing Jiancheng Bioengineering Institute, China). The amount of EOS was observed and counted by using a hemocytometer according to the manufacturer instructions under 100X magnification with microscope.

2.8 Inflammatory cells in BALF

After last challenge, cannulas were gently inserted into mice tracheae. BALF was performed with three aliquots of 0.5mL PBS withdrawn three times each. BALF samples were centrifuged (1000 r·min⁻¹) for 10 min. The supernatant was taken and stored for further tests. The pelleted cells were resuspended in 0.5 ml PBS, smeared and fixed by Wright and Giemsa's staining. Each stained slide was counted twice double-blindly with 200 cells per count using microscope under 100X magnification. Each kind of leukocyte was sorted out, then the percentage of EOS and neutrophils in BALF were analyzed.

2.9 Airway responsiveness determination

The mice tracheas were isolated by blunt dissection and connected to ventilator under anesthetization. With different concentration of ACH stimulation, the airway responsiveness was analyzed by Buxco FinePointeTM RC system. Airway resistance (RI) and dynamic lung compliance (Cdyn) in response to increasing concentrations of ACH (0, 6.25, 12.5, 25, 50mg·mL⁻¹) were recorded and analyzed.

2.10 Histopathological assessment

The right lung tissues were fixed in 10% formalin and embedded in paraffin. The sections were stained with Hematoxylin and Eosin (HE) and the histopathological changes in lung tissues were observed under a light microscope. Lung injury scores were determined using a semi-quantitative scoring method as previously described by Underwood et al. (1995), in which lung injury was graded from 0 (normal) to 4 (severe) based on inflammatory cell infiltration, thickened bronchial smooth muscle and increased tracheal secretions.

2.11 Measurement of cytokine and neurokinin levels in BALF

Levels of Th2-related cytokine interleukin-13 (IL-13), neurogenic inflammation mediator Substance P (SP), mast cell activation marker prostaglandin D₂ (PGD₂) and nerve growth factor (NGF) in BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer's instructions.

2.12 Real time PCR analysis

Total RNA was extracted from 50mg lung tissues following the Trizol reagent instructions as (Kang et al. 2011) previously described. Following the instructions of PrimeScript RT reagent Kit with gDNA Eraser kit, first-strand cDNA synthesis was performed from 20 µl of reaction mixture including 1µg of total RNA as a primer and reverse transcriptase. 50µl PCR reaction medium was used for the amplification. The primer sequences were TRPA1(NM177781.4):forward:5-ACGGCTACAGCAGGGAGACT-3, reverse:5-ATGTCAGTGGCTCCCTGGGT-3; TRPV1 (NM001001445.2): forward:5-CATTGCTCTCATGGGCGAG-3, reverse:5-AGTCATCCTTGCCCTCCGG-3; GAPDH(BC093508.1):forward:5-GGTGAAGGTCGGTGTGAAC-3, reverse:5-TCGCTCCTGGAAGATGGTG-3. Following the manufacturer instructions, the amplification protocol was performed by starting denaturation at 50°C for 2min then 95°C for 10min, followed by 40 cycles of denaturing at 95°C for 15s and annealing at 60°C for 1min. Data were analyzed by 2^{-ΔΔCT} method

2.13 Western blot analysis

The total protein in mice lung tissue was drawn according to the instructions of total protein extraction kit (Nanjing KeyGEN Biotech Co., Ltd.) and diluted by denaturing lysis buffer. The protein samples were measured and quantified to 50 µg each with Bradford method, then the equivalent samples were boiled for 5min in boiled water and subjected to SDS-PAGE (Nanjing KeyGEN Biotech Co., Ltd.). Proteins were transferred after electrophoresis and then blocked for 2h in 5% skim milk power block buffer and washed 4 times with PBST. Appropriate amount of mice TRPA1, TRPV1, and β-Tubulin antibodies were added to incubate the samples (overnight at 4°C), then the membranes were washed 4 times with PBST. The secondary antibodies were added to incubate the membranes by the shaker for 2h under room temperature, then the membranes were washed another 4 times with PBST. PVDF membranes with developing solution were laid aside under room temperature for 5min, and then exposed with G:BOX chemiXR5(Bio-Rad, USA). The results were analyzed with Gel-Pro32 software.

2.14 Statistical analyses

Data were evaluated using one-way analysis of variance (ANOVA). The T test was applied in the intergroup comparison. P<0.05 was considered as significant. Data were presented as $\bar{x} \pm sd$ (mean $\pm$ standard deviation). Statistical analysis was performed by SPSS22.0 software.

3. Results

3.1 Identified chemical components in SAD

The chemical components in SAD were analyzed by UPLC-QTOF-MS. The herbs in SAD formed a complex matrix. Under the optimized chromatographic and MS condition, the major chemical components were separated and validated. 21 signal peaks of the chemical components were identified according to retention time (tR), mass fragment ions (m/z), and relevant references. Details were shown in Table 1 and Figure. 1.

Table 1. Main components in San'ao decoction

No.	Assigned identity	Molecular formula	Molecular weight	tR(min)
1	Licoricone	C ₂₂ H ₂₂ O ₆	382.1416	0.68
2	[Norpseudoephedrine-CH ₃]-edrine	C ₉ H ₁₂ N	134.097	2.96
3	Norephedrine	C ₉ H ₁₃ NO	151.0997	3.2
4	7-methoxy-Liquiritin	C ₂₃ H ₂₄ O ₁₁	476.1319	3.27
5	Ephedrine	C ₁₀ H ₁₅ NO	165.1154	3.74
6	pseudoephedrine	C ₁₀ H ₁₅ NO	165.1154	3.9
7	Methylephedrine / Methylpseudoephedrine	C ₁₁ H ₁₇ NO	179.131	4.23
8	Epicatechin	C ₁₅ H ₁₄ O ₆	290.079	5.05
9	Amygdalin	C ₂₀ H ₂₇ NO ₁₁	457.1584	5.79
10	Vicenin	C ₂₇ H ₃₀ O ₁₅	594.1585	6.91
11	Schaftoside	C ₂₆ H ₂₈ O ₁₄	564.1479	7.52
12	Isoschaftoside	C ₂₆ H ₂₈ O ₁₄	564.1479	7.83
13	Liquiritigenin	C ₁₅ H ₁₂ O ₄	256.0736	8.95
14	Liquiritin apioside	C ₂₆ H ₃₀ O ₁₃	550.1686	9.00
15	Formononetin	C ₁₆ H ₁₂ O ₄	268.0736	12.17
16	Isoliquirtin	C ₁₅ H ₁₂ O ₄	256.0736	12.33
17	Licorice saponin A3	C ₄₈ H ₇₂ O ₂₁	984.4566	16.09
18	Licorice saponin G2	C ₄₂ H ₆₂ O ₁₇	838.3987	16.91
19	Hydroxyglycyrrhizic acid	C ₄₂ H ₆₂ O ₁₇	838.3987	18.13
20	Licorice saponin H2	C ₄₂ H ₆₂ O ₁₆	822.4038	19.29
21	Licorice saponin K2	C ₄₂ H ₆₂ O ₁₆	822.4038	20.51

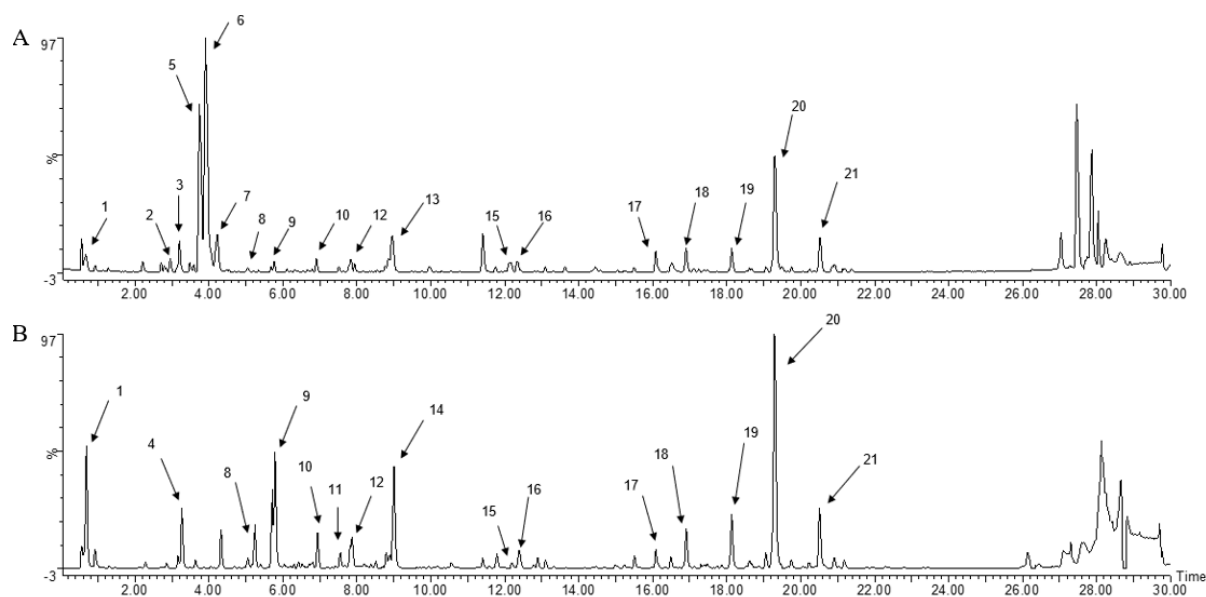


Fig. 1. Elementary particle flow graph chromatogram in positive (A) and negative (B) ion mode for the traditional San'ao decoction analyzed by UPLC-QTOF-MS

3.2 The changes of inflammatory cells in peripheral blood and BALF

The changes of inflammatory cells in both blood and BALF were measured and analyzed from mice of each group.

(1) The amount of EOS from peripheral blood were observed and counted as mixed with EOS count solution. The result showed that compared with normal group, the EOS count in blood increased significantly in model group ($P < 0.01$). Compared with model group, the EOS count decreased in DM group ($P < 0.01$), SAD-H and SAD-L groups ($P < 0.05$) (Figure 2. A).

(2) The inflammatory cells from BALF were fixed by Wright and Giemsa's staining. Then the percentage of EOS and neutrophil from BALF were counted and analyzed. The results showed that model group exhibited much higher EOS percent ($P < 0.01$) and neutrophil percent ($P < 0.05$) than that in normal group. Compared with model group, the EOS percent decreased significantly in SAD-H and DM groups ($P < 0.01$) as well as in SAD-L group ($P < 0.05$) (Figure 2. B). Also the neutrophil percent reduced remarkably in SAD-H and DM groups ($P < 0.05$) as compared to model group (Figure 2. C).

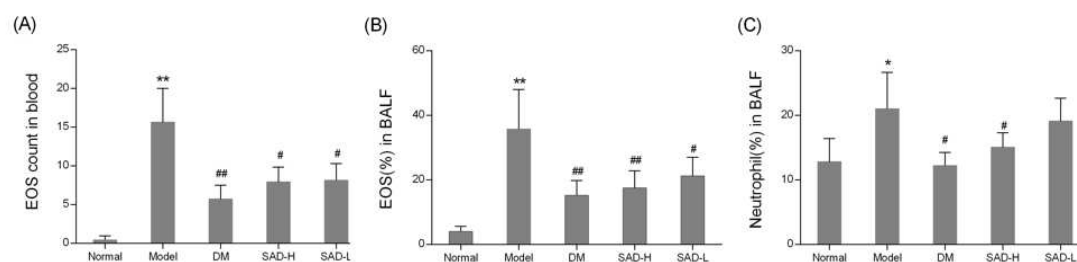


Figure 2. Inflammatory cells changes in both peripheral blood and BALF of each group. (A) the amount of EOS in peripheral blood measured with EOS count solution

(n=10, $\bar{x} \pm sd$); (B) EOS percent in BALF stained by Wright and Giemsa's staining
(n=10, $\bar{x} \pm sd$); (C) Neutrophil percent in BALF stained by Wright and Giemsa's
staining (n=10, $\bar{x} \pm sd$). Notes: *P<0.05 vs normal group, **P<0.01 vs normal group;
#P<0.05 vs model group, ##P<0.01 vs model group.

3.3 Airway responsiveness changes

the airway responsiveness of mice was analyzed when stimulated with the different concentrations of ACH. The results showed that with the increase of ACH concentration, RI value increased while Cdyn value decreased. There were no obvious difference of RI value and Cdyn value as challenged with PBS and 6.25mg/ml of ACH. The statistical differences were detailed as follows, also seen in Figure 3:

(1) 12.5mg/ml of ACH: Compared with normal group, RI value significantly increased (P<0.05) while Cdyn value decreased (P<0.05) in model group. Compared with model group, RI value obviously decreased in DM and SAD-H groups (P<0.05) while Cdyn value remarkably increased in SAD-H and DM groups (P<0.05)

(2) 25mg/ml of ACH: Compared with normal group, RI value largely increased (P<0.01) while Cdyn value decreased (P<0.05) in model group. Compared with model group, RI value significantly decreased in DM and SAD-H groups (P<0.05) while Cdyn value increased in SAD-H and DM groups (P<0.05).

(3) 50mg/ml of ACH: RI value notably increased in model group (P<0.01) as compared to that in normal group, while largely decreased in DM group (P<0.01) and SAD-H group (P<0.05) as compared to that in model group. Besides, Cdyn value in model group obviously decreased as compared to that in normal group (P<0.01), while increased in DM group (P<0.01), SAD-H and SAD-L groups (P<0.05) as compared to that in model group.

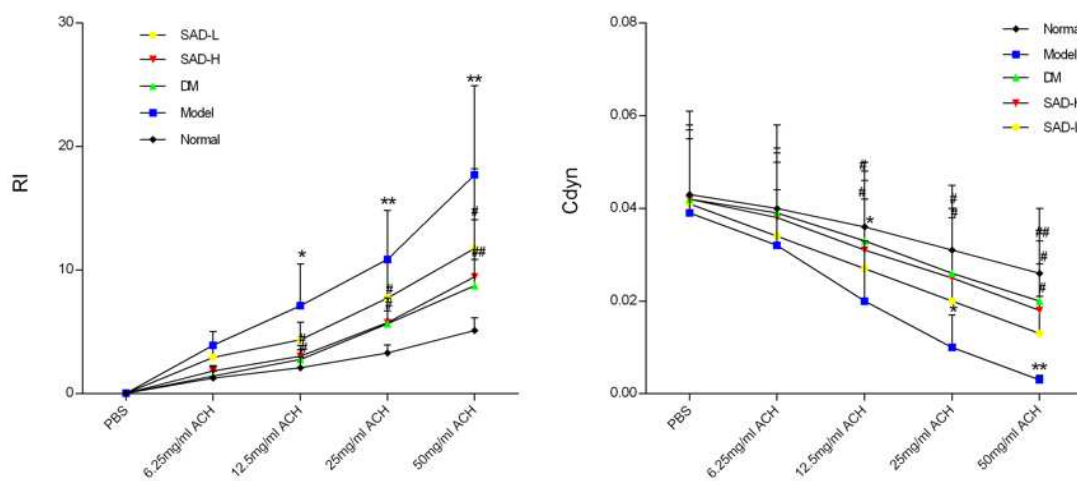


Figure 3. The changes of airway responsiveness of mice in all groups (n=6, $\bar{x} \pm sd$).

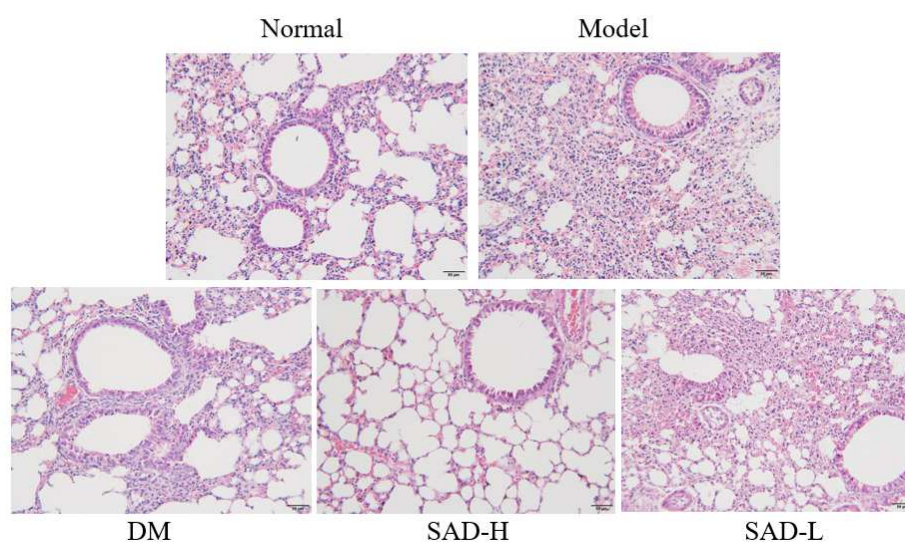
Airway resistance (RI) value and lung compliance (Cdyn) value were measured as

challenged by different concentrations of ACH (0, 6.25, 12.5, 25, 50mg/ml) and analyzed with Buxco FinePointe™ RC system. Notes: * $P < 0.05$ vs normal group, ** $P < 0.01$ vs normal group; # $P < 0.05$ vs model group, ## $P < 0.01$ vs model group.

3.4 Histopathological changes

Through HE staining, it was found that mice of the model groups had significant increase in the thickness of bronchial smooth muscle. There were increasing excretion in lumen. Large amount of inflammatory cells were found in bronchial submucosa, muscular layer and lung interstitium. Also, the average of lung injury score obviously increased ($P < 0.01$) in model group compared with normal group. However, the above symptoms were significantly improved in three medication groups, also lung injury score decreased in SAD-H and DM groups ($P < 0.05$) compared with model group (Figure 4)

(A)



(B)

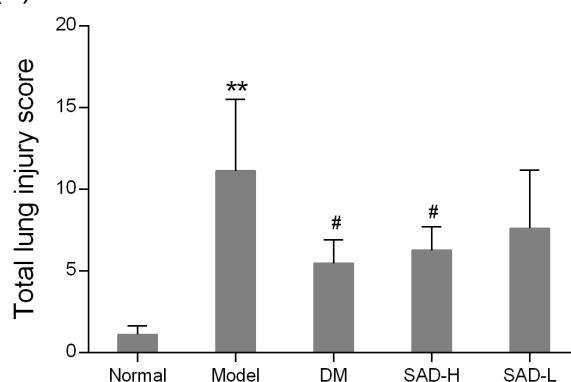


Figure 4. Histopathological changes in lung tissues ($n=8$, $\bar{x} \pm sd$). (A)pulmonary tissue slices stained by H&E of each group (x200); (B)total lung injury scores were graded from normal (0) to severe (4) and analyzed with semi-quantification method.

Notes: * $P < 0.05$ vs normal group, ** $P < 0.01$ vs normal group; # $P < 0.05$ vs model group, ## $P < 0.01$ vs model group.

3.5 IL-13、SP、PGD₂ and NGF levels in BALF

The relevant cytokines and neurokinines in BALF were measured with ELISA kits. The results showed that compared with normal group, levels of IL-13、SP、PGD₂ and NGF in BALF all significantly increased in model group ($P < 0.01$). Compared with model group, (1)IL-13 level obviously decreased in DM and SAD-H groups ($P < 0.01$); (2)PGD₂ and NGF levels remarkably decreased in DM and SAD-L groups ($P < 0.05$), as well as in SAD-H group ($P < 0.01$); (3)SP level largely decreased in DM group ($P < 0.05$), also decreased in SAD treated groups but the difference did not reach significance (Figure 5).

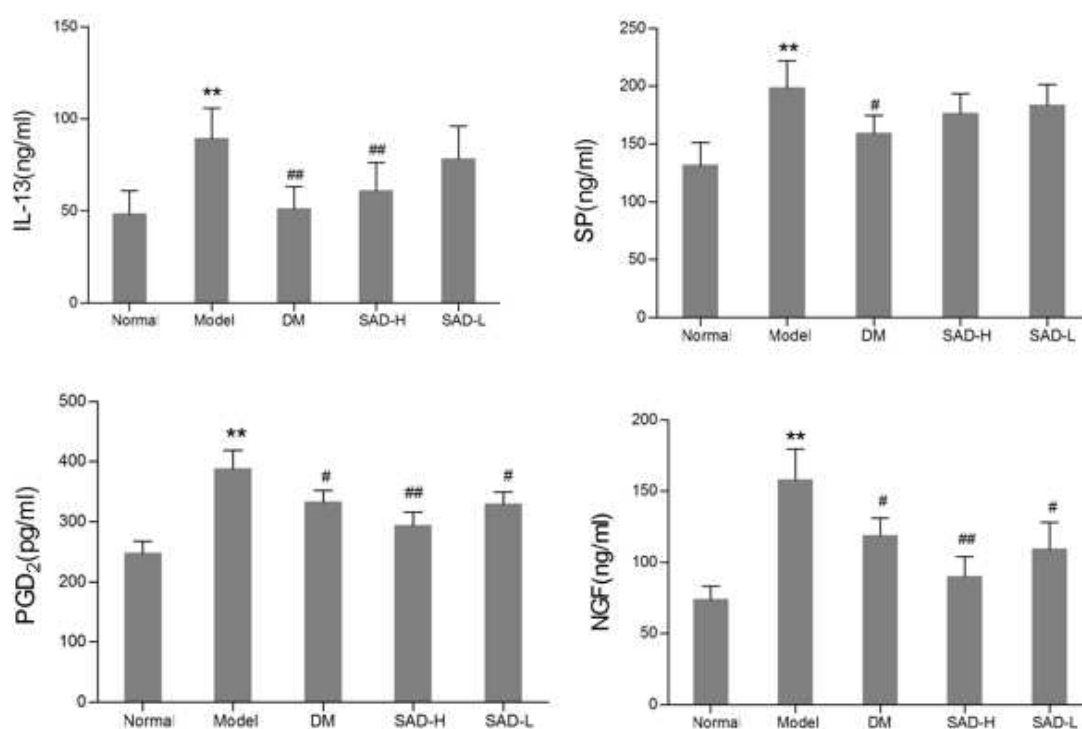


Figure 5. Changes of IL-13, SP, PGD₂ and NGF levels in BALF measured with

ELISA kits (n=10, $\bar{x} \pm sd$). Notes: * $P < 0.05$ vs normal group, ** $P < 0.01$ vs normal group; # $P < 0.05$ vs model group, ## $P < 0.01$ vs model group.

3.6 TRPA1 and TRPV1 mRNA expressions in lung tissues

The gene expressions were measured with RT-PCR analysis and analyzed by $2^{-\Delta\Delta CT}$ method. The results showed that compared with normal group, the mRNA expressions of TRPA1 and TRPV1 significantly increased in model group ($P < 0.05$); compared with model group, the TRPA1 mRNA expression obviously decreased in SAD-H and SAD-L groups ($P < 0.05$), and TRPV1 mRNA expression obviously decreased in DM group ($P < 0.05$) (Figure 6).

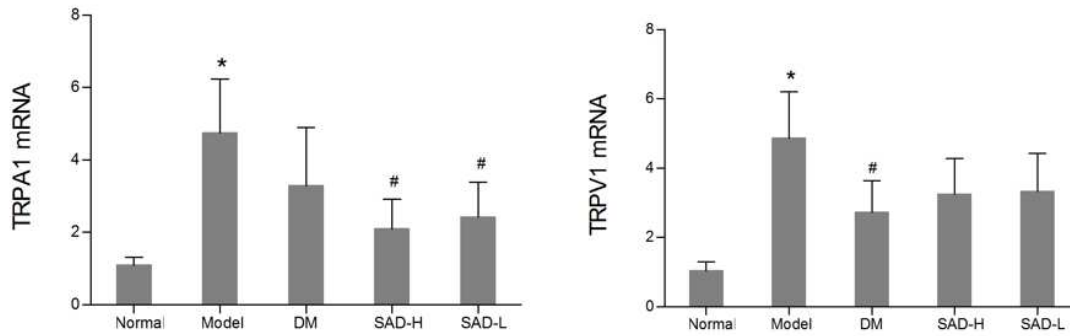


Figure 6. TRPA1 and TRPV1 mRNA expressions in lung tissues of each group measured by RT-PCR analysis and analyzed by $2^{-\Delta\Delta CT}$ method (n=6, $\bar{x} \pm sd$). Notes: *P<0.05 vs normal group, **P<0.01 vs normal group; #P<0.05 vs model group, ##P<0.01 vs model group.

3.7 TRPA1 and TRPV1 protein expressions in lung tissues

Based on the results mentioned above, it was found that SAD high dose had better effect on improving asthma exacerbation and adjusting the levels of cytokines and neurokines. Therefore, this study mainly focused on the effect of SAD high dose on TRPA1 and TRPV1 protein expressions measured with Western blot analysis. The results showed that the protein expressions of TRPA1 and TRPV1 in model group were significantly higher than that in normal group (P<0.01). Compared with model group, TRPA1 protein expressions obviously decreased in DM group (P<0.05) and SAD-H group (P<0.01); TRPV1 protein expressions largely decreased in DM and SAD-H groups (P<0.05) (Figure 7).

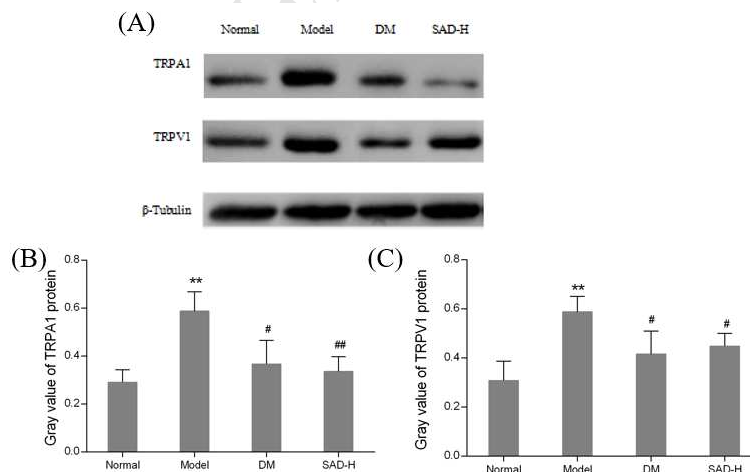


Figure 7. Expressions of TRPA1 and TRPV1 protein in lung tissues. (A) Individual representatives of TRPA1 and TRPV1 protein expressions from each group by Western blot analysis. β -Tubulin was used as the internal reference control. (B) Quantification of the gray value of TRPA1 protein expressions (n=6, $\bar{x} \pm sd$). (C) Quantification of the gray value of TRPV1 protein expressions of all groups (n=6, $\bar{x} \pm$

sd). Notes: Gray values of protein expressions were measured as integrated density divided by the area pixels compared to those in control. * $P < 0.05$ vs normal group, ** $P < 0.01$ vs normal group; # $P < 0.05$ vs model group, ## $P < 0.01$ vs model group.

4. Discussion

In this study, the effect and mechanism of SAD on aggravated asthma mice model induced by OVA plus PM2.5 were explored and evaluated. The results showed that SAD, especially SAD high dose, could improve asthma exacerbation through lowering EOS count in blood and inflammatory cells in BALF, decreasing airway hyperresponsiveness, relieving lung injuries, etc. Also, SAD-H group significantly reduced the gene expressions of TRPA1, the protein expressions of TRPA1 and TRPV1, as well as the levels of relevant cytokines and neurokines.

Airways are tended to be irritated by inhaling the stimulating substances, which may provoke allergic reactions for developing asthma. Studies found PM2.5 could worsen asthma by increasing EOS ratio, aggravating pulmonary inflammation, increasing airway Penh value, as well as increasing the levels of IL-4, IL-5, IL-10 and TNF- α while decreasing INF- γ level in BALF (Wagner et al. 2012; Zhang et al. 2015). Asthma initiated by PM2.5 is mainly through immunoreaction and inflammatory reaction. Some researches (Jiang et al. 2014; Ogino et al. 2014; Dobrev et al. 2015) found out PM2.5 could aggravate the imbalance of Th1/Th2 and Th17/Treg pathways through inflammation in airway and lung. Therefore, in this study OVA plus PM2.5 was used to establish the aggravated asthmatic model in mice, showing increased inflammatory cells, aggravated lung pathological injury, and severe airway hyperresponsiveness, etc.

The roles of some cytokines and neurokines have been recognized in the process of asthma. IL-13, secreted by Th2 cells, is the mediator of airway susceptibility and contributed to asthma aggravation. It was reported that IL-13 was produced exaggeratedly in asthmatic lung tissues when affected by allergen or inflammation (Gour and Wills, 2015). Relevant clinical researches also investigated asthmatic exacerbations in patients with type 2 inflammation were reduced by the IL-3-directed therapies (Fahy, 2015). Substance P, a kind of neuropeptide, is mainly involved in a variety of biological processes such as neurogenic inflammation and nociceptive sensation (Xanthos and Sandkuhler, 2014). SP and its receptor NK1 were highly expressed on goblet cells in asthmatic airways, stimulating mucus secretion in a positive correlation (Khansaheb et al. 2011; Atanasova and Reznikov, 2018). Besides, the Th1/Th2 balance could be regulated by SP with its ability to bias the inflammatory response to Th17 immunity (Cunin et al. 2011). Another neurotrophic factor NGF was studied to reinforce the TRPV1 channel transporting to plasma membrane in dorsal root ganglion, resulting in the increased Ca^{2+} responses stimulated by bronchoconstrictor (Abcejo et al. 2012). In this study, increased levels of IL-13, SP, NGF and PGD_2 in aggravated asthma model also confirmed the above findings that relevant cytokines and neurokines were related to the inflammation and immunity regulation during asthma occurrence. And the administration with SAD significantly reduced the IL-13, NGF and PGD_2 levels in BALF. Although SP level was also

reduced by SAD, a larger sample size might be needed for the differences to reach the statistical significance.

In TRPs family, TRPA1 and TRPV1 were considered to be closely related to the mechanism of asthma. TRPA1/TRPV1 channel could up-regulate Th2 type cytokines, such as IL-13 to cause the immunomodulatory imbalance, the increase of immunoglobulin synthesis and the inflammatory cells gathering around airway (Wu et al. 2013). TRPA1 channel could be activated by low temperature (less than 17°C) and cause pain, injury, neuroinflammation, etc., while TRPV1 channel was mainly stimulated by heat (over 43°C), causing calcium influx and tachykinin release, which led to airway neurogenic inflammation (Gouin et al. 2017; Boillat et al. 2014). It was considered that some factors relevant to TRPA1/TRPV1 channel, such as SP, PGD₂, NGF, played significant roles in airway inflammation (Mickle et al. 2016; Skaper, 2017; Shibata et al. 2016). In this study, the high gene and protein expressions of TRPA1 and TRPV1 in PM2.5 aggravated asthmatic mice model indicated that TRP channels were activated and caused lung function damage during asthma occurrence, which was relieved by treated with SAD.

In traditional Chinese medicine, SAD is well known in treating exogenous cough and asthma. As an exogenous pathogenic factor, PM2.5 plus OVA may lead to lung dysfunction and obstruction. In this study, the 42 days aggravated asthmatic mice model induced by OVA plus PM2.5 was successfully established. Also the effect and mechanism of SAD were evaluated and explored. Yet there are still some questions need further investigation. The mechanism of SAD in improving OVA plus PM2.5 induced aggravated asthma model through mediating TRP channel need further confirmation. Besides, the effects of the three single herbs in SAD need to be discovered and studied as well.

We need to point out that this research investigated the effect and mechanism of SAD through TRP channel based on our earlier studies. However, it should be noted that asthma is a complex disease related to multiple pathogenesis. Besides, The anti-asthmatic action of SAD in vitro need to be examined further as well so that our conclusions could be supported sufficiently.

5. Conclusions

This study observed and evaluated the effect and mechanism of SAD on aggravated asthma mice model induced by OVA plus PM2.5. In this study, SAD, especially SAD high dose, was proved to improve asthma exacerbation by reducing inflammatory cell infiltration, decreasing airway hyperresponsiveness and relieving pathological injury in lung tissues. The effect and mechanism of SAD might be associated with the regulation of Th2 related cytokine IL-13 and relevant neurokines through inhibiting TRPA1 and TRPV1 channels.

Conflict of interest

The authors declared no conflict of interest.

Authors' contributions

Xinsheng Fan (fanxsh126@126.com) designed and conceived the experiments Pengli Wang (wangpengli2012@163.com) and Hong Liu (751445206@qq.com) collected and analyzed the data. Hong Liu, Pengli Wang and Zhenhua Zhu (2296880372@qq.com) performed the experiments. Zhenhua Zhu and Yue Zhu (244370559@qq.com) contributed the analysis tools and techniques. Pengli Wang wrote the original manuscript. All authors have read and approved the final manuscript.

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Figure legends

Figure 1. Elementary particle flow graph chromatogram in positive (A) and negative (B) ion mode for the traditional San'ao decoction analyzed by UPLC-QTOF-MS

Figure 2. Inflammatory cells changes in both peripheral blood and BALF of each group. (A) the amount of EOS in peripheral blood measured with EOS count solution (n=10, $\bar{x} \pm sd$); (B) EOS percent in BALF stained by Wright and Giemsa's staining (n=10, $\bar{x} \pm sd$); (C) Neutrophil percent in BALF stained by Wright and Giemsa's staining (n=10, $\bar{x} \pm sd$). Notes: *P<0.05 vs normal group, **P<0.01 vs normal group; #P<0.05 vs model group, ##P<0.01 vs model group.

Figure 3. The changes of airway responsiveness of mice in all groups (n=6, $\bar{x} \pm sd$). Airway resistance (RI) value and lung compliance (Cdyn) value were measured as challenged by different concentrations of ACH (0, 6.25, 12.5, 25, 50mg/ml) and analyzed with Buxco FinePointe™ RC system. Notes: *P<0.05 vs normal group, **P<0.01 vs normal group; #P<0.05 vs model group, ##P<0.01 vs model group.

Figure 4. Histopathological changes in lung tissues (n=8, $\bar{x} \pm sd$). (A) pulmonary tissue slices stained by H&E of each group (x200); (B) total lung injury scores were graded from normal (0) to severe (4) and analyzed with semi-quantification method. Notes: *P<0.05 vs normal group, **P<0.01 vs normal group; #P<0.05 vs model group, ##P<0.01 vs model group.

Figure 5. Changes of IL-13, SP, PGD₂ and NGF levels in BALF measured with ELISA kits (n=10, $\bar{x} \pm sd$). Notes: *P<0.05 vs normal group, **P<0.01 vs normal group; #P<0.05 vs model group, ##P<0.01 vs model group.

Figure 6. TRPA1 and TRPV1 mRNA expressions in lung tissues of each group measured by RT-PCR analysis and analyzed by 2^{-ΔΔCT} method (n=6, $\bar{x} \pm sd$). Notes: *P<0.05 vs normal group, **P<0.01 vs normal group; #P<0.05 vs model group, ##P<0.01 vs model group.

Figure 7. Expressions of TRPA1 and TRPV1 protein in lung tissues. (A) Individual representatives of TRPA1 and TRPV1 protein expressions from each group by Western blot analysis. β-Tubulin was considered as the internal reference control. (B) Quantification of the gray value of TRPA1 protein expressions (n=6, $\bar{x} \pm sd$). (C) Quantification of the gray value of TRPV1 protein expressions of all groups (n=6, $\bar{x} \pm sd$). Notes: Gray values of protein expressions were measured as integrated density divided by the area pixels compared to those in control. *P<0.05 vs normal group, **P<0.01 vs normal group; #P<0.05 vs model group, ##P<0.01 vs model group.