



Article

The Impact of Fine Particulate Matter on Embryonic Development

Chia-Ta Wu ^{1,2}, Ting-Shuan Wu ^{3,*,†}  and Min-Sho Ku ^{1,4,*,†} 

¹ School of Medicine, Chung Shan Medical University, No. 110, Sec. 1, Chien-Kuo N. Road, Taichung 402, Taiwan; allenpig1102@gmail.com

² Department of Emergency Medicine, Changhua Christian Hospital, Changhua 500, Taiwan

³ Department of Biomedical Sciences, Chung Shan Medical University, Taichung 402, Taiwan

⁴ Division of Allergy, Asthma and Rheumatology, Department of Pediatrics, Institute of Allergy, Immunology, and Rheumatology, Chung Shan Medical University Hospital, No. 110, Sec. 1, Chien-Kuo N. Road, Taichung 402, Taiwan

* Correspondence: ab118tw@gmail.com (T.-S.W.); a129184@yahoo.com.tw (M.-S.K.); Tel.: +886-4-3609-5913 (T.-S.W.); +886-4-2473-9595 (ext. 34947) (M.-S.K.)

† These authors contributed equally to this work.

Abstract: Airborne fine particulate matter (PM_{2.5}) in air pollution has become a significant global public health concern related to allergic diseases. Previous research indicates that PM_{2.5} not only affects the respiratory system but may also induce systemic inflammation in various tissues. Moreover, its impact may vary among different populations, with potential consequences during pregnancy and in newborns. However, the precise mechanisms through which PM_{2.5} induces inflammatory reactions remain unclear. This study aims to explore potential pathways of inflammatory responses induced by PM_{2.5} through animal models and zebrafish embryo experiments. In this study, zebrafish embryo experiments were conducted to analyze the effects of PM_{2.5} on embryo development and survival, and mouse experimental models were employed to assess the impact of PM_{2.5} stimulation on various aspects of mice. Wild-type zebrafish embryos were exposed to a PM_{2.5} environment of 25–400 µg/mL starting at 6 h after fertilization (6 hpf). At 6 days post-fertilization, the survival rates of the 25, 50, 100, and 200 µg/mL groups were 100%, 80, 40%, and 40%, respectively. Zebrafish embryos stimulated with 25 µg/mL of PM_{2.5} still exhibited successful development and hatching. Additionally, zebrafish subjected to doses of 25–200 µg/mL displayed abnormalities such as spinal curvature and internal swelling after hatching, indicating a significant impact of PM_{2.5} stimulation on embryo development. In the mouse model, mice exposed to PM_{2.5} exhibited apparent respiratory overreaction, infiltration of inflammatory cells into the lungs, elevated levels of inflammatory response-related cytokines, and inflammation in various organs, including the liver, lungs, and uterus. Blood tests on experimental mice revealed increased expression of inflammatory and chemotactic cytokines, and GSEA indicated the induction of various inflammatory responses and an upregulation of the TNF-α/NFκB pathway by PM_{2.5}. Our results provide insights into the harmful effects of PM_{2.5} on embryos and organs. The induced inflammatory responses by PM_{2.5} may be mediated through the TNF-α/NFκB pathway, leading to systemic organ inflammation. However, whether PM_{2.5}-induced inflammatory responses in various organs and abnormal embryo development are generated through different pathways requires further study to comprehensively clarify and identify potential treatment and prevention methods.

Keywords: air pollution; fine particulate matter; inflammatory responses; pathway



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1. Introduction

Air pollutants refer to one or more substances present in the atmosphere, including particulate matter, gases, and chemicals. These pollutants originate from various human activities and natural phenomena. The concentration, quantity, and duration of exposure

to air pollutants may pose health hazards to humans [1]. The primary route of contact and exposure to air pollutants is through inhalation into the respiratory system. These pollutants may lead to cell inflammation, oxidative stress, immune responses, gene mutations, and the onset of diseases [1,2].

The World Health Organization (WHO) has identified air pollution as a significant factor severely affecting human health. Not only is it classified as a carcinogen, but it also poses a high risk and impact on individuals with allergies [1]. According to a 2013 study by the International Agency for Research on Cancer (IARC), particulate matter is listed as one of the primary environmental factors causing cancer-related deaths [3]. A more recent WHO report indicates that approximately 7 million people die each year due to indoor and outdoor air pollution, with a strong correlation between air pollutants and cardiovascular diseases, acute and chronic respiratory diseases, and cancer [1].

Our previous studies [4,5] and others [6–9] suggest a close correlation between air pollutants, especially particulate matter (such as PM_{2.5} and PM₁₀), gases (such as nitrogen dioxide and ozone), and the generation and exacerbation of various diseases. Diseases induced by exposure to air pollutants include respiratory system disorders such as chronic obstructive pulmonary disease (COPD), bronchitis, and asthma [1]; cardiovascular diseases such as heart disease, stroke, and heart failure [10]; and neurological disorders such as brain damage, stroke, Alzheimer's, and Parkinson's disease [11], as well as potential impacts on child development and fetal health [12,13].

In our daily lives, we unavoidably inhale or come into contact with particulate matter through both the nasal cavity and skin. While most particulate matter can be cleared by mechanisms such as nasal mucosal cilia and the skin barrier, PM_{2.5}, with a diameter smaller than 2.5 µm, can linger within lung tissues upon inhalation or contact. It can breach the respiratory barrier, gaining access to the circulatory system and causing systemic diffusion. Consequently, a growing body of research suggests that PM_{2.5} not only influences respiratory organs but also potentially affects the health of diverse tissues [14].

Previous studies [10,14,15] have pointed to several potential health effects that PM_{2.5} may have, such as increasing the incidence of diabetes and cardiovascular diseases [10,14]. Concerning the adverse effects of PM_{2.5} exposure on pregnancy, it has been proposed that exposure during gestation could disrupt the endocrine balance in pregnant women [15]. Concerning the adverse effects of PM_{2.5} exposure on pregnancy, it has been proposed that exposure during gestation could disrupt the endocrine balance in pregnant women. This disruption may affect the delivery of oxygen and nutrients to the fetus through the placenta, potentially resulting in preterm birth and low birth weight [15]. Additionally, prenatal exposure to PM_{2.5} may lead to impaired neurodevelopment in newborns [16].

Other studies [17,18] have demonstrated that maternal exposure to PM_{2.5} during pregnancy could impact placental growth factor binding, consequently affecting placental function. Moreover, prenatal exposure to PM_{2.5} has been linked to an elevated risk of childhood asthma and a potential association with neonatal jaundice induction, as indicated by our prior investigation [19].

Even with low PM_{2.5} exposure levels, potential health risks persist. A prior study [20] suggested that PM_{2.5} can induce inflammation and elevate oxidative stress levels in the body, resulting in damage to proteins, lipids, and even deoxyribonucleic acid (DNA). However, the exact mechanisms underlying the effects of PM_{2.5}, oxidative stress, and respiratory inflammatory diseases still require further elucidation.

An experimental study [21] observed that exposure to PM_{2.5} triggers inflammatory responses, alters gene expression, and enhances protein secretion in both human and animal cells in a dose-dependent manner. This results in elevated levels of proinflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1) [22,23]. Additionally, PM_{2.5} exposure has been associated with a significant increase in eosinophils and elevated levels of albumin, urea, and α 1-antitrypsin in asthmatic children [24].

Exposure to PM_{2.5} has also demonstrated an increase in macrophage infiltration and the upregulation of pro-inflammatory genes such as TNF- α , MCP1, and leptin in mice [25]. Although one study [26] suggests that PM_{2.5} exposure may reduce the expression of toll-like receptor 4 (TLR4) or toll-like receptor 2 (TLR2), type 2 T helper (Th2)-related cytokines like interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10), and interleukin-13 (IL-13) were found to remain significantly elevated. However, type 1 T helper (Th1) cytokines such as interferon gamma (IFN- γ), which balance Th2 responses, showed a decrease [27]. Therefore, exposure to PM_{2.5} is likely to cause changes in the immune system.

Moreover, exposure to PM_{2.5} may activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway by phosphorylating nuclear p65 and cytoplasmic inhibitory- κ B kinase- α (IKK- α). This can lead to the dose-dependent binding of nuclear p65/p50 DNA complexes in human lung epithelial cells, triggering inflammatory responses and potentially causing abnormalities in embryonic development [28,29]. One study also indicates that exposure to PM_{2.5} in children may lead to elevated levels of various inflammatory biomarkers in the bloodstream [30]. Furthermore, the NF- κ B activation induced by PM_{2.5} and its associated inflammatory responses could potentially be prevented proactively.

We have encountered additional studies on PM_{2.5} [31–37], which highlight its undeniable impact on the respiratory system and lungs [35]. Concerningly, its effects on children's health might outweigh those observed in adults [33,36]. Moreover, there is evidence suggesting that PM_{2.5} exposure can induce chronic inflammation in women, potentially disrupting ovarian function [37]. This poses risks not only to fertility but also to the health of newborns born to affected women [33]. Regarding cardiovascular diseases, exposure to PM_{2.5} may worsen existing conditions or precipitate their onset [34]. Additionally, some research underscores the adverse effects of PM_{2.5}, whether it infiltrates through food contamination or indoor work environments, on human health [31,32].

Viewing this comprehensively, exposure to PM_{2.5} extends its influence beyond specific organs, potentially triggering systemic inflammation. The impact of PM_{2.5} on diseases is broad and varied, with potentially more severe effects on specific demographics, including the elderly, pregnant women, children, individuals with respiratory allergies, and asthma patients [38,39].

Nonetheless, further investigation is imperative to elucidate the precise exposure levels or PM_{2.5} components responsible for severe inflammation.

To improve the effectiveness of preventive and interventional measures in both medical and preventive healthcare, it is crucial to conduct comprehensive research and analysis into the mechanisms by which air pollutants induce different diseases. This study aims to utilize embryo experiments and animal models to further explore the pathway analysis of PM_{2.5}-induced systemic inflammation. The goal is to provide more accurate insights and guidelines for preventing and managing PM_{2.5}-induced diseases in the future.

2. Results

2.1. Survival Analysis of Zebrafish Embryos Exposed to PM_{2.5}

Figure 1 illustrates the condition of zebrafish embryos under PM_{2.5} stimulation, including survival status, heart development, hatch rate, and overall morphology. Zebrafish embryos at 6 hpf were exposed to various PM_{2.5} concentrations to determine the optimal concentration and exposure time for subsequent experiments. Wild-type zebrafish embryos were exposed to PM_{2.5} concentrations ranging from 25 to 400 μ g/mL. The survival rate was observed until 6 days post-fertilization (dpf). As shown in Figure 1A, embryos exposed to 400 μ g/mL all died by 2 dpf, while at 6 dpf, the survival rates for the 25, 50, 100, and 200 μ g/mL groups were 100%, 80%, 40%, and 40%, respectively. Embryos exposed to 25 μ g/mL did not show any mortality during the experiment. However, embryos treated with concentrations greater than 25 μ g/mL demonstrated lower heartbeats, spinal curvature, or death at the stage of 6 dpf (Figure 1B,D). The hatch rate in each group did not show a significant decrease (Figure 1C). Despite successful hatching, zebrafish exposed to

PM2.5 stimulation exhibited some morphological abnormalities, such as spinal curvature (Figure 1E).

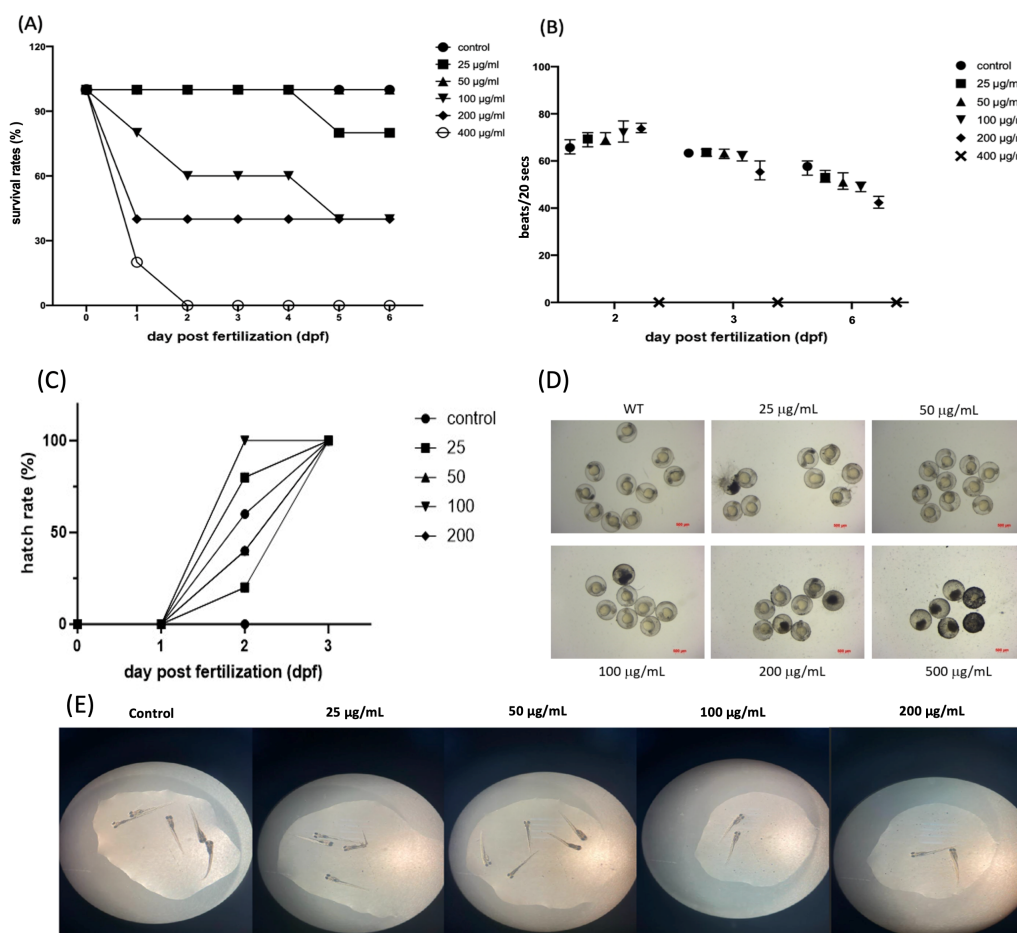


Figure 1. Illustration of the results of observing the survival rate of zebrafish embryos after exposure to PM2.5. (A) illustrates the embryo survival rate six days post-exposure, (B) shows the heartbeat count during embryo development, (C) presents the embryo hatch rate, (D) presents the appearance of zebrafish embryos under different dosage treatments, and (E) depicts the morphology of zebrafish after successful hatching.

2.2. Effect of PM2.5 in Mouse Model

Based on these results, exposure to PM2.5 indeed induces respiratory symptoms in experimental mice (Figure 2). From the airway resistance measurements in Figure 2B, it can be observed that exposure to PM2.5 leads to a significant increase in airway resistance, with a more pronounced effect observed in the pregnant group. The cell count results from the bronchoalveolar lavage fluid (BALF) in Figure 2C reveal a significant increase in inflammatory cells in the airways. It is worth noting that the increase in neutrophil count appears to be higher than that of eosinophils, suggesting that the inflammatory response triggered by airway pollutants may differ from that caused by typical allergens.

Airway hyperresponsiveness testing is based on clinical testing methods, including measuring respiratory values after inhaling different concentrations of methacholine.

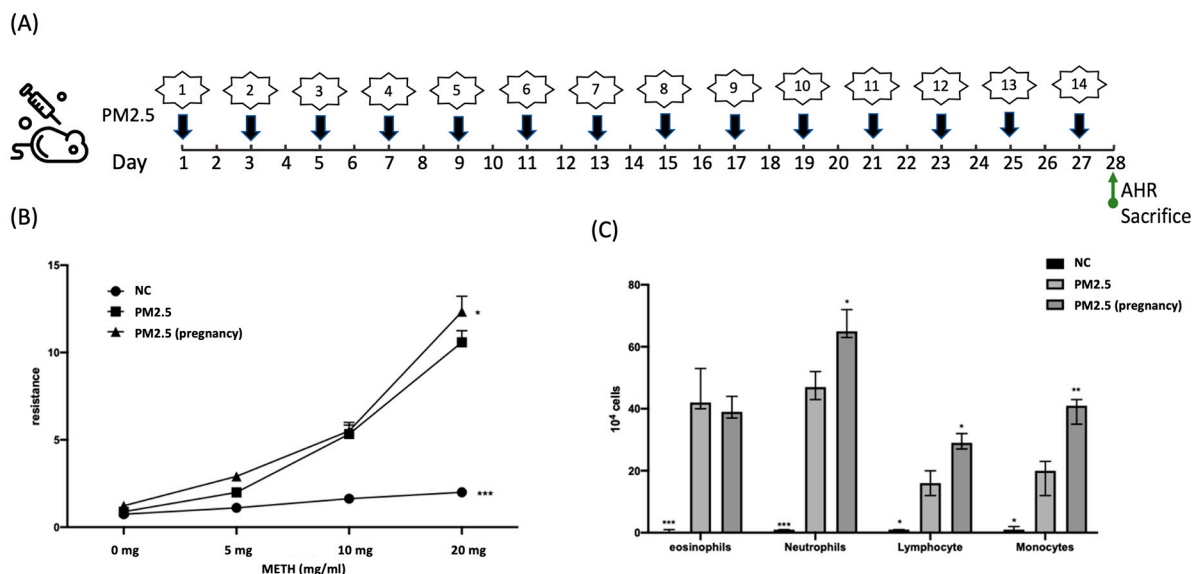


Figure 2. Illustration of the results of PM2.5 stimulation in wild-type BALB/c mice and pregnant mice. (A) outlines the experimental procedure for PM2.5 stimulation in mice. (B) displays respiratory resistance measurements, and (C) presents various inflammatory cell counts in the bronchoalveolar lavage fluid (BALF) of experimental mice. Statistical analysis compared all data with the group exposed only to PM2.5. Asterisks denote significance levels as follows: * indicates a p -value less than 0.05, ** indicates a p -value less than 0.01, and *** indicates a p -value less than 0.001.

2.3. Effect of PM2.5 on Gene Expression of Th1/Th2/Th17/Treg in Mouse Model

Figure 3 presents the polymerase chain reaction (PCR) analysis performed on the fresh lung tissues collected from experimental mice. PCR analysis revealed significantly upregulated expression of both GATA binding protein 3 (GATA3) and RAR-related orphan receptor- γ (RoR γ t) in mice exposed to PM2.5 stimulation, regardless of pregnancy status. Pregnant mice in the experimental group notably demonstrated markedly higher expression of the RoR γ t gene associated with T helper 17 cells (Th17) compared to other groups. In the pregnant mouse group stimulated by PM2.5, regulatory T (Treg) cells also exhibited higher expression levels. These results suggest that different populations undergo distinct inflammatory responses following exposure to PM2.5 stimuli.

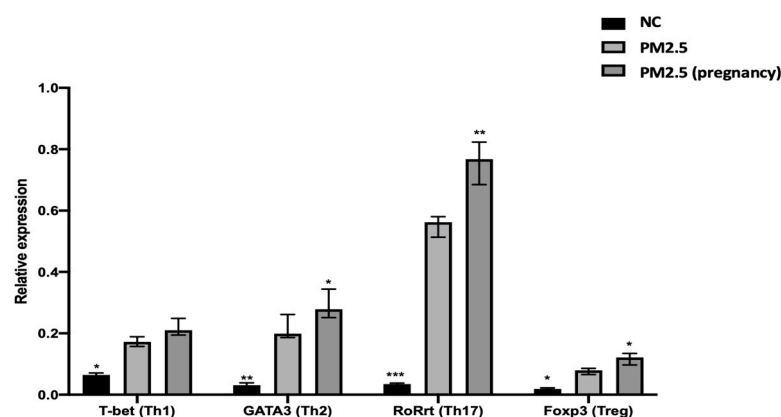


Figure 3. Depiction of the PCR identification analysis of Th1/Th2 and Th17/Treg in the lungs of wild-type BALB/c mice and pregnant mice after exposure to PM2.5. Statistical analyses were conducted, comparing all data with the PM2.5-exposed group. Asterisks denote significance levels as follows: * indicates a p -value less than 0.05, ** indicates a p -value less than 0.01, and *** indicates a p -value less than 0.001.

2.4. Effect of PM2.5 on Inflammation in Various Tissues

Figure 4 shows the liver, lung, and uterus tissues collected and processed into pathological sections. These sections were stained with H&E to assess tissue inflammation. In the liver, compared to the normal control (NC) mice and non-pregnant mice stimulated with PM2.5, pregnant mice showed a more pronounced inflammatory condition, as indicated by black arrows. In the lung, airway inflammation was evident with H&E staining. Pregnant mice exposed to PM2.5 exhibited significantly more severe goblet cell hyperplasia and airway inflammation compared to non-pregnant mice exposed to PM2.5. In the uterus, H&E staining revealed signs of inflammation under PM2.5 stimulation. Additionally, sections of the uterus from pregnant mice displayed multiple areas of inflammatory conditions.

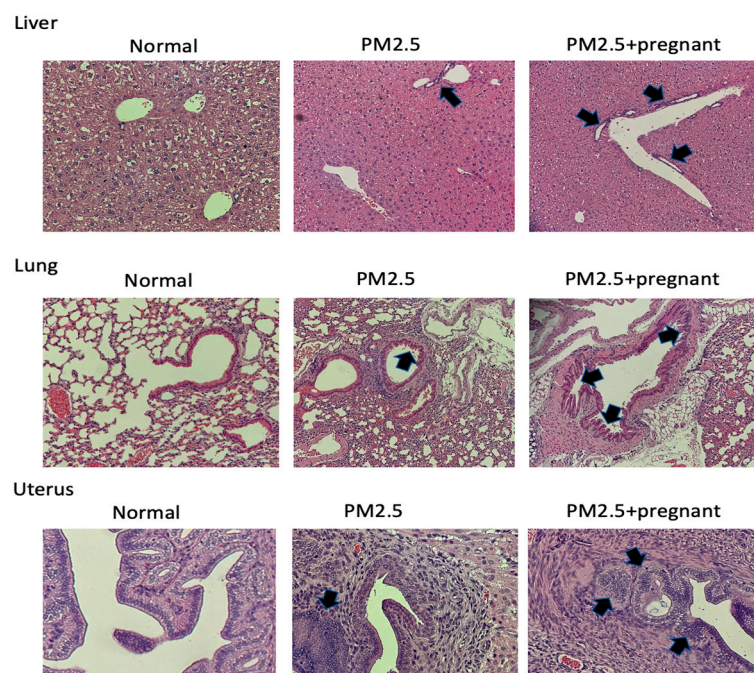


Figure 4. Display of the histological sections stained with hematoxylin and eosin (H&E) of the lungs, liver, and uterus of experimental mice. According to these research findings, it is evident that PM2.5 significantly induces inflammation in various tissues of the experimental mice.

2.5. GSEA of PM2.5 in Animal Model

Gene set enrichment analysis (GSEA) was used to analyze the impact of PM2.5 on various cytokine and chemokine indicators in mice. Pathways identified include INFLAMMATORY_RESPONSE and TNFA_SIGNALING_VIA_NFKB. The summarized potential participating biological markers for each pathway are detailed in Table 1. In this part, a cytokine array assay was employed to assess the peripheral blood from mice by testing approximately 111 cytokines and chemokines. Subsequently, GSEA was conducted to deduce factors influencing the observed effects.

Table 1. Illustration of the various participating genes classified through gene set enrichment analysis (GSEA) after conducting a cytokine array test on peripheral blood from experimental mice.

HALLMARK_INFLAMMATORY_RESPONSE		HALLMARK_TNFA_SIGNALING_VIA_NFKB	
PM2.5	PM2.5P	PM2.5	PM2.5P
Csf1	Cxcl5	Csf1	Cxcl5
IL12b	IL12b	IL12b	IL12b
Csf3	Csf1	Cxcl5	Csf1
Cxcl5	Ccl17	Cxcl11	Vegfa
Cxcl11	IL10	Serpine1	Cxcl2

Table 1. Cont.

HALLMARK_INFLAMMATORY_RESPONSE		HALLMARK_TNFA_SIGNALING_VIA_NFKB	
Cxcl9	Serpine1	Ptx3	Serpine1
Serpine1	Sele	Cxcl10	Ptx3
Sele	Csf3	Vegfa	Cxcl1
Ccl17	Cxcl9	Ccl5	Il23a
Cxcl10	Ccl5	Ccl20	Ccl5
Ccl5	F3	Cxcl12	Csf2
Ccl20	Ccl22	F3	F3
Il10	Icam1	Il23a	Icam1
F3	Cxcl11	Csf2	Cxcl11
CCl22	Cxcl10	Icam1	Cxcl10
Icam1	Ccl20	Cxcl1	Ccl20

“PM2.5P” represents mice that were pregnant and exposed to PM2.5 stimulation.

3. Discussion

Based on our established air pollution animal experimental model, we observed various inflammatory conditions, confirming the success of this model. The research results indicate that the inflammatory response induced by ambient PM2.5 may differ among different populations.

Furthermore, in the experimental model involving pregnant mice, we gained insights into the impact of PM2.5 exposure on the health of pregnant mothers and the repercussions on the overall health of offspring when respiratory allergic inflammatory diseases are induced during pregnancy. Our study found that PM2.5 may induce inflammation in various tissues and organs of mice, extending beyond respiratory tract inflammation.

Based on the current research results, we propose that in the presence of PM2.5, the induced inflammatory response may shift from the Th2 inflammatory response to non-Th2 inflammatory mechanisms. Pathway analysis using GSEA reveals potential involvement of inflammatory responses and the TNF- α /NF- κ B pathway. However, further investigation is needed to determine whether these pathways predominantly contribute to the occurrence and exacerbation of respiratory inflammatory allergic diseases or induce systemic inflammation. Additionally, exploring potential alternative mechanisms is essential.

In our study, we extensively utilized zebrafish embryo experiments. This approach allows for a more precise analysis of the impact of PM2.5 stimulation during the embryonic period on later development. It also aligns with animal welfare considerations, using species with similar but distinct responses for experimentation, thereby enhancing experimental accuracy.

GSEA results indicate that exposure to PM2.5 in mice may induce the elevation of various cytokines, which are still believed to be inflammatory responses triggered by the NF- κ B pathway. Although numerous fragmentary studies continuously reveal the influence of PM2.5 on various diseases, the specific mechanisms of its effects remain unclear due to a lack of direct evidence from animal modeling experiments and even fewer embryonic experiments. Our results provide insights into the harmful effects of PM2.5 on embryos and organs. However, whether PM2.5-induced inflammatory responses in various organs and abnormal embryo development are generated through different pathways requires further study to comprehensively clarify and identify potential treatment and prevention methods.

In terms of study limitations, the composition of PM2.5 may indeed vary between batches. The current research findings demonstrate that PM2.5 obtained from the manufacturer and used in our study can indeed induce systemic inflammatory responses in experimental mice and zebrafish embryos. These findings underscore the similarity in the principal pathways activated by PM2.5 exposure, highlighting the undeniable impact of PM2.5-induced inflammation. However, in the future, we will further explore the effects of different compositions of PM2.5 on health through compositional analysis studies.

4. Materials and Methods

4.1. Mice

Wild-type female BALB/c mice aged 6 weeks and pregnant mice (National Laboratory Animal Center, Taipei, Taiwan) were used for the study. The mice were housed individually in cages with ad libitum access to food and water. All animal care and housing procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chung Shan Medical University (Animal Experiment Approval code: 2855).

4.2. Test Species and Husbandry

Wild-type (WT) AB strain zebrafish (*Danio rerio*) were provided by the Taiwan Zebrafish Core Facility at Academia Sinica (TZCAS) and maintained at 28 °C with a 14-h light/10-h dark cycle. Adult care and breeding were performed in compliance with relevant laws and institutional guidelines, and the Institutional Animal Care and Use Committee approved the experiments (IACUC approval no. 113021). Fish were fed three times per day with dry feed (Taikong Inc., Taipei, Taiwan). To generate embryos, adults were placed in spawning tanks in the afternoon and then spawned following the onset of light the next day. The embryos were collected and maintained in embryo media (60 µg/mL Instant Ocean® (Blacksburg, VA, USA) sea salts) until the time of exposure. Embryos were staged using the criteria of Kimmel [40].

4.3. Protocol for PM2.5 Exposure and Challenge in Mice

PM2.5 was purchased from the Sigma-Aldrich Company, U. S. (NIST® RM 8785, St. Louis, MO, USA). The PM2.5 challenge experiment lasted for a total of 28 days. Beginning with the first day of the experiment, each experimental mouse received PM2.5 stimulation through nasal inhalation every other day, resulting in a total of 14 inhalations throughout the experiment (as shown in Figure 2A). The dosage of PM2.5 administered in each inhalation was 20 µg/mL. Subsequently, on the day following the 14th PM2.5 inhalation, the mice's airway hyperresponsiveness (AHR) was evaluated using the Buxco research system, and the animals were sacrificed after the assessment.

4.4. Toxin Exposure in Zebrafish

PM2.5 was purchased from the Sigma-Aldrich Company, U. S. (NIST1650B). PM2.5 was first dissolved in normal saline at a concentration of 200 mg/mL. The zebrafish embryos were exposed to vehicle (0.01 M phosphate buffered saline (PBS)) or various concentrations of PM2.5 (0, 25, 50, 100, 200, and 400 µg/mL) at 6 h post-fertilization (hpf).

4.5. Airway Resistance Assessment

The FinePointe RC system (Data Sciences International, DSI™) was used to measure airway resistance. To establish a baseline, mice were initially challenged with aerosolized normal saline, followed by ascending doses of methacholine (METH) (0, 5, 10, and 20 mg/mL). Compliance values were recorded for a 3-min period following each challenge.

4.6. Preparation of Bronchoalveolar Lavage (BAL) Fluid and Analysis of Cellularity

Following the measurement of airway responsiveness, BAL was performed immediately. Mice were deeply anesthetized with 50 mg/kg of pentobarbital sodium administered intraperitoneally and euthanized by exsanguination from the abdominal aorta. The trachea was cannulated with a polyethylene tube, and the lungs were lavaged three times with 1.0 mL of physiological saline, resulting in a total fluid removal of 3.0 mL.

The BAL fluid was filtered through wet gauze (4 × 4 inches), and total cell count and viability were determined by trypan blue exclusion. The BAL fluid was then centrifuged at 150× g for 10 min, and the obtained pellet was suspended in 4 mL of physiological saline. Total cell counts in the BAL fluid were determined in duplicate using a hemocytometer (improved Neubauer counting chamber). Additionally, a 100 µL aliquot was centrifuged in a cytocentrifuge (model 2 Cytospin; Shandon Scientific Co., Pittsburgh, PA, USA),

and differential cell counts were performed on the centrifuged preparations stained with Diff-Quick; 500 or more cells were counted for each animal at a magnification of $\times 1000$ (oil immersion).

4.7. Cytokine Measurement

Cytokine and chemokine levels in serum were assessed using the Proteome Profiler Mouse XL Cytokine Array (ARY028), following the manufacturer's protocol (R&D Systems, Inc., San Diego, CA, USA). All reagents and samples were equilibrated to room temperature prior to use, and the cytokine array kit was operated according to the manufacturer's instructions.

4.8. Histological Analysis

Following the final exposure, the mice were euthanized to obtain lung tissue samples for morphological analysis. The trachea and right lung were filled with a fixative solution containing 0.8% formalin and 4% acetic acid via a ligature around the trachea. The right lung tissue was then embedded in paraffin and cut into 5 μm sections for examination. Hematoxylin and eosin (H&E) staining was performed to evaluate airway inflammation.

4.9. Statistical Analysis

All data were analyzed using GraphPad Prism 6 for Windows. The data are expressed as median $\pm$ IQR. For variables, the Kruskal–Wallis test (non-parametric version of ANOVA) was used for comparison. Differences with p -values less than 0.05 were regarded as statistically significant.

5. Conclusions

Our air pollution animal experimental model revealed that ambient PM_{2.5} can induce diverse inflammatory conditions, affirming the model's success. Variations in the inflammatory response to PM_{2.5} among different populations were also evident. Additionally, our zebrafish embryo experiments provided precise insights into the embryonic impact of PM_{2.5}, aligning with animal welfare considerations and enhancing experimental accuracy.

In pregnant mice, our studies illuminated the impact of PM_{2.5} exposure on maternal health and highlighted potential implications for offspring health, especially concerning respiratory allergic inflammatory diseases during pregnancy. We further confirmed PM_{2.5}-induced inflammation across multiple tissues and organs in mice, extending beyond the respiratory tract. PM_{2.5} exposure may lead to elevated cytokines, indicating NF- κ B pathway-triggered inflammatory responses.

While our findings contribute to understanding the harmful effects of PM_{2.5}, elucidating the involved pathways and identifying potential treatments require further investigation, particularly in animal modeling and embryonic studies.

Author Contributions: Conceptualization was primarily undertaken by C.-T.W. and M.-S.K.; methodology was the responsibility of T.-S.W.; software development was overseen by M.-S.K.; validation efforts were jointly conducted by C.-T.W. and T.-S.W.; the original draft of the manuscript was prepared by C.-T.W.; and the review and editing process was carried out by T.-S.W. and M.-S.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), and the IACUC approval nos. are 2855 and 113021.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest: The study had no conflicts of interest.

Abbreviations

AHR	airway hyperresponsiveness
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
COPD	chronic obstructive pulmonary disease
DNA	deoxyribonucleic acid
dpf	days post-fertilization
GATA3	GATA binding protein 3
GSEA	gene set enrichment analysis
H&E	hematoxylin and eosin
hpf	hours post-fertilization
IARC	International Agency for Research on Cancer
IFN- γ	interferon gamma
IKK- α	inhibitory- κ B kinase- α
IL-1 β	interleukin-1 beta
IL-4	interleukin-4
IL-5	interleukin-5
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IL-13	interleukin-13
TLR4	toll-like receptor 4
TLR2	toll-like receptor 2
MCP-1	monocyte chemoattractant protein-1
METH	methacholine
NC	normal control
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RoRyt	RAR-related orphan receptor- γ
TNF- α	tumor necrosis factor-alpha
Th1	type 1 T helper
Th2	type 2 T helper
Th17	T helper 17 cells
Treg	regulatory T
WHO	World Health Organization
WT	wild-type

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