Gene expression profiling to explore the protective effect of *Antrodia cinnamomea* compound decoction on PM2.5-induced lung inflammation in rats

Guishu Wang

Beijing University of Chinese Medicine

Weicheng Chen

kingland property Co. Ltd.

Yufeng Meng

Peking University People's Hospital

Caijuan Zhang

Beijing University of Chinese Medicine

Jiakun Zheng

Beijing University of Chinese Medicine

Yaqian Liu

beijing university of chinese medicine

Changle Zhu

beijing university of chinese medicine

Liangtian Shi

Beijing University of Chinese Medicine

Yongjie Wei

Chinese Research Academy of Environmental Sciences

Jenhua Kuo

E-DA hospital

Cuiling Feng (✉ fengcuiling@sina.com)

peking university people's hospital <https://orcid.org/0000-0001-5559-8431>

Research

Keywords: Antrodia cinnamomea, traditional Chinese medicine, PM2.5-induced lung inflammation, genomics

Posted Date: October 27th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-96135/v1>

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Abstract

Background

Antrodia cinnamomea compound decoction (ACCD), a Chinese herbal extract composed of *Antrodia cinnamomea*, *Rhinacanthus nasutus* (L.) Kurz and *Phellinus igniarius* (L. ex Fr.), were widely used with anti-inflammatory activity. However, the effects and mechanisms of ACCD on the lung inflammation induced by PM2.5 are not fully understood. This study aims to reveal the effect of ACCD on PM2.5-induced lung inflammation and analyze the possible mechanisms through gene chip expression profiling.

Methods

Male Wistar rats were subjected to lung damage by stimulation with 10% PM2.5, with/without ACCD (0.67g/kg/d) intervention. The HE, AB-PAS staining and IL-1 β were performed to evaluate the effect of ACCD in the treatment of lung inflammation caused by PM2.5. In addition, the extracted lung genes were detected based on the gene chip expression profile. Bioinformatics analysis and quantitative real-time polymerase chain reaction assay (Q-PCR) were used to analysis and verify the related differentially expressed genes (DEGs).

Results

Results showed that instillation of PM2.5 increased the inflammatory cells immersion in the airways and the acid mucus content in the tracheal epithelium compared with the sham group, and it increased the IL-1 β levels in Bronchoalveolar lavage fluid (BALF). ACCD reduced inflammatory cell immersion in airways, alleviated goblet cell metaplasia in tracheal epithelium and reduced IL-1 β levels in BALF. A total of 99 genes with significant differential expression were identified ($p < 0.05$) after ACCD treatment compared with the model group, including 48 up-regulated and 51 down-regulated genes. Bioinformatics analysis showed that these DEGs were mainly related to inflammatory response and significantly enriched in cancer signaling pathway. Five of the DEGs were subjected to Q-PCR and three of them (Plc β -1, Axin2, Ccbe1) were significant difference ($p < 0.05$).

Conclusion

Our study suggests that ACCD may exert anti-inflammatory effects on PM2.5-induced lung inflammation via the cancer pathway, and that Plc β -1, Axin2, and Ccbe1 may be key targets of ACCD action on PM2.5-induced lung inflammation. This work could provide genomic clues for the continued study of ACCD for the therapy of PM2.5-induced lung inflammation.

Background

Morbidity and mortality from air pollution remains a growing problem worldwide and the leading environmental cause of premature death [1, 2]. Fine particulate matter with an aerodynamic diameter less than 2.5 μ m (PM2.5) is a common air pollutant associated with a number of adverse health

consequences [3, 4]. The chemical profile of PM_{2.5} is known to have an impact on a variety of respiratory diseases such as bronchus inflammation, asthma, pulmonary fibrosis, chronic obstructive pulmonary disease (COPD), and even lung cancer [5–7]. A study in China showed that in 2015 PM_{2.5} contributed up to 33.1% of deaths from acute lower respiratory tract infections (< 5 years), 23.9% of lung cancer deaths, 18.7% of COPD deaths, and 30.2 of total deaths from combined stroke, COPD, and lung cancer [8]. The prior studies reported that PM_{2.5}-exposure can trigger lung inflammatory damage with increased inflammatory cell infiltration and inflammatory cell numbers, as well as increased levels of inflammatory cytokines [9].

Antrodia cinnamomea compound decoction (ACCD) is a traditional Chinese medicine composed of *Antrodia cinnamomea* (AC), *Rhinacanthus nasutus* (L.) Kurz (RN) and *Phellinus igniarius* (L. ex Fr.) (PI) in a ratio of AC: RN: PI = 6: 2: 2. Numerous useful biological properties compounds of AC were found, including triterpenoids, maleic/succinic acid derivatives, polysaccharide, benzenoids, and benzoquinone derivatives [10, 11]. Rhinacanthone, rhinacanthins C, D, N, Q, and E, and Polysaccharides are the main components of RN and PI [12, 13]. AC is a unique fungus that grows only on the brown heartwood of *Cinnamomum kanehirae* Hayata (Lauraceae) in Taiwan [14]. Previous experiments have proved its potential to reduce inflammation [15–17]. However, the ability of oral ACCD treatment to mitigate PM_{2.5}-induced lung damage in vivo and the evidence for mechanisms associated with ACCD protection of the lung are unclear.

Many studies[18, 19] have shown that many genes change after lung injury caused by PM_{2.5}, which were closely related to complex pathophysiological processes. Genomics has become a common method for the study of respiratory diseases [19]. Comparing differences in gene expression to reveal biological phenomena or molecular mechanisms of disease occurrence is a common strategy for high-throughput studies [20]. Agilent expression profiling microarrays have been widely used in basic research, disease diagnosis and drug development in biology and medicine, etc [21]. With a probe length of 60 mer, Agilent expression profiling microarrays can strike a good balance between sensitivity and specificity, and due to their long probe design, they can capture the low abundance of genes that are more important for life activities [22]. Therefore, genomics can be used to investigate the mechanism of action of ACCD intervention in PM_{2.5}-induced lung inflammation. However, there is no genomics to explore the mechanism of ACCD treatment of PM_{2.5}-induced lung inflammation.

In this study, we focused on the genomic changes of ACCD on PM_{2.5}-induced lung protection in rats. Based on PM_{2.5}-induced lung inflammation and ACCD-treated rat in vivo models, a series of tests were performed, including HE and AB-PAS staining, inflammatory factor analysis, gene expression profiling display, functional pathway analysis and quantitative real-time polymerase chain reaction assay (Q-PCR) analysis. We studied the therapeutic role of ACCD for PM_{2.5}-induced lung inflammation through a genomic approach to reveal the future potential of ACCD in the treatment of PM_{2.5}-induced lung inflammatory.

Methods

ACCD preparation

ACCD was obtained from Kingland Property Co., Ltd., Taiwan, China (production batch number: ACX Jing 1707-1). The herbal extract was manufactured by providing a mixture consisting of 60 wt% of *Anrodia cinnamomea*, 20 wt% of *Rhinacanthus nasutus* and 20 wt% of *Phellinus igniarius*, extracting the mixture by 95% ethanol in 50 °C for 8 hours with a weight-volumetric ratio of 1: 2, followed by concentrating to obtain the herbal extract. At least half of the ethanol was removed by concentration under reduced pressure by No. 3 filter paper vacuum filtration, and freeze-dried at -60 °C (Taiwan patent No. TWI555529B).

Component determination of ACCD using liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The herbal extract was put in a test tube with 5 ml of methanol, sonicated for 15 minutes (3000 rpm, 10 minutes) and heated to dryness in a 100°C water bath without the supernatant. The chromatographic analysis was performed on a Purospher STAR (Merk) RP-18e (5 µm) 250mm × 4 mm column. Acetonitrile and 0.085% phosphoric acid solution were mixed at a flow rate of 1 mL/min with a volume of 47:53 as the mobile phase.

PM2.5 preparation

PM2.5 quartz filters were given by Chinese academy of environmental sciences as a friendship gift. The filter membrane was cut into a size of 1 × 1 cm and placed in a centrifuge tube, then poured into deionized water. Afterwards, the centrifugal tube was placed in an ultrasonic cleaning machine and vibrated (10 min, 3 times). The liquid was filtered with eight-fold gauze, frozen and placed in a freeze-dryer. Deionized water was added to the frozen powder to prepare PM2.5 suspension at a concentration of 650 µg/ml, giving 0.1 ml/rat every 3 days.

Animal experimental design

Specific pathogen free (SPF) male Wistar rats (150 ± 10 g) were obtained from Beijing Vital River Laboratory Animal Co., Ltd (Beijing, China). Rats were placed in polypropylene cages in an air-conditioned animal center with unrestricted access to food and water (temperature of 24 ± 2 °C, humidity of 40%±5%). Experiments began after a week period of acclimatization. The rats were randomly divided into three groups: (a) Sham group (Sham): rat from sham group served as the control throughout the experiment; (b) PM2.5 group (PM2.5): rats underwent the PM2.5 procedure; (c) ACCD group (ACCD): PM2.5 rats received ACCD (0.67 g/kg/d) orally.

The PM2.5 suspension was injected into the rat airway through a trocar needle 0.1 ml/rat every 3 days. Then the rat was to the left and right uprightly for 20 seconds to make sure the PM2.5 suspension even distribution in the lungs. This operation was done every three days. The saline (10 ml/kg/d) was given to the Sham group and the PM2.5 group from the 1st day. Meanwhile, ACCD group rats would take ACCD

(10 ml/kg/d) with the concentration of 0.67 g/ml every day. On the 28th day in experiment, pentobarbital was administered intraperitoneally and all rats were sacrificed.

HE and AB-PAS staining

Ligation of the right external lung bronchus and resection of the right lung lobe were performed immediately. The right lung lobe was fixed in 4% paraformaldehyde buffer solution for 24 h, sliced to a thickness of 4 μ m after paraffin embedding and stained with haematoxylin-eosin (HE) to assess the degree of inflammatory cell infiltration and changes in bronchoalveolar structure. Alcian blue periodic acid-Schiff (AB-PAS) staining were performed to identify the degree of proliferation of goblet cells in the airway epithelial mucous metaplasia. This procedure was performed according to the instructions of the AB-PAS staining kit (Baso Biological Technology Co. Zhuhai, China).

Measurement of inflammatory cytokines in BALF

Bronchoalveolar lavage fluid (BALF) samples were acquired. The trachea was cut open, and the lungs were lavaged three times with 5 ml PBS, and the collected samples were centrifuged (4000 rpm, 10 min) and stored at -80 °C. The IL-1 β content of the supernatant was detected using a specific ELISA kit (MULTISCIENCES (LIANKE) BIOTECH, CO., LTD) according to the manufacturer's instructions. All tests were performed in duplicate.

RNA microarray detection

We extracted total RNA from the lung tissue of the three groups mentioned above through the mirVana^{TM} RNA separation kit according to the manufacturer's specifications. Agilent's low-input rapid-amplifying marker kits produce fluorescent complementary RNA and sample input total RNA for monochrome processing. In the experiment, nine samples (3 per group) were analyzed using the Agilent SurePrint G3 rat GE V2.0 microarray (8 $\times$ 60K, design ID: 074036). After generating a microarray scan image, the image is extracted using feature extraction software. NanoDrop ND-2000 (Thermo Scientific) was used to quantify total RNA and assess RNA integrity. Total RNA was transcribed into double-stranded cDNA, followed by synthesis of cRNA and labeling with cyan-3-CTP. The labelled cRNAs were hybridised to the microarrays. After cleaning, the arrays were scanned with an Agilent scanner G2505C. Basic analysis of the raw data was completed using Genespring (version 13.1, Agilent Technologies). First, the raw data were normalized by a quantitative algorithm. Under any one condition, at least 100% of the values had a "detected" flag for the probe selected for further data analysis.

Identification of differentially expressed genes

Differentially expressed genes (DEGs) of different groups were analyzed according to the expression levels of mRNAs, and the data were extracted by the Feature Extraction software. The raw data files were standardized by Genespring for subsequent data analysis. A fold change (FC) of gene expression ≥ 2 and p-value < 0.05 were analyzed. To understand more about the DEGs and pathways that are responsive to ACCD treatment in PM_{2.5} induced-lung inflammation, we performed GO enrichment and KEGG

enrichment analysis of DEGs using the DAVID bioinformatics resources ([https://david-ncifcrf.gov/home.jsp](https://david.ncifcrf.gov/home.jsp), version 6.7).

Quantitative real-time polymerase chain reaction (Q-PCR)

Quantitative real-time polymerase chain reaction (Q-PCR) were validated of the relevant DEGs. Total RNA was isolated and purified as described above. PCR reaction settings: pre-incubation (95 °C, 30 s); amplification (95 °C, 10 s; 60 °C, 30 s; 40 cycles); melting curve (Slowly warm up from 60 °C to 97 °C and collect 5 fluorescent signals per °C). This was performed triplicate independently to analyze. After the PCR cycle was completed, melting curve analysis was performed to verify the generation of the expected PCR product specifications. Primer sequences were designed in the laboratory and synthesized based on mRNA sequences obtained from the NCBI database, as shown in Table 1.

Table 1
primer sequence used for Q-PCR.

Gene Symbol	Forward primer (5'→3')	Reverse primer (3'→5')
ACTB	GCGAGTACAACCTTCTTGC	TATCGTCATCCATGGCGAAC
Il6r	TCGAGCTTCGATACCGAC	CATGGATGACACATTGATGC
Ppbp	CTGGAATCCCACTGAACTC	GGCTATCACTTCCACATTGTC
Plcβ1	GTACCTCTTCCAAGCCCTAT	TTACCACTTCCCTCTGACG
Axin2	CGCCACCAAGACCTACATA	TCCATCACCGCCTGAATC
Ccbe1	GTGGACGAGTGAAACGAC	GCCCTTCCAAACGTGACATA
The 2 ^{-ΔΔCT} method (Livak and Schmittgen 2001) was used to calculate expression levels of mRNAs and ACTB to normalized.		

Statistical analysis

We performed independent t-test or one-way analysis of variance (ANOVA) by SPSS 25.0 software. When p-value < 0.05, the differences were considered significant.

Results

LC-MS/MS analysis of ACCD components

The triterpenoids of the bovine camphoraceae have six index components, namely, antcin K, antcin C, zhankuic acid C, dehydrosulphurenic acid, zhankuic acid, dehydroeburicoic acid, and the results of each group are shown in Fig. 2.

Effects of ACCD on airway inflammation and mucus secretion in PM2.5 induced-lung inflammation mice

Histopathological examination by HE staining showed that after PM2.5 inhalation, airway cilia were inverted, alveolar walls were thickened, inflammatory cells were infiltrated, and erythrocytes were dispersed in the alveolar region. But these conditions improved after ACCD treatment. ACCD significantly reduces inflammatory cell immersion in PM2.5-induced lung inflammation rats, as shown in Fig. 3A.

AB-PAS positive staining was sporadic in the airway epithelium of rats in the Sham or ACCD groups, whereas the airway epithelium of the PM2.5 group contained many large particles of positive material in storage. As shown in Fig. 3B, AB-PAS stained lung sections from the PM2.5 group showed overproduction of mucus compared with the Sham groups. In the ACCD group, much lighter positive staining areas were observed.

Effect of ACCD on Levels of IL-1 β in BALF

To investigate the role of ACCD in reducing PM2.5-induced airway inflammation, we tested the levels of IL-1 β in BALF. The results showed that IL-1 β levels were obviously increased in the PM2.5 group compared with the Sham group ($P < 0.01$). The ACCD-treated group showed a significant decrease in IL-1 β ($P < 0.05$). It seemed that ACCD could inhibit IL-1 β triggered by PM2.5.

Gene expression profiling analysis

A total of 99 genes were found to be markedly differentially expressed after ACCD treatment, including 48 up-regulated genes and 51 down-regulated genes. In addition, to show the significant difference between the two groups of probes, volcanic plots (Fig. 4) and heatmaps to distinguish between the expression patterns of the were plotted based on the P-value and FC values obtained from the t-test analysis.

GO enrichment and functional pathway analysis

Count the number of differential genes contained in each GO enrichment and calculate the significance of the differential gene enrichment using statistical tests. The calculated result returns a p-value for enrichment significance, with a small p-value indicating that the differential gene is enriched in this GO entry. As shown in Fig. 4A, we found significantly enriched BP including response to lipopolysaccharide (GO:0032496), inflammatory response (GO:0006954), negative regulation of cell proliferation (GO:0008285), positive regulation of protein processing (GO:0010954).

The KEGG pathway analysis showed that the significantly differentially expressed DEGs ($p < 0.05$) were mainly enriched in the pathways such as Amoebiasis(rno05146), Pathways in cancer (rno05200), Wnt signaling pathway(rno04310) and PI3K-Akt signaling pathway(rno04151), as shown in Fig. 4B.

Table 2
DEGs regulated by ACCD

Term	Count	P-Value	Genes
GO-BPs			
GO:0032496-response to lipopolysaccharide	6	3.31E-03	CXCL11/ORM1/ACOD1/PPBP/CD14/IL6R
GO:0032957-inositol trisphosphate metabolic process	2	1.78E-02	ITPKB/PLCB-1
GO:0006954-inflammatory response	5	2.20E-02	CXCL11/CYP26B1/PPBP/CD14/FPR-RS3
GO:0007612-learning	3	2.35E-02	NRXN3/CNTN2/PLCB-1
GO:0010954: positive regulation of protein processing	2	3.88E-02	CCBE1/CNTN2/
GO:0008285: negative regulation of cell proliferation	5	4.54E-02	DUXBL1/SCIN/ZBTB16/RARRES1/AXIN2
KEGG pathways			
has05146: Amoebiasis	4	4.77E-03	COL4A3/CD14/PLCB-1/LOC102554659
has05200: Pathways in cancer	6	6.45E-03	WNT10A/ZBTB16/COL4A3/AXIN2/PLCB-1/LOC102554659
has04310: Wnt signaling pathway	3	6.99E-02	WNT10A/AXIN2/PLCB-1
has04151: PI3K/Akt signaling pathway	4	8.52E-02	IRS1/COL4A3/IL6R/LOC102554659

Q-PCR analysis

Our results identified key genes contributing to model group via involvement in response to inflammatory response. The downregulation of genes including Ppbp1, Axin2, Ccbe1 was detected (Fig. 6). This is consistent with the microarray chip data and further experiments targeting these genes are required.

Discussion

There are two major new findings in this study. First, we demonstrated that ACCD could effectively attenuate PM2.5-induced lung inflammation in rats, including a reduction in inflammatory infiltration and

cupocytosis of the airways and a decrease in the inflammatory factor (IL-1 β) in BALF. Second, by gene microarray technology, we found that the protective effect of ACCD on rats exposed to PM2.5 was closely related to the inflammatory response. Five genes related to inflammation were selected for validation among which three genes, Plc β -1, Axin2 and Ccbe1, significantly changed between PM2.5 and ACCD treated group. Our results suggest that ACCD can play a protective role against PM2.5-induced lung inflammation, and Plc β -1, Axin2 and Ccbe1 may be its key targets of action.

PM2.5 exposed is always associated to excessive production of pro-inflammatory cytokines and a pronounced inflammatory responses in the lungs [23]. Our findings are generally concordant with a previous report that characterized the airway inflammatory reaction associated with PM2.5 [24]. Significant inflammatory variations in lung tissue and BALF can be observed rapidly after PM2.5 treatment. IL-1 β is considered to be a key pro-inflammatory cytokine that plays an important role in coordinating local and systemic inflammation of infection and inflammation [28, 29]. Several in vitro studies have found that PM2.5 can induce IL-1 β secretion from macrophages through multiple pathways [27]. We examined the expression of IL-1 β in inflammatory locations. Consistent with previous studies, PM2.5 significantly increased the production of IL-1 β in BALF [28].

99 DEGs were identified between PM2.5 group and ACCD group. GO enrichment of DEGs in present study showed that the top 3 terms with the highest number of DEGs in BP were related to lipopolysaccharide, inflammation and inhibition of cell proliferation. Both animal and human research have described that exposure to PM2.5 may cause a respiratory inflammation. However, the inflammatory response is a diversified, comprehensive and complex process. It is associated not merely with genetic members of the inflammatory process, but also with a great number of genes belonging to an atypical inflammatory language. Lipopolysaccharide response and inhibition of cell proliferation belongs to the most basic inflammatory processes, involving multiple aspects such as airway inflammation and goblet cell metaplasia in respiratory diseases. Meanwhile, the lung-protection of ACCD against PM2.5 induced lung inflammation was mainly involved in cancer signaling pathway. Research has shown that there was a significant positive correlation between PM2.5 concentration and cancer mortality [29]. Inflammation, particularly chronic inflammation, is strongly associated with the development of cancer [30]. ACCD may provide the prevention and treatment of inflammation through cancers-associated pathways in PM2.5-induced lung inflammation.

In order to study the potential role of these dysregulated genes in the treatment of PM2.5-induced lung inflammation, we then examined the mRNA (IL6r, Ppbb, Plc β -1, Axin2, Ccbe1) expression levels through Q-PCR and validated that Plc β -1, Axin2, and Ccbe1 were the highly downregulated genes treated with ACCD. The results are consistent with the microarray data, further confirming the key dysregulation genes play roles in ACCD to reduce PM2.5-induced lung damage through anti-inflammatory. Phospholipase C- β (PLC- β) is a key player in a signaling cascade that links G protein coupled receptors (GPCR) to an intracellular signaling network [31]. The PLC- β class of phospholipases is composed of four isozymes (β 1– β 4) encoded by different genes [32]. PLC- β 1 is highly expressed in neuronal tissue and may play an important role in neuroendocrine growth factor-stimulated cell proliferation [37, 38]. PLC- β blockade of signaling

may be a valuable method to inhibit cell growth and metastasis. The signal transduction role of PLC- β constitutes a possible target for pharmacological interventions to treat disease. In our research, PLC- β 1 was upregulated in the model group, which is consistent with previous literature reports.

Axis inhibition protein 2 (AXIN2) is a downstream target of the Wnt signaling pathway, which plays an important role in the regulation of β -catenin stability and its involvement in various cellular functions [39, 40]. Previous studies suggest that AXIN2, one of the downstream targets of T cytokines/lymphopoietic enhancers of the Wnt signaling pathway, may have oncogenic effects [37]. AXIN2 has been shown to be upregulated in various types of cancer [38]. The present study demonstrated that AXIN2 were significantly upregulated after PM2.5-inducing and downregulated by the ACCD treatment. This has not been reported in previous studies on PM2.5 causing lung damage.

Collagen and calcium-binding epidermal growth factor domain calcium-containing protein 1 (CCBE1) can promotes proteolysis of vascular endothelial growth factor (VEGF) [39]. In this study, we reveal CCBE1 overexpressed in PM2.5 induced lung tissue. Nevertheless, it is a future question to explore how CCBE1 transcription is activated and which of the transcription factors augment the transcription of CCBE1 in PM2.5.

Herbs have long been used to prevent or treat diseases and are indeed valuable chemical compounds that have developed into indispensable medicines in medical practice [40]. The extraction of natural compounds from Chinese herbal medicine is increasingly accepted and used in clinical research as a novel drug discovery method. TCM is traditionally used to treat diseases with the characteristics of multi-targeting signaling pathways and even meditate the multi-organ collaborative work in whole body system [41].

Conclusion

Our study provides the protective effect of ACCD treatment on PM2.5-induced lung inflammation and a comprehensive bioinformatics analysis associated with DEGs. In addition, *Plcb1*, *Axin2* and *Ccbe1* were validated by Q-PCR. We focused on the involvement of these genes in the pathways and their roles in pathological process. Considering the complex mechanism of lung inflammation after PM2.5 intervention, more molecular tests or clinical trials are needed to support the gene expression profiling data of our rat model.

Abbreviations

AB-PAS

Alcian blue periodic acid-Schiff

AC

Antrodia cinnamomea

ACCD

Antrodia cinnamomea compound decoction
AXIN2
Axis inhibition protein 2
BALF
Bronchoalveolar lavage fluid
CCBE1
Collagen and calcium-binding epidermal growth factor domain calcium-containing protein 1
COPD
chronic obstructive pulmonary disease
DEGs
differentially expressed genes
HE
haematoxylin-eosin
PI
Phellinus igniarius (L. ex Fr.)
PLC-β
Phospholipase C-β
Q-PCR
quantitative real-time polymerase chain reaction assay
RN
Rhinacanthus nasutus (L.) Kurz
VEGF
vascular endothelial growth factor

Declarations

Acknowledgements

This work was funded by Corporate and Society-sponsored projects (2017-Z-14) and National Natural Science Foundation of China (No. 81673921).

Authors' Contributions

Cuiling Feng supervised the whole research and helped to coordinate funding. Yufeng Meng designed the research. Guishu Wang and Lingtian Shi conducted animal model establishment. Guishu Wang, Caijuan Zhang and Jiakun Zheng carried out data analysis. Yongjie Wei and Weicheng Chen provided resources of PM2.5 Teflon filter membrane and herbal and information on preparation. Yaqian Liu, Jenhua Kuo and Changle Zhu helped sample collection. Guishu Wang carried out data visualization and manuscript writing. We are very grateful for the detailed information on the preparation of ACCD offered by Weicheng Chen and Kingland Property Co., Ltd.

Funding

This work was funded by Corporate and Society-sponsored projects (2017-Z-14) and National Natural Science Foundation of China (No. 81673921).

Ethics approval and consent to participate

All animal handling procedures were approved by the Institutional Animal Care Committee of Peking University People's Hospital (2017PHC021).

Consent for publication

All authors consented to the publication of this manuscript.

Competing interests

The authors report no conflicts of interests in this work.

Author details

¹Dongzhimen Hospital Affiliated to Beijing University of Chinese Medicine, Beijing 100029, China.

²Kingland Property Co., Ltd., Taiwan 11143, China. ³Peking University People's Hospital, Beijing 100044, China. ⁴School of Life Science, Beijing University of Chinese Medicine, Beijing 102488, China. ⁵State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences, Beijing 100012, China. ⁶Pharmacy, E-DA Hospital, Kaohsiung City, Taiwan 82445, China

Corresponding authors

Correspondence to Cuiling Feng.

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Figures

Figure 1

Schematic design for Gene expression profiling of rats. Rats were randomly divided into three groups: Sham, PM2.5, ACCD. ACCD (0.67g/kg/d) or equal distilled water was given continuously for 28 days while PM2.5 (650µg/3d) were tracheal dripped. The lung tissue and BALF were taken off for pathology testing and gene extraction. LC-MS/MS analysis were applied to obtain fingerprints of ACCD and GO and KEGG were used to analysis bioinformatics. Q-PCR was used to verify the related DEGs

Figure 2

The LC-MS/MS fingerprints of ACCD. (1a) (1b) Actcin K; (2a) (2b) Antcin C; (3) Zhankuic acid C; (4) Dehydrosulphurenic acid; (5a) (5b) Zhankuic acid A; (6) Dehydroeburicoic acid

Figure 3

The therapeutic effects of ACCD in treating PM2.5-exposure rats. (A) HE staining ($\times 200$) of pulmonary histopathology in rats. In PM2.5 group, we found airway cilia were inverted, alveolar walls were thickened, inflammatory cells were infiltrated, and erythrocytes were dispersed in the alveolar region. But these conditions improved after ACCD treatment. (B) AB-PAS staining ($\times 200$) of bronchial. AB-PAS stained lung sections from the PM2.5 group showed overproduction of mucus compared with the Sham groups. In the ACCD group, goblet cells in the airway epithelium reduced. (C) IL-1 β expression levels in the BALF were significantly decreased in the ACCD group compared to PM2.5 group (* $p < 0.05$, ** $p < 0.01$).

Figure 4

Volcanic map and heat map and Heatmap of DEGs. (A) Volcanoes are plotted from the P and FC values obtained from t-test analysis. The horizontal axis represents the FC values of the probes and the vertical axis represents the degree of difference between the probes ($-\log_{10} p$ value). (B) The heat map shows the hierarchical clustering of differentially expressed mRNAs in the PM2.5 and ACCD groups. Red represents up-regulated genes and green represents down-regulated genes.

Figure 5

GO and KEGG pathway enrichment of DEGs. (A) GO enrichment analysis of molecular functions. The results show a set of DEGs and related molecular function terms. The left side indicates the DEGs names and the right with different colors means the enrichment terms. (b) KEGG Pathway analysis. Dot plot of the significant KEGG pathways. The horizontal axis indicates the gene counts of each pathway and the darker the color, the more pronounced the expression ($-\log_{10} P$ value).

Figure 6

Data were analyzed by Q-PCR against multiple genes. To further validate the differentially expressed mRNAs, Q-PCR assays were performed. The $2^{-\Delta\Delta CT}$ method was applied to calculate the relative transcript levels. The relative expression of ACTB was used to standardize the expression of IL6r, Ppbp, Plcβ-1, Axin2, Ccbe1 and other assayed genes. Independent sample Student's t-test was also performed for statistical analysis. Description of significant differences. *, $P < 0.05$; **, $P < 0.01$.