Content

1 OTHER MAIN INSTRUMENTS AND REAGENTS

The main instruments and reagents used in this study are already provided in the manuscript and the remaining major instruments are listed in the SI.

1.1 Main Instruments

Supplementary Table S1. Main instruments in the experiment

Instrument/Model	Company
High Flow Air Sampler/ HY-1000WS	Qingdao Hengyuan Technology Development Co., Ltd., China
Low Temperature High Speed Centrifuge/ Centrifuge 5430R	Eppendorf, Germany
Fluorescence Inverted Microscope/IX70-112	OLYMPUS Corporation, Japan
Slicer	Leica, Germany
Embedding Machine	Leica, Germany
NanoDrop™ Lite Spectrophotometer	Thermo Fisher Scientific, USA
Fluorescence Quantitative PCR Instrument/ZFX96 touch	Bio-Rad Laboratories, USA
Thermal Cycler/EDC-810	Beijing Dongsheng Innovative Biotechnology Co., Ltd., China
Cell Culture Incubator/HERAcell 240I	Thermo Fisher Scientific, USA
Microscope/IX70-112	OLYMPUS Corporation, Japan
Cell Counter	Eppendorf, Germany
Sxhlet Extractor/B-811	BUCHI Corporation, Switzerland

1.2 Main Reagents

Supplementary Table S2. Main reagents used in the experiment

Experimental Reagents	Company
Pentobarbital Sodium	American Sigma
PBS Buffer Solution	American Gibco
Anhydrous Ethanol	China National Pharmaceutical Group Corporation
Formalin	Canadian Biosharp
Eosin	Canadian Biosharp
Safranin	Canadian Biosharp
Paraffin	Canadian Biosharp
Neutral Resin	Canadian Biosharp
Protease Inhibitor Cocktail (general purpose)	Shanghai Bioyuntian Biotechnology Co., Ltd.
Enhanced BCA Protein Concentration Determination Kit (enhanced version)	Shanghai Bioyuntian Biotechnology Co., Ltd.
RIPA Lysis Buffer Proteinase	Shanghai Bioyuntian Biotechnology Co., Ltd.

2 EXPERIMENTAL METHODS

2.1 Whole-body Ambient Inhalational Protocol

Six- to eight-week-old male C57BL/6J mice were randomly assigned to three groups for

exposure to filtered air (FA), unfiltered air (UA), and concentrated $PM_{2.5}$ air (CA) using real-time ambient particulate matter exposure chambers. The FA group was exposed to clean air in chambers equipped with HEPA filters, the UA group was exposed to ambient air in chambers without HEPA filters, and the CA group was exposed to concentrated $PM_{2.5}$ atmosphere. Post-exposure, all animals were housed in cages with HEPA filters to maintain consistent environmental conditions. The exposure schedule consisted of 6 hours per day, 6 days per week, from November 19, 2021, to March 8, 2022, totaling 16 weeks.

The animal experiments were carried out at the Animal Facility of the School of Public Health, Hebei Medical University, which is equipped with advanced real-time inhalation exposure chambers and monitoring devices. The concentrated $PM_{2.5}$ used in this study was generated by a $PM_{2.5}$ concentration enrichment system (VACES) located in Shijiazhuang, China. During the exposure period, $PM_{2.5}$ was collected on Teflon filters using high-volume air samplers. The extraction process involved cutting the filters into small pieces and placing them into 50 mL centrifuge tubes. The filters were subjected to ultrasonic treatment in ice water for 30 minutes, followed by shaking for 20 minutes on a shaker. This process was repeated three times. The resulting extracts were then passed through a 40 μm sterile nylon filter and lyophilized. The extracted $PM_{2.5}$ powder was stored at -20 ℃ until it was ready for analysis.

2.2 Collection and Preparation of Lung Tissue Samples

The heart and lungs were exposed after anesthesia and blood was removed by perfusion through the left ventricle with pre-chilled PBS or physiological saline. The entire lungs are weighed, and a portion of lung tissue was placed in a sterile dish containing PBS or physiological saline, while the remaining tissue was stored in liquid nitrogen. Lung tissues were fixed in 4% paraformaldehyde for 24 hours, dehydrated in 50%, 70%, 80%, 90%, and 95% ethanol sequentially, followed by thrice in absolute ethanol, and then cleared in chloroform or xylene for 1.5 hours. The cleared lung tissues were embedded in paraffin and sectioned into 5 μm slices before being baked.

Slices are dewaxed by xylene (twice, each time for 5 min) and hydrated by alcohol with different concentrations (100%, 80%, and 70%, each time for 10 min). Then stained with hematoxylin and eosin sequentially, the slices are treated with alcohol with increasing

concentrations (first 95% alcohol, then absolute ethanol, twice each, 1 min each time) and cleared in xylene (twice, each time for 5 min). After covered with xylene-based mounting media and cover glass, slices are observed under a microscope for image acquisition.

2.3 Comprehensive Targeted Metabolomic Analysis of Lung Tissue

200 μL of 80% methanol and 20 mg of lung tissue are added into a 1.5 mL Eppendorf tube pre-chilled to -80 ℃ and the tissue is ground for 1–2 min. After shaken at 4℃ for 1 min, the tissue is plated at -80 ℃ for 2 h. Centrifuged at 14,000g for 20 min, supernatant is dried with a low-temperature low-flow nitrogen blower. 200 μL of 70% methanol is used for precipitate redissolution. 120 μL of the supernatant into a sample vial for analysis after ice bath (10min) and centrifugated (12,500 rpm for 10 min at 4 ℃) twice -20 ℃ for 20 min.

2.4 Detection of Cytokines and Redox Related Indicators in Lung Tissue

ELISA kits are used to detect the expression of TNF-α, IL-1β, MIP-1α, GSH, GSH-Px, and GPX4 in tissues. After collecting the supernatant of tissue homogenates, 50 μL of standard, 1:4 diluted sample and and 50 μL of sample diluent are added into wells, respectively. 100 μL of antibody is added into the wells except the blank well. Sealed with a sealing tape, the wells are incubated at 37 ℃ in the dark for 1 hour. After five times of removing the liquid from the wells, washing or discarding, reagent A and reagent B in a 1:1 ratio is added into each well, and incubated in a microplate incubator for 15 minutes. Finally, 50 μL of stop solution is added to stop the reaction. The OD value is measured at 450 nm and the concentration of inflammatory factors in lung tissue using a standard curve is calculated. Divided by the protein concentration of lung tissue, concentration of inflammatory factors per unit protein is calculated.

2.5 Determination of Fe^{2+} in Lung Tissue

Following the removal of lung tissues from the -80 ℃ freezer, the tissues are weighed and washed three times with pre-chilled PBS (100 mg lung tissue/1 mL PBS). Five volumes of Assay Buffer and beads are then added, and ice bath sonication is performed for 5 minutes. The mixture is centrifuged at 16,000 g for 10 minutes, and the tissue supernatant is collected. The supernatant is transferred to microtubes.

To prepare a 100 μmol/L standard solution, 100 μL of a 1 mmol/L standard solution is added to a microtube containing 900 μL of detection buffer. Based on this concentration, consecutive serial dilutions are conducted to generate a series of standard solutions with varying

concentrations of 50, 25, 12.5, 6.25, 3.125, 1.5625, and 0 μmol/L.

A volume of 300 μL of Fe^{2+} solution is to be added to the Fe^{2+} sample tube, followed by the addition of 15 μL of 5% detection buffer. In the same manner, 300 μL of total iron solution is to be added to the total iron sample tube, along with 15 μL of 5% detection buffer. The sample tubes are then placed in a microplate incubator shaker for 15 minutes. Subsequently, other reagents are to be added according to the kit instructions, and the samples are to be incubated in the microplate incubator shaker for 1 hour to measure the OD values of each well at 593 nm. The concentrations of Fe^{2+} and Fe^{3+} in lung tissue are to be calculated using the standard curve equation, as indicated in Equation 1:

$$[Fe^{3+}] = [\text{Total Fe}] - [Fe^{2+}] \quad (1)$$

2.6 Expression of Iron Death-related Genes in Lung Tissues

2.6.1 Extraction of Total RNA from Lung Tissue and Cells A total of 20 mg of lung tissue from each group is transferred to 350 μL of Buffer RL and thoroughly mixed with beads. The mixture is centrifuged at 14,000 g for 3 minutes, after which 300 μL of the supernatant is removed and transferred to a new tube. Subsequently, 300 μL of 70% ethanol is added and vortexed. The collected cells are washed three times with pre-chilled PBS. Following this, 350 μL of Buffer RLT is added to each well and mixed thoroughly. Again, 350 μL of 70% ethanol is added and vortexed. An additional 350 μL of 70% ethanol is added to each well and vortexed again. Next, 600 μL of the sample solution from the lung tissue and 700 μL from the BEAS-2B cells are added to the adsorption column, which is then centrifuged at 10,000 g for 20 seconds, and the liquid in the collection tube is discarded. Subsequently, 600 μL of Buffer RW1, followed by 500 μL of Buffer RPE, and another 500 μL of Buffer RPE is added to the adsorption column, with centrifugation at 10,000 g for 20 seconds for the first two buffers and 10,000 g for 1 minute for the last buffer. A new tube is then used to add 40 μL of RNA-free water, which is centrifuged at 10,000 g for 1 minute to elute the RNA. The concentration and purity of the extracted RNA are measured using a NanoDrop™ Lite spectrophotometer.

2.6.2 mRNA Reverse Transcription In accordance with the guidelines provided by the PrimeScript RT Reagent Kit, the reverse transcription reaction system should be prepared (refer to Supplementary Table S3), and 1 μg of RNA should be reverse transcribed into cDNA. The

samples are then to be placed in a PCR thermal cycler and incubated at 50 ℃ for 15 minutes, followed by 85 ℃ for 5 minutes.

Supplementary Table S3. Reverse transcription system preparation table

Reagent	Amount
RNA	1 g
Oligo(dT)18 primer	1 μL
Water, nuclease-free	Up to 12 μL
5X Reaction Buffer	4 μL
Ribolock RNase Inhibitor (20 U/mL)	1 μL
10mmol/L dNTP Mix	2 μL
RevertAid M-MuLV RT (200 U/mL)	1 μL

2.6.3 qPCR Reaction The PCR reaction system is to be prepared according to the standard protocol for SYBR Green RT-qPCR (as detailed in Supplementary Table S3), and analysis is to be conducted using a real-time fluorescence quantitative PCR instrument (the primer sequences for the target genes are provided in Supplementary Table S4). The program settings include 40 reaction cycles, which consist of a denaturation step at 95 ℃ for 5 seconds, an annealing step at 55 ℃ for 30 seconds, and an extension step at 72 ℃ for 30 seconds. Changes in gene expression levels are to be evaluated using the $2^{-\Delta\Delta Ct}$ method.

Supplementary Table S4. qPCR reaction system

Reagent	Volume (μL)
SYBR qPCR SuperMix Plus	5
RNAae-free ddH2O	3.2
Forward Primer	0.4
Reverse Primer	0.4
cDNA	1

Supplementary Table S5. Mouse target gene sequences

Upstream Primer (5' - 3')	Downstream Primer (3' - 5')
AGCTGGGGCCGTCTGAGCCG	ATGTCCTTGGCTGAGAATTCGT
TGCAATCTGCATCTCCATGGCTCT	AAGCAGGAGAGGGCAACAAA
TGGAGTTGTATGCCTCCTACG	TGGAGAAAGTATTTGGCAAAGTT
CCTTTGGCTCATGTGCTGGAACT	CAGCGGCC ATAAGTGTGGGTTT
GAAGATGGGCAACCACCTGACC	TAGTCGTGCTTCAGAGTGAGGCG
TGACCTCAACTACATGGTCTACA	CTTCCCATTCTCGGCCTTG

2.7 Ethics Approval and Consent to Participate

2.7.1 Adherence to ARRIVE Guidelines We have provided a detailed explanation that we strictly followed the requirements of the ARRIVE guidelines throughout our experimental process.

This includes comprehensive reporting of our experimental design, sample size calculation randomization methods, blinding procedures(See Manuscript).

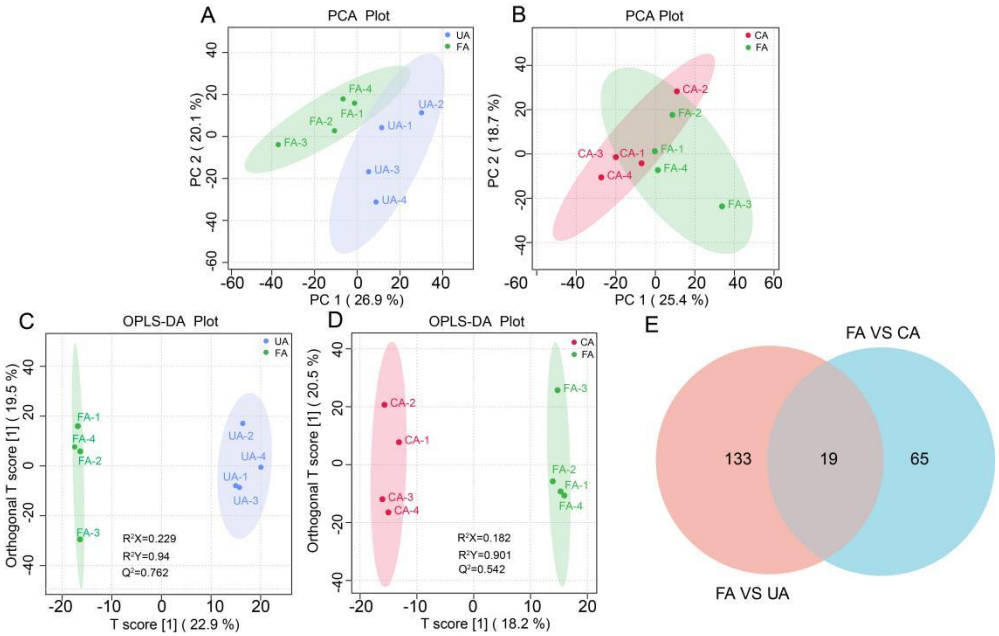
2.7.2 Specific Measures to Minimize Animal Suffering To minimize animal suffering, we implemented the following specific measures: We used appropriate anesthetics (e.g., isoflurane) prior to any procedures that could cause pain or distress, such as surgery or sampling. Post-procedural analgesics (e.g., meloxicam) were administered as needed to manage pain. Throughout the experiment, animals were regularly monitored for signs of distress or abnormal behavior. Any animals showing severe discomfort or unusual symptoms received immediate veterinary evaluation and treatment as necessary. Euthanasia was performed according to pre-approved criteria by the ethics committee when animals exhibited irreversible illness or distress. The euthanasia process was designed to be quick and painless, ensuring humane endpoints. To promote the well-being of the animals, we provided environmental enrichment, such as toys, nesting materials, and social housing where appropriate, to reduce stress and improve their quality of life during the study period. All personnel involved in handling the animals were trained in proper techniques to minimize stress and ensure gentle handling. Regular training sessions were conducted to maintain high standards of animal care.

3 RESULTS

3.1 Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant (OPLS-DA)

Perform PCA analysis on each group after deleting QC samples. The results showed a significant separation of metabolites between the FA group and the UA group, with the main components PC1 and PC2 explaining 26.9% and 20.1% of the changes, respectively (Supplementary Figure S1A). The main components PC1 and PC2 of metabolites in the FA and CA groups explained 25.4% and 18.7% of the changes, respectively (Supplementary Figure S1B). The score plot of orthogonal partial least squares discriminant analysis (OPLS-DA) shows that the FA group and UA group, as well as the FA group and CA group, are all distributed in different regions. Fit goodness value and predictive ability value (FA group and UA group: R2X = 0.229, R2Y = 0.94, Q2 = 0.762； The FA group and CA group (R2X = 0.182, R2Y = 0.901, Q2 = 0.542) indicate that the OPLS-DA model has good fitting effect and predictive ability (see Supplementary Figure S1C-D). There were a total of 19 differential metabolites between the FA and CA groups (Supplementary

Figure S1E).



Supplementary Figure S1. Using PCA and OPLA methods to analyze differential metabolites and volcano map. (A)Comparing the FA group with the UA group, the primary components PC1 and PC2 explained 26.9% and 20.1% of the variance, respectively. (B)Comparing the FA group with the CA group, the primary components PC1 and PC2 explained 25.4% and 18.7% of the variance, respectively. (C) In the comparison between the FA group and the UA group, the model explained 22.9% of the variance in the independent variable and 94% of the variance in the dependent variable. (D) In the comparison between the FA group and the CA group, the model explained 18.2% of the variance in the independent variable and 90.1% of the variance in the dependent variable.(E)Comparison between groups: The Venn diagram displays the shared differential metabolites found in both groups.

3.2 Enrichment Analysis of Metabolic Pathways

Perform pathway enrichment analysis on differential metabolites in the UA and CA groups using the KEGG database. The results showed that there were 7 differential metabolic pathways associated with ferroptosis in the UA group, including glycolysis/gluconeogenesis, pentose phosphate pathway, xanthine and xanthine recovery, glutamate degradation II, phenylalanine metabolism, riboflavin metabolism, and acetyl CoA biosynthesis III; Four differential metabolic pathways were identified in the CA group, all of which are associated with ferroptosis,

including glycolysis/gluconeogenesis, pentose phosphate pathway, riboflavin metabolism, and xanthine metabolism.

Supplementary Table SI 6. Metabolic pathways and enriched metabolites

Group	Pathway	Enriched Metabolites
FA VS UA	Lycolysis/gluconeogenesis	D1-glyceraldehyde-3-phosphate
	Phosphopentose pathway	D1-glyceraldehyde-3-phosphate
	Xanthine and xanthine recovery	Xanthine
	Glutamate degradation II	Oxalacetic acid
	Riboflavin metabolism	Riboflavin
	Phenylalanine metabolism	L-phenylalanine, L-tyrosine, L-methionine
	Acetyl CoA biosynthesis III	Biotin
FA VS CA	Lycolysis/gluconeogenesis	D1-glyceraldehyde-3-phosphate
	Phosphopentose pathway	D1-glyceraldehyde-3-phosphate
	Riboflavin metabolism	Riboflavin
	Xanthine and xanthine recovery	Xanthine

3.3 The Expression Levels of Intracellular Iron

As shown in Supplementary Table S7, compared with the control group, the intracellular Fe^{2+} and Fe^{2+}/Fe^{3+} levels in the UA and CA groups were significantly upregulated, indicating that $PM_{2.5}$ can significantly induce iron overload, potentially leading to ferroptosis in cells.

Supplementary Table S7. Different levels of intracellular iron

Groups	Fe^{2+}	Fe^{3+}	Fe^{2+}/Fe^{3+}
FA	0.20 ± 0.09	0.47 ± 0.03	0.43 ± 0.18
UA	$0.43 \pm 0.18^{***}$	$1.17 \pm 0.12^{***}$	$1.02 \pm 0.18^{**}$
CA	$0.96 \pm 0.32^{**}$	0.46 ± 0.13	$2.08 \pm 0.26^{**}$

Note. Significance was determined at $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$.