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Abstract:

Background: Substantial evidence links PM2.5 to the development of inflammatory lung diseases such as chronic airways, but effective treatments are lacking. Gan-Du-Qing Decoction is a traditional Chinese medicine formula for chronic airway inflammation. However, whether GDQ can ameliorate PM2.5-induced lung injury and its mechanism are unknown and we will further investigate.

Study Design/Methods: Male Sprague-Dawley (SD) rats weighing 120 grams were utilized to establish a rat model of lung injury through systemic exposure to PM2.5. Gan Du Qing (GDQ) was administered via gavage starting four weeks post-exposure. Morphological changes were observed through Hematoxylin and Eosin (HE) staining. Inflammatory cell infiltration was detected using immunohistochemical staining, while scanning electron microscopy was employed to observe ultrastructural changes in the lung trachea. Levels of inflammatory cytokines in bronchoalveolar lavage fluid were quantified using Enzyme-Linked Immunosorbent Assay (ELISA). The main components of GDQ were identified through Ultra-High-Performance Liquid Chromatography-High-Resolution Mass Spectrometry (UHPLC-HRMS). Additionally, a combination of serum metabolomics and 16S gene sequencing of lung microbiota was employed to pinpoint key targets mediating the therapeutic effects of MGMD in the treatment of PM2.5-induced lung injury.

Results: The findings indicated that GDQ had the capability to reduce the pathological changes of lung tissue and mitigate inflammatory exudation in the lungs. 16S rRNA gene sequencing revealed that GDQ effectively reduced the richness and diversity of the pulmonary microbiome.
induced by PM2.5 and restored the overall structure of the pulmonary microbiome.

Metabolomic analysis identified 65 potential differential metabolites that may contribute to GDQ's attenuation of PM2.5-induced lung injury. These metabolites were mainly enriched in the Phospholipase D signaling pathway, Metabolism of xenobiotics by cytochrome P450, and Glutathione metabolism.

**Conclusion:** Our research offers valuable insights into how GDQ operates to mitigate PM2.5-induced lung injury through the modulation of lung microbiota and serum metabolome. These findings may have important implications for the development of effective strategies to protect against lung injury caused by PM2.5.

**Key words:** PM2.5, Lung injury, Ganduqing, Lung microbiota, Serum metabolome
Abbreviations:

BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; FA, filtered air; FOT, forced oscillation technique; GDQ, Ganduqing; PBS, phosphate-buffered saline; IHC, Immunohistochemical; LC-MS, liquid chromatography-mass spectrometry; PM2.5, Ambient air fine particulate matter; PEF, peak expiratory flow; TCM, Traditional Chinese medicine
1 Introduction

Fine particulate matter (PM2.5) in ambient air has become a significant contributor to air pollution in recent times. A growing body of evidence suggests that exposure to components of PM2.5 in ambient air pollution can lead to or worsen lung diseases such as asthma. (Tripathy et al. 2021), chronic obstructive pulmonary disease (COPD)(Wang et al. 2022; Lo et al. 2022), bronchitis(Horne et al. 2018), and idiopathic pulmonary fibrosis(Yang et al. 2020). Therefore, it is crucial to explore the precise impact of PM2.5 on lung diseases, especially in areas with high levels of air pollution. The incidence of these diseases is also higher in contaminated areas(Nakao et al. 2016), emphasizing the crucial character of PM2.5 in the progression or worsening of underlying lung diseases. Lungs are the organs that first encounter PM2.5 particles in the human body and can communicate directly with the outside world. Consequently, pulmonary functions can be compromised due to continuous exposure to PM2.5. Research has shown that fine particle deposition in the lungs can lead to a significant inflammatory response and a decrease in pulmonary function (Zhang et al. 2019; Jiang et al. 2021).

Recent advances in high-throughput sequencing technology have challenged the traditional view of lungs as sterile organs, revealing instead a diverse and dynamic microbial community (Cheng et al. 2020; Ashley et al. 2020). The pulmonary microbiota is crucial in maintaining lung health, playing a vital role in preserving respiratory physiology and immune homeostasis (Wypych, Wickramasinghe, and Marsland 2019). Commensal pulmonary microbiota protects against invasion by foreign or pathogenic microorganisms through various mechanisms, including space-occupying effects, nutrient competition, and secretion of...
However, in the presence of disease, the microbiota structure can be altered, leading to microecological imbalance (Huang and Boushey 2015; Enaud et al. 2020). Limited research has examined the effects of particulate matter on respiratory microbes. In one study, Li et al. (Li et al. 2017) demonstrated that exposure to biofuels and motor vehicle exhaust altered lung microbial and immune dynamics in rats. In human volunteer studies, exposure to PM2.5 was found to have a profound impact on respiratory microbiota, with sputum bacterial load and microbiota profiles associated with respiratory function profiles (Wang, Cheng, et al. 2019). Intratracheal instillation of PM2.5 into mice lungs was shown to not only significantly alter the composition of the microbiota but also perturb metabolites involved in various metabolic pathways (Li et al. 2020). There is a growing body of evidence connecting exposure to PM2.5 with the onset of metabolic disorders (Zhao et al. 2022; Longhin et al. 2013; Chu et al. 2021), which may be a crucial link between environmental factors and the increased incidence of respiratory diseases.

Metabolic disorders affect about 20% of the world's population, and their incidence continues to rise rapidly (López-Gil et al. 2022; Agus, Clément, and Sokol 2021). Hence, it is imperative to explore the impact of inhaling ambient PM2.5 on the host's microbiota and the resultant metabolic alterations.

Ganduqing is a widely used clinical formula developed by our group specifically for treating PM2.5-induced lung injury. This formula has received approval from the China Food and Drug Administration (No.: 2016L05320) for its efficacy in addressing this condition (Hao 2021) (WANG Zhenxing 2018). Through a comprehensive transformation process, Ganduqing...
has been developed into a compound traditional Chinese medicine that integrates the principles
of invigorating qi and detoxification with insights from modern pharmacological research,
clinical practice experiences, and advanced pharmaceutical processes. GDQ, the compound
traditional Chinese medicine, is comprised of *Astragalus mongholicus* Bunge and *Rhizoma
Belamcandae*. Extensive research has demonstrated the effectiveness of GDQ in alleviating
pulmonary inflammation by enhancing vital energy, detoxifying the body, and eliminating
pathogenic factors. Furthermore, studies have shown that GDQ can effectively improve chronic
bronchitis by suppressing the inflammatory response, regulating intestinal flora, and
modulating immunity. In summary, Ganduqing is a highly valued clinical formula that has been
rigorously developed and authorized for the management of lung injury induced by PM2.5 (Hao
2021). Its transformative journey into a compound traditional Chinese medicine reflects the
integration of traditional knowledge with modern scientific advancements. The therapeutic
benefits of GDQ, derived from *Astragalus mongholicus* Bunge and *Rhizoma Belamcandae,*
embrace its ability to alleviate pulmonary inflammation, strengthen vital energy, and regulate
the immune system while addressing chronic bronchitis (Wu et al. 2021). Despite its proven
therapeutic efficacy in clinical settings, the exact mechanism underlying GDQ's mitigation of
lung injury is not yet fully understood, largely due to the intricate composition of traditional
Chinese medicine compounds. Hence, additional research is required to clarify GDQ's
mechanism of action concerning lung injury.

In this investigation, male SD rats were subjected to either filtered air (FA) or ambient
PM2.5 for a period of three months, employing an authentic environmental PM2.5 exposure
system. The main objective was to examine the influence of PM2.5 exposure on the lung microflora and serum metabolism of rats, as well as to evaluate the effects of GDQ on lung flora and serum metabolites. To our knowledge, this is the initial investigation into the impact of traditional Chinese medicine (TCM) on serum metabolic changes and their association with lung microbiota in rats exposed to ambient PM2.5 throughout their entire bodies. This study employed a non-targeted metabolomics approach to achieve this goal. The outcomes of this investigation are anticipated to advance our understanding of the potential mechanisms responsible for PM2.5-induced metabolic disturbances. Furthermore, they are expected to provide a fundamental framework for future research into the prevention and treatment of PM2.5-induced lung injuries using traditional Chinese medicine (TCM).

2 Material and method

2.1 Animals

We acquired male Sprague-Dawley rats, aged 6 weeks and weighing 90-110 g, from Chengdu Dashuo Experimental Animal Co., Ltd in Chengdu, China. The rats were kept in standard housing conditions, adhering to the guidelines provided by the Animal Experimental Centre. The research project was adhered to the guidelines established by the Experimental Animal Research Ethics Committee at Chengdu University of Traditional Chinese Medicine. Ethical approval for these experiments was granted under the reference number 2022-43.

2.2 Whole-body inhalation exposure to real environmental PM 2.5

In line with the methodology described in our prior research, a real environmental PM2.5
exposure system was established (Wu et al. 2022). Initially, air samples were obtained from the environment through a negative pressure pump and catheter device. A PM2.5 detector (DT-9881M) was used to validate the PM2.5 content in the air, which indicated high levels of PM2.5. Subsequently, the air containing PM2.5 was directed into two separate rooms: an air filter control room and a PM2.5 exposure room. To effectively eliminate PM2.5 from the air, a 3-layer filter was installed at the entrance of the air filtration control room. However, these filters were not implemented in the PM2.5 exposure chamber. A monitoring system was put in place in both rooms to maintain uniform environmental conditions, including temperature (22-24 °C), humidity (40-60%), pressure (18-24 PA), ventilation frequency (20-22/h), and air velocity (0.18 m/s). To maintain a consistent PM2.5 concentration within the exposure chamber, a 47 mm Teflon filter with a constant airflow of 0.18 L/min was used.

2.3 Experimental protocols

After one week of acclimation to the feeding environment, all rats were subjected to the PM2.5 exposure system. The rats were randomly allocated to either the AF exposure chamber or the PM2.5 exposure chamber, where they were exposed to a 7-day cycle of continuous 24-hour exposure for a duration of 16 weeks. For detailed experimental procedures, please refer to Supplemental Data Attachment 1.

2.4 Preparation of Ganduqing

GDQ is composed of two botanicals: *Astragalus mongholicus* Bunge (Huangqi) 20g and *Rhizoma Belamcandae* (shegan) 10g. The mass ratio of *Astragalus* to *Radix Astragali* is 2:1. Both botanicals were sourced from the herbal pharmacy of Chengdu University of TCM.
Hospital. Initially, the two herbs were immersed in pure water at a volume eight times greater for a duration of 1 hour, and then they were decocted three times for 30 minutes each. The three decoctions were combined, filtered, and left to stand, and the supernatant was gathered. The supernatant was then concentrated to a concentration of 1.0 g/mL at 60°C. The concentrate was frozen at -80°C for 12 hours, followed by evacuation at -30°C for 48 hours, and finally dried at 30°C for 24 hours to obtain the lyophilized powder. It should be noted that 10g of the original herb is equivalent to 1g of lyophilized powder. Based on the results of our preliminary clinical study, the recommended daily dose of GDQ lyophilized powder for adults is 0.016 g/kg. The daily dosage for rats was determined at 0.105g/kg by applying a conversion factor of 6.3 for surface area equivalence between rats and humans. (Wu 2021).

2.5 UPLC-MS/MS Analysis of Ganduqing Decoction Components

To analyze the chemical constituents in Ganduqing liquid, UPLC-HRMS (Ultra-performance liquid chromatography-high resolution mass spectrometry) was employed. A total of 600 μL of Ganduqing concentrate sample was taken and mixed with 400 μL of methanol by vortexing. From this mixture, 200 μL was diluted with 200 μL of a 40% methanol aqueous solution. The resulting mixture was vortexed and then centrifuged at 16,000 g for 15 minutes at 4 °C. The supernatant was collected for further analysis. The GDQ extracts were analyzed using a Vanquish UHPLC system (Thermo Scientific, Waltham, MA) equipped with an HSS-T3 column (100 × 2.1 mm, 1.8 μm particle size; Waters) at a column oven temperature of 35 °C. The mobile phase A consisted of water with 0.1% formic acid, while the mobile phase B was acetonitrile with 0.1% formic acid.
acid (both solvents were of LC-MS grade and from Fisher chemical). The samples were separated at a flow rate of 0.3 mL/min using the following gradient: 5% B for 1 minute, a linear increase to 98% B within 16 minutes, returning to 5% B in 0.5 minutes, and then maintaining isocratic conditions at 5% B for 2.5 minutes.

A Q-Exactive HFX mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was utilized in combination with the UHPLC system for the analysis. Mass spectrometry acquisition was carried out in both electrospray ionization (ESI) positive and negative modes. Data-dependent acquisition (DDA) mode was employed, wherein the top 10 MS1 ions were selected for obtaining MS/MS spectra. Collision energies (CEs) were set to normalized energy levels of 20, 40, and 60 using a step-wise approach.

The data acquisition range spanned from m/z 90 to 1300. The spray voltages used were 3800 (for positive mode) and -3000 (for negative mode), with a sheath gas flow rate of 45. The capillary temperature was maintained at 320 °C, and the probe heater temperature was set to 370 °C.

2.6 Sample collection

Rat lung tissue and bronchoalveolar lavage fluid (BALF) were obtained following previously described methods (Wu et al. 2021). Rats were first anesthetized, and an incision was made in the anterior cervical skin to expose the left and right main bronchi. The right main bronchus was ligated using sutures, and a 5 mL syringe was used to inject 2 mL of phosphate-buffered saline (PBS) into the left lung. The left lung was gently shaken, and the injected PBS was subsequently aspirated back into the syringe. The collected PBS was centrifuged at 3000g.
for 5 minutes at 4 °C, and the resulting supernatant was collected. Additionally, the right lung's middle lobe was subjected to fixation using 4% paraformaldehyde and subsequently underwent staining utilizing hematoxylin and eosin (H&E). The remaining lung tissue was stored at -80 °C.

2.7 Measurement of cytokine levels

BALF was retrieved to quantify inflammatory cytokines. The levels of IL-6, TNF-α, IL-10, and IL-1β in BALF were assessed using ELISA kits obtained from MULTI SCIENCES (Hangzhou, China). According to the manufacturer's guidelines, the absorbance values of the standard samples were measured, and a standard curve was established by correlating the absorbance values and the corresponding known concentrations of the standard samples. Based on the provided standard curve, the concentrations of the unknown samples were determined through calculation.

2.8 Identification of Th17/Treg cells using flow cytometry

Seven fresh samples of lung tissues and mesenteric lymph nodes (MLNs) from every group were collected and positioned in RPMI 1640 sochilled containers. To adjust cell density to 10⁶ cells per milliliter, lung tissues and MLNs underwent chopping, filtration using a 300 mesh filter cloth, and the elimination of tissue cell remnants. Draw out 100 μL of cells into a sanitized EP tube, introduce 1μL of Anti-Rt CD4eBiosciencesTM FITC and Anti-Mo CD25eBiosciencesTM PE antibodies into every EP tube, blend thoroughly, and apply a stain for 30 minutes at 4 °C to shield them from light. Then wash the tube with 1mL PBS, centrifuge the tube and discard the supernatant. After stabilizing the cells and subsequently rupturing the membranes, 1 μL of anti-Mo/Rat/IL-17A eBio-scienceTM Percp-cy5.5 and Alexa Fluor 647
anti-rat FoxP3 Biolegend antibodies were introduced as per the guidelines, thoroughly blended, and dyed at 4 °C for half an hour in lucifugal position. To the mixture, 1 mL of PBS (PH = 7.4) at 4 °C was introduced, thoroughly blended, spun in a 300 g centrifuge for 5 minutes, and discarded the supernatant; then, 400 μL of PBS solution was added, thoroughly mixed, identified using a CytoFLEX flow cytometer, and examined with Kaluza 2.1 software.

2.9 Analysis via Western blotting

The total proteins found in lungs of seven rats per group were gathered using a radioimmunoprecipitation assay (RIPA), and their concentration was measured with a BCA kit. Total proteins underwent a separation process using sodium twelve-acetate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, followed by their transfer to polyvinylidene fluoride membranes. Membranes underwent blocking with 5% fat-free milk for two hours at room temperature, followed by an incubation night at 4 °C with primary antibodies ROR-γt (1:1000), Foxp3 (1:1000), GAPDH (1:10000). Proteins underwent incubation with their respective secondary antibodies for a duration of 2 hours at ambient temperature. Visualization of the immunoblots was achieved through advanced chemiluminescence (Thermo Fisher Scientific), and their comparative protein levels were assessed via a gel imaging device. Protein levels were determined using the Image PRO Plus 6.0 software, developed by the NIH in Bethesda, MD, USA.

2.10 Assessment of pulmonary function

At the conclusion of the experiment, lung function was assessed using the forced oscillation technique (FOT). Lung function tests were performed on rats in each group. Prior to
the tests, the rats were anesthetized with a 1% sodium pentobarbital solution. The anterior neck skin was incised to expose the trachea. A minor tracheal incision was created, and a Y-shaped plastic tube was inserted, firmly secured with sutures. Following this, the rat's airway was connected to a ventilator (AniRes2005 Version 3.5) via the Y-shaped tube. Following the configuration of the program, the ventilator automatically monitored and recorded the lung function parameters of the rats.

2.11 Histopathological staining of lung tissue

Fresh lung tissue samples were fixed and embedded in paraffin. The paraffin-embedded lung tissue was subsequently cut into sections that were 5 mm thick for hematoxylin and eosin (H&E) staining. These lung sections were observed under a light microscope by three pathologists. The pathologists evaluated the lung sections and assigned a pathological score using a previously established scoring scale for assessing lung injury (Pei et al. 2021).

2.12 Immunohistochemical (IHC) staining of lung tissue

The lung tissue sections were subjected to dewaxing and washing processes, followed by antigen retrieval using citric acid repair buffer. Subsequently, the sections were blocked with 3% BSA at room temperature for 30 minutes. We acquired the Anti-Galectin 3 (BS-20700R) antibody and the Anti-Neutrophil (BS-6982R) Elastase antibody from Bioss (Beijing, China). Additionally, the secondary antibodies were procured from Thermo Fisher Scientific (Waltham, MA, USA). The sections were incubated overnight at 4 °C with an anti-galectin-3 antibody (diluted at 1:400) and a neutrophil antibody (diluted at 1:500). After washing the primary antibody, the sections were incubated with HRP-conjugated secondary antibody at room
temperature for 50 minutes. After washing, sections were stained sequentially with 3,3' diaminobenzidine (DAB) chromogenic solution and hematoxylin. Finally, the sections were dehydrated and fixed. Sections were observed microscopically and assessed quantitatively by Image-pro Plus 6.0 software.

2.13 Scanning electron microscopy of lung tissue

Fresh lung tissue blocks were fixed with fixative at room temperature for 2h and then transferred to a 4 ℃ refrigerator for storage. Next, the tissue blocks were washed three times with phosphate buffer (pH 7.4) for 15 minutes each. Then, these tissue blocks were fixed with 1% osmium tetroxide at room temperature in the dark for 2 hours. After washing again, the tissue blocks were dehydrated with alcohol and isoamyl acetate. These lung tissue blocks are attached to metallic stubs and sputter-coated with gold for the 30s. Finally, we observed lung tissue by scanning electron microscopy and collected images.

2.14 16S rRNA gene sequencing for the lung microbiome

Bronchoalveolar lavage fluid (BALF) were collected from rats. The lung microbiome was analyzed using 16S rRNA high-throughput sequencing to investigate the impact of GDQ. The sequencing assay was conducted at Novo-gene Biotechnology (Beijing, China). In brief, total bacterial DNA was first extracted using CTAB. The purity and concentration of the DNA were then detected by agarose gel electrophoresis experiments. The DNA sample was then diluted to 1 ng/µL with sterile water as the sample to be tested. DNA templates are amplified using specific primers with barcodes. The V4 region of the bacterial 16S rRNA gene was used as the amplification region. The sequences of the primer genes used are as follows: 515 forward:
GTGCCAGCMGCGGTAA, 806 reverse: GGACTACHVGGGTWTCTAAT.

The library construction was performed using the TruSeq® DNA Free PCR Sample Preparation Kit. The constructed libraries were quantified by Qubit and Q-PCR. After the library is qualified, NovaSeq6000 is used to perform sequencing. The Illumina NovaSeq sequencing platform was used to perform paired-end sequencing of the libraries. The obtained reads were clustered into Operational Taxonomic Units (OTUs) for species annotation and abundance analysis. The species composition and community structure differences between samples were assessed using α-Diversity and β-Diversity measures.

2.15 Serum metabonomics analysis

To begin the sample preparation, measure 100 μL of the sample and transfer it to an EP tube. Add 400 μL of an 80% methanol-water solution, and then vortex and shake the mixture. Let it sit in an ice bath for 5 minutes to facilitate precipitation of the proteins. Following this, centrifuge the samples at 15,000 g, 4°C for 20 minutes. Once centrifuged, collect a certain amount of the supernatant and dilute it with mass spectrometry-grade water to obtain a methanol content of 53%. After dilution, centrifuge the samples again at 15,000 g, 4°C for 20 minutes to remove any remaining impurities. Collect the supernatant and analyze it using liquid chromatography-mass spectrometry (LC-MS) for further analysis. The chromatographic conditions used for LC are as follows: Hypesil Goldcolumn (C18) is used as the chromatographic column, with the column temperature maintained at 40°C, and a flow rate of 0.2 ml/min. For the positive mode, mobile phase A is 0.1% formic acid, and mobile phase B is methanol. Conversely, for the negative mode, mobile phase A is 5 mM ammonium acetate with
a pH of 9.0, and mobile phase B is methanol. Regarding the mass spectrometry conditions,
scans were performed between m/z 100 to 1500. An electrospray ionization (ESI) source was
used with the following settings: spray voltage of 3.2 kV, sheath gas flow rate of 40 arb, aux
gas flow rate of 10 arb, and a capillary temperature of 320°C. The polarity can be set in either
positive or negative mode. For tandem mass spectrometry (MS/MS), data-dependent scans are
performed.

The raw data files (.raw) obtained from the spectrometry analysis were imported into the
advanced software Compound Discoverer 3.1 (CD3.1, Thermo Fisher) for further analysis.
Initial screening of the data was performed based on parameters such as retention time and
mass-to-charge ratio. To improve identification accuracy, peak alignment was performed on
different samples, using a retention time deviation of 0.2 min and a mass deviation of 5
ppm. Peak extraction was carried out based on the set parameters, which included a mass
deviation of 5 ppm, signal intensity deviation of 30%, signal-to-noise ratio of 3, minimum
signal intensity of 100,000 and summing ions. The obtained peak areas were then quantified,
with target ions integrated accordingly. To improve identification accuracy further, molecular
formula prediction was performed. This prediction relied on both molecular ion peaks and
fragment ion information, and the results were cross-referenced with databases including
mzCloud (https://www.mzcloud.org/), mzVault, and Masslist. To enhance data quality, blank
samples were utilized for background ion removal. Subsequently, the quantification results
underwent normalization, ultimately yielding data that included both identification and
quantification outcomes. This analysis should provide you with a comprehensive and reliable
overview of the analyzed substance.

2.16 Statistical analysis

GraphPad Prism software was employed for data analysis. (version 8.0, United States). The value is expressed as Mean ± SEM. Statistical significance of group differences was assessed using one-way analysis of variance (ANOVA) followed by Tukey's post-test. For data that did not follow a normal distribution, the Kruskal-Wallis one-way ANOVA was employed. Statistical differences are represented by $P$ values less than 0.05.

3 Results

3.1 Identification of components in GDQ by LC-MS/MS

In this experiment, UHPLC-HRMS was used to collect the data of GDQ solution and compare its positive and negative ion BPC maps. In the Ganduqing sample (GDQ positive and negative ion BPC diagram, the chromatographic peaks with higher abundance were confirmed for peak shape and examined for secondary spectrum, and then the peak numbers were labeled, and each peak in the positive and negative ion diagram was labeled in digital order as shown in the Figure1A -B.

The collected data were imported into the local standard map database of traditional Chinese medicine for secondary mass spectrometry search and comparison, 1051 chemical components in Ganduqing solution were analyzed and identified, and a total of 99 categories were identified according to the classification method in the literature ClassyFire. Among them, the top 6 compounds and their subclasses are: Benzene and substituent derivatives, Carboxylic acids and derivatives, Fatty Acyls, Flavonoids, Organooxygen compounds, and Prenol lipids.
3.2 Real-ambient PM2.5 exposure

Throughout this experiment, rats were continuously exposed to PM2.5 for 16 weeks, 24 hours a day, 7 days a week. The detailed experimental procedure is illustrated in Supplementary Data Figure 1A-B. During the exposure period in Jinniu District, Chengdu, the mean PM2.5 concentration in the surrounding environment was 117.8 ug/m$^3$, while the PM2.5 concentrations in the dedicated PM2.5 exposure chambers were recorded as 110.5 mg/m$^3$ (Figure 2A). Statistical analysis revealed that the PM2.5 concentrations in the exposure chambers were comparable to those in the ambient environment. Moreover, Pearson correlation analysis confirmed that the concentrations in the exposure chambers exhibited a significant correlation with the ambient PM2.5 concentrations (Refer to Supplemental Data Attachment 1). Following filtration through the filters, the PM2.5 concentration in the AF room decreased significantly, indicating the filters’ effectiveness in acting as a barrier against PM2.5. Previous studies have also investigated the composition of PM2.5 in indoor settings, which closely resemble the components found in the ambient environment.

3.3 GDQ inhibit systemic inflammatory response induced by PM2.5 exposure

During the entire study, we observed that the rats exposed to PM2.5 inhaled air showed a slower increase in body weight compared to the rats in the AF chamber (Figure 2B). However, this trend was effectively reversed following the intervention of GDQ. Additionally, following GDQ intervention, the rats displayed significantly shinier fur in comparison to the PM2.5 exposed indoor rats, indicating an improvement in their overall health. Furthermore, the activity
levels of the rats were notably enhanced after GDQ intervention, illustrating a greater sensitivity and vitality.

Since the lungs are the main organ affected by PM2.5 exposure, our initial emphasis was on evaluating the inflammatory responses occurring in the respiratory tract. To investigate the effect of PM2.5 on lung tissue inflammation, we measured the expression levels of inflammatory cytokines, including IL-10, IL-6, IL-1β, and TNF-α, in lung tissues using ELISA (Figure 2C-F). The results revealed significantly elevated cytokine levels in the PM2.5 exposed group, in comparison to the AF group. Moreover, GDQ intervention successfully reduced cytokine levels when compared to the PM2.5 group. Immunohistochemical staining with anti-neutrophils and galectin-3 (a macrophage specific marker) demonstrated considerable infiltration of neutrophils and macrophages in the lungs of rats exposed to PM2.5 (Figure 5B). Notably, the lung tissue of rats in the PM2.5 exposed group exhibited severe damage, with a marked increase in macrophage and neutrophil infiltration when compared to the AF group. However, GDQ intervention significantly alleviated inflammatory cell infiltration induced by PM2.5 exposure. Collectively, these findings provide compelling evidence that GDQ effectively mitigates inflammatory responses in PM2.5 exposed rats.

3.4 GDQ regulated systemic and local immune response of PM2.5 induced lung injury

The lung's immune system was partly propelled by the growth and diversification of immune cells within the lung and MLNs. Investigating GDQ's impact on immune cells within lung tissues and MLNs, the specific subset of T cells in these areas was identified using flow cytometry. The results showed a decrease in Treg count in CD4+ T cells and an increase in Th17
cells in the PM2.5 group relative to rats in the AF group. GDQ escalated the proliferation of Treg cells within lung tissues and MLNs, whereas their reduction in Th17 cell expression mirrored that observed in AF rats. Consequently, we simultaneously analyzed how ROR-γt and Foxp3 are expressed in lung tissues. Relative to the AF group, there was a notable surge in ROR-γt expression in the PM2.5 group, whereas Foxp3 expression in the PM2.5 group exhibited a slight decrease relative to the AF group. Alterations in the previously mentioned variables can be reversed following GDQ therapy (Figure 4A-C).

3.5 GDQ ameliorate pulmonary dysfunction caused by PM2.5 exposure

To further investigate the detrimental impacts of PM2.5 exposure on lung health, we conducted lung function measurements in rats. Currently, invasive lung function tests represent the most reliable method for evaluating lung function in rats, serving as the gold standard for such assessments (Glaab et al. 2007). In our study, lung function was assessed using the forced oscillation technique (FOT). The PM2.5-exposed rats exhibited significantly reduced Forced Vital Capacity (FVC) in comparison to the AF group, accompanied by reductions in the 0.2 s forced expiratory volume (FEV0.2), as well as reductions in the ratio of 50% FVC to 25% FVC (F50/F25%) in the PM2.5 exposed rats (Figure 3A-E). Peak Expiratory Flow (PEF) is a vital measure, as it reflects the highest rate of exhalation through the bronchial tree during forced expiration. PEF is frequently employed to assess the presence of airway obstruction (Figure 3F). PEF serves as an indicator of bronchial hyperresponsiveness and mirrors alterations in airway caliber (Chinn, Jarvis, and Burney 2002). PEF exhibited a statistically significant increase in PM2.5-exposed rats when compared to AF control rats. However, GDQ intervention
could increase lung function parameters compared with control group. These results further illustrate that GDQ intervention reversed PM2.5 exposure-induced lung function decline in rats. **3.6 GDQ Alleviates PM2.5-Induced Lung Tissue Damage**

To further examine the histological changes in lung tissues, we conducted a pathological analysis. The obtained lung histopathology data revealed that exposure to PM2.5 induced severe bronchitis and interstitial pneumonia, characterized by thickening of the alveolar wall, congestion in the alveolar space, and significant pulmonary edema. However, following GDQ intervention, both bronchitis and pulmonary inflammatory cell infiltration were mitigated. These results indicate a clear association between PM2.5 exposure and lung injury, as evidenced by the decline in lung function, while GDQ intervention proved effective in alleviating this injury (Figure 5A).

Scanning electron microscopy was employed to examine the ultrastructural changes in lung tissue. In the Ctrl group, lung tissue exhibited a pristine structure, with evenly distributed round-shaped alveoli. The type I epithelial cells of the alveolar wall were flat and firmly attached, displaying a wide coverage area. The cell membranes were intact, and no visible damage was observed. The alveolar septa were thin and uniform in thickness, with scattered red blood cells in the capillary lumens. In contrast, in the PM2.5 group, lung tissue sections exhibited more pronounced structural damage. Alveoli varied in size, with significant atrophy and irregular collapse. The type I epithelial cells of the alveolar wall were flat and attached to hypertrophied pulmonary interstitium. Large areas of the alveolar septa were noticeably thickened, with scattered red blood cells in the capillary lumens. In the GDQ group, the
structural damage to lung tissue sections was mild. Alveoli were evenly distributed, with only a few varying in size. While individual alveoli showed slight atrophy, most of them maintained a round shape. The type I epithelial cells in the alveolar wall were flat, adherent, and displayed a wide coverage area. The cell membranes appeared intact, and no observable damage was detected. Mild pulmonary interstitial hypertrophy was observed, along with localized thickening of the alveolar septa. Notably, the pulmonary interstitium was mildly hypertrophic, while focal areas of the alveolar septa displayed thickening (Figure 3G).

3.7 GDQ’s Effect on Lung Microbiota Composition Induced by PM2.5 Exposure in Rats

We utilized 16S rRNA gene sequencing to explore how PM2.5 exposure affected the lung microbiome and to assess the potential effects of GDQ intervention. Alpha diversity metrics were utilized to gauge the diversity and richness of the lung microbial communities. The results demonstrated a significant increase in microbial community diversity in rats exposed to PM2.5 compared to the Ctrl group, as indicated by both the Shannon index and Simpson index (Figure 5A-B). However, GDQ intervention led to a reduction in lung microbiome diversity. Additionally, the richness estimates of Chao1 and ACE were substantially higher in the PM2.5 group than in the Ctrl group, and GDQ intervention resulted in decreased values of Chao1 and ACE (Figure 6C-D).

Weighted UniFrac principal component analysis (PCoA) was utilized to evaluate the similarity of microbiota and detect variations in lung microbiota composition among the groups. The PCoA results clearly demonstrated significant dissimilarities in the composition and structure of lung microbiota between the PM2.5 group and the other groups. This finding
strongly suggests that the stimulation of PM2.5 significantly perturbed the delicate equilibrium of lung microbiota, disrupting its dynamic balance (Figure 6E). Significantly, it was observed that the GDQ and Ctrl groups exhibited a closer distance, suggesting that GDQ administration partially alleviated the lung dysbiosis triggered by PM2.5 exposure. We used the beta diversity index in this research to assess potential variations in species diversity among the groups, applying the Wilcoxon rank-sum test for analysis. (Figure 6F). After analyzing the box plots presented in the figure, it can be observed that the width of the box within each group suggests good reproducibility. Moreover, the PM2.5 group displayed a notable rise in microbiota diversity in contrast to the control group. However, following GDQ intervention, the diversity decreased, and this difference was statistically significant.

To further investigate the variation in species, we examined the lung bacterial composition and their relative abundance. The taxonomic composition was assessed at both the phylum and genus levels to identify significant differences among the groups. The taxonomic composition at the phylum level mainly comprises: Proteobacteria, Tenericutes, Firmicutes, Actinobacteria, Notably, the administration of GDQ could ameliorate the alterations in lung microflora induced by PM2.5 exposure (Figure 7A). At the genus level, Pseudomonas, Mycoplasma, and Lactobacillus were the main dominant genera. Lactobacillus was higher than PM2.5 group after GDQ intervention, while Pseudomonas and Mycoplasma were lower than PM2.5 group. These findings suggest that GDQ intervention can effectively modulate the bacterial structure in the lungs of PM2.5-exposed rats (Figure 7B).

3.8 Effects of GDQ on the Serum Metabolome in Rats Exposed to PM2.5
Analysis of the serum metabolome in rats from each group was conducted using LC-MS. The metabolites exhibited distinct separation between the Ctrl and PM2.5 groups, as evidenced by the principal components PC1 and PC2, which accounted for 24.41% and 11.15% of the total variation, respectively (Figure 8A). Subsequently, the metabolites that exhibited differences among the experimental groups were further analyzed using the partial least squares-discriminant analysis (PLS-DA) model (Figure 8B). The validation results demonstrated the model's robust predictive ability, thus providing additional evidence for the experiment's reliability (Figure 8C). The PLS-DA scores revealed distinct dispersion between the control and PM2.5 groups, indicating significant differences in their metabolic profiles. Additionally, the PCA integral plot demonstrated clear separation of the metabolic profiles between the PM2.5 and control groups. Notably, the metabolic profiles of the GDQ group exhibited closer proximity to those of the blank group, suggesting that GDQ intervention could ameliorate the metabolic disruptions caused by PM2.5 exposure (Figure 8D). Furthermore, the PLS-DA score plot exhibited complete separation of the metabolic trajectories among the three groups of rats. The permutation test was employed to evaluate the effectiveness of the model (Figure 8E).

In this study, we employed variable importance projection (VIP) as a threshold to discern metabolites that exhibited notable variances between the Ctrl and PM2.5 groups. Metabolites that met the criteria of VIP > 1.0 and fold change (FC) > 1.5 were considered as differential metabolites. A total of 290 metabolites displayed significant differences between the Ctrl and PM2.5 groups in the comparative analysis.
Out of the 290 differential metabolites, 146 metabolites showed an elevation in the PM2.5 group, while 144 metabolites exhibited a decrease compared to the Ctrl group. To gain further insights into the differences in metabolite profiles, a qualitative and quantitative analysis was conducted on the major metabolites identified in each group. The relative content of metabolites at the same level was assessed using Z-Score, and the top 30 metabolites were identified (Figure 8A). When comparing the PM2.5 group with the GDQ group, a total of 312 distinctive metabolites were identified. Among these 312 metabolic species, 206 metabolites exhibited an elevation, while 106 metabolites showed a decrease in the GDQ group compared to the PM2.5 group. Additionally, a qualitative and quantitative analysis was performed on the major metabolites identified in each group. The relative content of metabolites at the same level was assessed using Z-Score, and the top 30 metabolites were identified (Figure 8B). The different metabolites were analyzed using the KEGG database, and metabolic pathways were constructed and analyzed to gain insight into the relevant metabolic pathways associated with the inflammatory response. The analysis revealed that these metabolites are involved in various metabolic pathways, including the Phospholipase D signaling pathway, Metabolism of xenobiotics by cytochrome P450, and Glutathione metabolism (Figure 9C-D). These findings shed light on the potential mechanisms underlying the action of the inflammatory response and provide valuable information for further investigation.

4. Discussion

With the rapid development of Chinese cities, the concentration of PM2.5 in the atmosphere has been increasing year by year, which in turn induces and aggravates the
occurrence and development of respiratory diseases, such as COPD (Yang et al. 2021), asthma (Zhao et al. 2020), and lung cancer (Hamra et al. 2014). The adverse effects of PM2.5 on human and possible underlying mechanisms have been documented, however the prevention and treatment methods are scarce. Therefore, it is necessary to explore preventive measures that can protect people from lung injury caused by PM2.5. In this study, we investigated the metabolic and pulmonary microbial changes in rats after systemic environmental PM2.5 exposure using untargeted metabolomics and 16S RNA sequencing in a real environmental exposure system, and the safeguarding impact of GDQ on PM2.5-triggered lung injury.

Increasing research suggests that the microbiome and its associated metabolites may help to understand possible underlying mechanisms of disease development (Aron-Wisnewsky et al. 2021; Wang et al. 2020). Recent studies had shown that PM2.5 exposure affected the metabolome composition of mouse in lungs, and 13 differential metabolites analyzed as potential biomarkers of PM2.5 induced lung injury using gas chromatography-mass spectrometry (GC-MS) (Wang, Gao, et al. 2019). Another study reported the effects of PM2.5 exposure on the lung microbiome and identified potential microbial markers of PM2.5 induced lung injury (Ran et al. 2021). However, the current prevention and treatment measures of PM2.5 induced lung injury are extremely scarce. Therefore, this study will investigate the mechanism by which GDQ alleviates PM2.5 induced lung injury in combination with lung microbiota and serum metabolomics.

In the present study, we developed the model of lung injury induced by PM2.5 through a systemic exposure system, this system has been introduced in detail in our previous study (Wu...
et al. 2022). A previous series of literature has reported the deleterious effects of PM2.5 exposure on lung function, while our study was also consistent with previous studies, lung function was decreased in rats exposed to PM2.5 (Jiang et al. 2021; Hou et al. 2020). However, GDQ improved the pulmonary function of rats to some extent. HE staining of lung histopathology showed that inflammatory cell infiltration was observed in the PM2.5 group, and immunohistochemistry also revealed neutrophil and macrophage invasion, suggesting the presence of airway inflammation. Scanning electron microscopy could more visually observe the morphological changes of inflammatory cells and alveoli, indicating that PM2.5 exposure could lead to airway epithelial cell damage, followed by inflammatory cell infiltration and structural damage. However, there were fewer inflammatory cells in the GDQ group, and the injured structure was recovered, indicating that GDQ can reduce lung tissue injury caused by PM2.5. In addition, Elisa results showed that PM2.5 exposure resulted in a significant up-regulation of inflammatory factors in lung tissue, while GDQ significantly reduced the level of inflammatory cells in lung tissue. According to the above results, we concluded that GDQ could effectively reduce lung injury induced by PM2.5.

Next, we analyzed the effect of PM2.5 exposure on lung microbiome composition using 16S rRNA gene sequencing and observed the regulatory effect of GDQ on lung microbiota. The results showed that lung microbiota diversity and richness were higher in the PM2.5 group than in the control group, which was also consistent with previous studies. However, GDQ intervention could reduce the increase in the abundance and diversity of lung microbiota caused by PM2.5. In addition, our results showed that the main differential species were Proteobacteria,
Bacteroidetes, Cyanobacteria, Firmicutes. The imbalance of these lung microbiome is present in a variety of respiratory diseases, and the increase of Proteobacteria is predominant, which is the main phylum leading to acute and chronic obstructive pulmonary disease, pulmonary fibrosis and pulmonary sarcoidosis (Gupta et al. 2021; Daniel et al. 2021). These results demonstrated that PM2.5 exposure disturbed the microbial structure of the lungs, and the microbial composition of the GDQ group was closer to that of Ctrl group, indicating that GDQ could restore the microbiota of the lungs. In fact, several studies have already shown that PM2.5 exposure can lead to lung injury, while lung microbes and airway immune system are intermediate mediators that increase individual sensitivity to PM2.5 (Wu et al. 2022; Wu et al. 2021). Moreover, studies had also shown that lung microbes could interact with the airway immune system, indicating that PM2.5 exposure causing lung microbiome disorders might be one of the causes of lung injury. Hence, GDQ could restore the lung microbiota disturbance caused by PM2.5, which may be one of the mechanisms by which GDQ alleviates PM2.5-induced lung injury.

Moreover, the LC-MS metabolomics analysis revealed notable alterations in the serum metabolic profile following PM2.5 exposure. Specifically, metabolites such as citric acid, α-ketoglutarate, fumaric acid, and indoleacetic acid exhibited significant reductions in the PM2.5 group compared to the control group. However, GDQ intervention effectively increased the levels of these metabolites and mitigated PM2.5-induced lung injury. Furthermore, KEGG enrichment analysis indicated that these metabolites were primarily associated with the Phospholipase D signaling pathway, Metabolism of xenobiotics by cytochrome P450, and
Glutathione metabolism. These findings highlight the potential metabolic pathways involved in the response to PM2.5 exposure and the protective effects of GDQ intervention. Phospholipase D signaling pathway has been shown to play a key role in pulmonary vascular endothelial barrier dysfunction and can directly lead to lung injury (Usatyuk et al. 2013). The Metabolism of xenobiotics by cytochrome P450 signaling pathway is known to play a crucial role in the metabolism of polycyclic aromatic hydrocarbons and nitro compounds in the lung. Activation of this pathway can result in DNA damage and contribute to the development of lung tumors (Ma and Ma 2002). Additionally, the glutathione metabolism pathway has been identified as a significant biomarker of pulmonary endoplasmic reticulum (ER) stress and is crucial in the pathogenesis of lung injury (Dunigan-Russell et al. 2020). The restoration of metabolic disorders observed in rats following GDQ intervention further supports the findings of metabolomics analysis, which partially elucidate the underlying mechanisms of lung injury induced by PM2.5 and highlight the protective effects of GDQ against PM2.5-induced lung injury.

Despite the promising findings of this study, it is important to acknowledge several limitations. Firstly, there is a potential for non-uniform distribution of PM2.5 in systemic exposures compared to tracheal instillation. However, previous research has demonstrated that both exposure methods result in comparable pulmonary toxicity in rats following exposure to refractory particulate matter (Driscoll et al. 2000). Secondly, due to the complex composition of PM2.5, it was not possible to identify the specific components responsible for the observed changes. Furthermore, using rats with a standardized rat microbiota could provide a more...
comprehensive model to investigate individual variations in lung microbiota.

Conclusions

In summary, this study employed 16S rRNA gene sequencing and LC-MS-based metabolomics analysis to investigate the impact of PM2.5 exposure on the lung microbiome and metabolic profile in rats. Furthermore, the protective effect of GDQ on PM2.5-induced lung injury was examined. The results demonstrated that PM2.5 exposure significantly altered the composition of the lung microbiota and disrupted the levels of certain metabolites involved in various metabolic pathways. However, GDQ administration was found to restore the composition of the lung microbiota and maintain metabolic homeostasis, thereby mitigating PM2.5-induced lung injury. Overall, these findings provide novel insights into the potential preventive and therapeutic strategies for addressing disturbances in the lung microbiome and associated metabolome caused by PM2.5 exposure, highlighting the potential of traditional Chinese medicine (TCM) interventions in mitigating PM2.5-induced lung injury.

Ethics approval and consent to participate

All experimental protocols were approved by the Animal Ethics Committee of Chengdu University of Traditional Chinese Medicine (2022-43). Animals were handled in accordance with the provisions of the National Animal Welfare Act of China. Animal welfare complied with international regulations on laboratory animals. Animals were euthanized following the AVMA Guidelines for Euthanasia of Animals 2020 published by the American Veterinary Medical Association. All the participants gave written
informed consent.

**Consent for publication**

Not applicable.

**Competing interests**

All the authors have no conflicts of interest to declare.

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**Authors’ contributions**

**Zhenxing Wang and Yongcan Wu:** Conceptualization, Methodology, Writing–original draft. **Yongcan Wu and Sijing Zhao:** Formal analysis. **Xinzhou and Caixia Pei:** Data curation. **Shihua Shi:** Software. **Demei Huang:** Software. **Yilan Wang and Xin Zhou:** Visualization, Investigation. **Xiaomin Wang:** Methodology. **Zherui Shen:** Methodology. **Jianwei Wang and Fei Wang:** Visualization, Investigation, Supervision, Writing–review & editing. **Zhenxing Wang and Xin Zhou and Biao Zuo:** Supervision, Writing–review & editing.

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Availability of data and materials

Data will be made available on request.
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Figure 1. GDQ Positive and Negative BPC Plot. (A) BPC Plot in Positive Ion Mode of GDQ - Standard Peak. (B) BPC Plot of GDQ in GDQ Negative Ion Mode — Standard Peak.
Figure 2. GDQ inhibit systemic inflammatory response induced by PM2.5 exposure.

(A) Concentrations of PM2.5 in PM2.5 exposure chambers and ambient air recorded during the experiment. Dashed and dotted lines represent the average daily limits and
levels of severe air pollution for the air quality guidelines in China. (B) Body weights of rats were recorded during the experiment (n = 20). (C-F) The expression of IL-10, IL-6, TNF-α, and IL-1β were determined by ELISA. Data are presented as mean ± SEM.

# p < 0.05, ## p < 0.01, ### p < 0.001 compared to control group. * p < 0.05, ** p < 0.01, *** p < 0.01, compared to PM2.5 group.

Figure 3. GDQ protected against PM2.5-induced lung injury. (A-F) Pulmonary function instruments were used to detect pulmonary function parameters under anesthesia in rats (n = 7), Lung function parameters: FVC, forced vital capacity; FEV0.2, forced
expiratory volume in the first 0.2s; FEF25%, maximal flow at 25% of forced vital capacity; FEF50%, maximal flow at 50% of forced vital capacity; PEF, peak expiratory flow. (G) PM2.5 induces lung damage to the ultrastructure of alveolar macrophages in lung tissue. (The first row of Figure 2G: scale bar = 1 μm, SEM × 500, The second row of Figure 2G: scale bar = 2 μm, SEM × 1000). Data are presented as mean ± SEM. # p < 0.05, ## p < 0.01, compared to control group. * p < 0.05, compared to PM2.5 group.

Figure 4. GDQ rescued Th17/Treg imbalance in PM2.5-induced lung injury. (A) Representative FACS plots of IL-17A+ cells in the CD4+ T-cell subset and Foxp3+ cells in CD4+CD25+ T-cell subset in the lung tissues and MLNs. (B) Statistical chart of
expression of Th17 and Treg cells. (C) The expression of Foxp3 and ROR-γt protein were determined by Western blot. Data are presented as mean ± SEM (n = 7). ## p < 0.01, ### p < 0.001 compared to control group. **p < 0.01, ***p < 0.001 compared to PM2.5 group.

Figure 5. GDQ attenuates lung tissue damage induced by PM2.5. (A) HE staining of lung tissue in each group. (The first row of Figure 3A: magnification 200 ×, Scale bar = 50μm, The second row of Figure 3A: magnification 400 ×, Scale bar = 50μm). Arrows
indicate classical lung injury. The blue, red and green arrows indicate inflammatory cell infiltration, hyperemia, and thickening of the alveolar walls respectively. (B) Immunohistochemical staining (magnification × 200) was used to observe the effect of GDQ on neutrophil and galectin-3 expression in lung tissue of PM2.5 induced lung injury in rats. (C) Statistical plots of semiquantitative histological scores and positive expression of neutrophils and galectin-3 assessed for lung injury induced by PM2.5. Data are presented as mean ± SEM (n=7). ### p < 0.001, compared to control group. * p < 0.05, *** p < 0.001 compared to PM2.5 group.
Figure 6. The effect of GDQ on the composition of the lung microbiota induced by PM2.5 exposure in rats. (A) Shannon index, (B) Simpson index, (C) chao1 index, (D) ACE index, (E) Weighted UniFrac principal coordinate analysis (PCoA), (F) β-diversity of lung microbiota in each group (n = 6). ## $p < 0.01$, ### $p < 0.001$, compared to control group.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to PM2.5 group.
Figure 7. The effect of GDQ on the composition of the lung microbiota induced by PM2.5 exposure in rats. The lung microbiota composition profiles at the phylum (A) and genus (B) level.
Figure 8. The effect of GDQ on the serum metabolome induced by PM2.5 exposure in rats.

(A) PCA score plots. (B) PLS-DA model validation diagram. (C) Validation Plot of PLS-DA Sorting. (D) Total PCA score plot. (E) Total PLS-DA score plots.
Figure 9. The effect of GDQ on the serum metabolome induced by PM2.5 exposure in rats. (A) Zscore plot of differential metabolite, (B) KEGG enriched bubble map.
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