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Expression of HMGB1 in maternal exposure to fine particulate air pollution induces lung injury in rat offspring assessed with micro-CT

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ABSTRACT

Objectives: Maternal particulate matter with less than 2.5 μm in diameter (PM2.5) is associated with an increased risk for acute lower respiratory infections and allergic airway inflammation; however, its effect on the developing lung remains unclear. The aim of this study is to determine the effect of maternal PM2.5 during pregnancy on lung development in offspring.

Methods: Timed pregnant Sprague-Dawley rats were treated with PM2.5 (0.1, 0.5, 2.5, or 7.5 mg/kg) once every 3 days from day 0–18 of pregnancy and delivered at term. Lungs were obtained on postnatal day 0, the structure of the lung was analyzed by quantitative micro-computed tomography (CT) and the levels of proinflammatory cytokines were analyzed using enzyme-linked immunosorbent assay (ELISA). The expression of high mobility group box-1 (HMGB1) was also detected by immunohistochemistry, Western blotting, and quantitative RT-PCR.

Results: Ground-glass opacity and high-density volumes in CT slice images of maternal PM2.5-exposure rats were observed. The concentrations of IL-1, IL-6 and TNF-α were significantly increased by 2.36-, 3.91- and 4.36-fold, respectively, in the rats of the PM-7.5 group compared with the rats in the control group. The PM2.5-treated rats showed a significant upregulated expression of HMGB1 in lungs.

Conclusions: PM2.5 exposure during pregnancy results in lung inflammation in offspring mediated by increased HMGB1 expression, followed by upregulated IL-1, IL-6 and TNF-α secretions, which may contribute to the development of inflammatory lung diseases in later life.

1. Introduction

Gestation is a sensitive period regarding exposure to poisonous substances. The developmental origins of health and disease, also called Barker's Hypothesis, emphasized the concept that the intrauterine environment has widespread consequences for later health, from the oocyte to the infant and adult [1–3].

Fine particular matter with an aerodynamic diameter of 2.5 μm (PM2.5) is a heterogeneous mixture of solid and liquid particles emitted from a variety of sources, such as street traffic exhaust, diesel exhaust and cigarette smoke. Epidemiological studies have reported that maternal exposure to various air pollutants including street traffic exhaust [4], cigarette smoke [5] and particulate matter [6] during pre- and post-natal stages of life are risk factors for the development of acute lower respiratory infections and allergic airway inflammation. Other studies also indicated that maternal exposure to particulate matter increased fetal placental cytokine expression and augmented ozone exposure-induced pulmonary proinflammatory cytokine secretion [7]. In addition, experimental studies have shown that the offspring of pregnant mice exposed to diesel particles exhibit stronger allergic immune responses or airway inflammation in the offspring of pregnant exposed dams [8]. Therefore, prenatal exposure to environmental contaminants may induce pulmonary inflammation.

High mobility group box-1 (HMGB1), discovered in the late 1990s as a nonhistone protein binding with DNA, has been considered a prototypic alarmin [9]. Growing evidence shows that HMGB1 is an important mediator of lung inflammation and tissue damage [10,11]. HMGB1 can modulate cigarette smoke-induced inflammatory mediator expression in human macrophages [12], as well as promote the chemotaxis and production of cytokines interacting with Toll-like receptors

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(TLR-2, TLR-4) and receptors for advanced glycation end-products to induce the nuclear translocation of NF-κB [13–15].

Although the detrimental impacts of environmental contaminant exposure are well documented, only a few studies have reported the impact of prenatal exposure to fine particulate matter, especially in animal models. Our previous study showed that maternal exposure to PM2.5 induced epithelial-to-mesenchymal transition (EMT), resulting in postnatal pulmonary dysfunction [16]. Several studies have demonstrated that cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and transforming growth factor (TGF)-β, are capable of inducing EMT [17–19]. The purpose of this study was to explore the role of inflammation in maternal PM2.5-exposed offspring. Micro-computed tomography (CT) was used to investigate lung injury in offspring rats, which was a more intuitive method. Furthermore, whether HMGB1 was involved in lung inflammation following maternal exposure to PM2.5 was examined. The levels of proinflammatory cytokines that are associated with chronic inflammation and lung injury, including TNF-α, IL-6, and IL-1, were also measured.

2. Materials and methods

2.1. PM2.5 sampling and processing

PM2.5 samples used for this study were collected on nitrocellulose filters using particulate sampler by the Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, from December 2012 to February 2013 at Kehua Road, Guangzhou, China. Filters were weighed and cut into squares of 1–2 cm^2, and then the substances on the surface of these particle cores were extracted by agitation for 20 min × 3 times in ultrapure water with ultrasonic shaker. The PM2.5 extract contains PAHs (e.g., benzo [b]fluoranthene, chrysene, and pyrene), carbon (e.g., organic carbon) and metals (e.g., Mg, Ca, Fe and Zn), which was stored in the dark at −80 °C. Before the whole experiment, PM2.5 was re-suspended in 99.9% dimethyl sulfoxide (DMSO) and stored at 4 °C. Prior to exposure, we diluted the PM2.5 (99.9% DMSO) to different concentrations of PM2.5 (5% DMSO) with 99.9% DMSO and ultrapure water and immediately injection.

2.2. Animals and PM2.5 exposure

Fifty female and twenty-five male Sprague Dawley rats (10–12 weeks old) were purchased from Guangdong Medical Laboratory Animal Center and housed in a specific pathogen-free condition laboratory of the experimental animal center under a 12/12-h light/dark cycle and provided with standard rodent chow and water ad libitum. All rats used in this study were treated humanely with regard for the alleviation of suffering.The Institutional Animal Care and Use Committee of Guangzhou Medical University approved all of the animal experimental proce-dures.

Female rats were timed-mated with Sprague Dawley males. The day that the vaginal plug was detected was considered the embryonic day 0 (E0), and the pregnant females were housed in individually ventilated cages. At E0, pregnant the females were randomly assigned to one of the following groups (n = 10): a) control group (5% DMSO); b) 0.1 mg/kg PM2.5 group; c) 0.5 mg/kg PM2.5 group; d) 2.5 mg/kg PM2.5 group; or e) 7.5 mg/kg PM2.5 group. PM2.5 exposure was achieved *via* intraperitoneal (i.p.) injection at time points of E0, E3, E6, E9, E12, E15, and E18. Detailed descriptions of the methodology and justification for i.p. injection as experimental route of exposure are published elsewhere [16]. The administered PM2.5 did not increase with increasing body weight during the experimental period. Pregnant females were allowed to deliver naturally, and newborn pups were termed PM-0.1, PM-0.5, PM-2.5, and PM-7.5, respectively. No significant differences in litter size were observed between the control and PM2.5-exposed rats. Fetal rats were sacrificed on postnatal day 1 (PND1).

2.3. Tissue collection and preparation

Lung tissue was collected on PND1. The left lungs were fixed in 4% paraformaldehyde for immunohistochemistry assays. The right lungs were isolated, immediately washed with phosphate buffer solution (PBS) 3 times, and either homogenized in protein homogenization buffer with glass-homogenizer or used for isolation of total RNA for mRNA detection. Aliquots (300 μl) were frozen and stored at −80 °C for further analysis.

2.4. Micro-CT analysis

For micro-CT scanning, one female and one male pup randomly selected from the litters were used. All imaging was obtained from spontaneously breathing animals anesthetized with 3% pentobarbital sodium (0.3 ml/kg, i.p.) to immobilize them during scanning and then positioned on the scan platform. CT images were acquired using a quantitative analysis micro-CT scanner for small lab animals (Aloka Latheta LCT-200, Hitachi, Japan) with a current of 220 μA, voltage of 80 kVp, exposure time of 20 ms, and acquisition resolution of 48 μm. Computerized analysis of tissue density of CT images was processed with the Image-Pro Plus (IPP) 6.0 software.

2.5. ELISA

Lung tissue levels of IL-1, IL-6 and TNF-α were measured in triplicate by a commercially available ELISA kit from R&D Systems (Minneapolis, MN). The assays were performed according to the manufacturer's protocol. All indexes were expressed as ng/L. No significant cross-reactivity or interference was observed.

2.6. Immunohistochemistry (IHC)

Immunohistochemistry was performed with a Super Sensitive TM IHC Detection System Kit (Bioworld Technology Inc. Louis Park, MN, USA) according to the manufacturer's protocols. Tissue sections were immersed in 0.01 M sodium buffer (pH 9.0) and heated in a microwave for 15 min at 90 °C for antigen retrieval, then cooled to room temperature. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min. Slices were washed with PBS, treated with 5% normal goat serum, and then incubated with the primary antibody against HMGB1 (1:10000; Abcam, Cambridge, UK) overnight at 4 °C. A horseradish peroxidase-conjugated secondary antibody was used to visualize the antibody signal with diaminobenzidine. The sections were evaluated by light microscopy using a built-in DC750 digital camera system (Leica Microsystems, Wetzlar, Germany).

2.7. Western blot analysis

Lung tissue homogenates were centrifuged at 13,000 rpm for 15 min at 4 °C. Protein concentrations in the lysates were quantified using a BCA Protein Assay Kit according to the manufacturer's directions. Lung lysates were adjusted to equal the protein concentration, boiled in loading buffer, loaded on 12% or 6% gels (Bio-Rad, Hercules, CA, USA), and further processed for blotting onto polyvinylidene difluoride membranes (PVDF, Merck Millipore, Darmstadt, Germany). Membranes were blocked in Tris-buffered saline/Tween buffer (10 mM Tris-base, 150 mM NaCl, 0.25% Tween 20) containing 5% skim milk for 2 h at room temperature. Membranes were incubated with primary antibodies against HMGB1 (1:10000) overnight at 4 °C. GAPDH was used as a loading control. All primary antibodies were from Abcam (Cambridge, UK). Protein bands were developed by chemiluminescence (ECL, Pierce, Rockford, IL, USA) and obtained using the Gel Logic 1500 imaging system (Kodak, Rochester, New York, USA). Intensity of the bands was quantified using IPP 6.0 software.

2.8. Quantitative real-time PCR (QRT-PCR)

TriPure reagent (Roche, Indianapolis, IN, USA) was used to isolate the total RNA from rat pup lungs according to the manufacturer's instructions. Reverse transcription was performed with 2 μg of total RNA using Transcriptor 1st strand cDNA Synthesis Kit (Roche, USA). qRT-PCR was performed using SuperReal PreMix (SYBR Green, Roche, USA) on Light Cycler 1.5 (Roche, USA). The primers (Invitrogen, Shanghai, China) were CGGATGCTTCTGTCAACT and TCAGCTTGGCAGCTTTCT for HMGB1; and AGCCCAGAACATCATCCCTG and CACCACCTTCTTG ATGTCATC for GAPDH. The cycling program was 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the internal control.

2.9. Statistical analysis

Data were expressed as the means ± standard error (SE) or the means ± standard deviation (SD). Statistical analysis was performed using SPSS software (version 13.0, SPSS Inc., Chicago, IL, USA). Data among groups were assessed by one-way analysis of variation (ANOVA), followed by the Tukey post hoc test to compare all groups at 95% confidence intervals. Data with differences between groups were further determined using Student's t-test. $p < 0.05$ was defined as statistical significance.

3. Results

3.1. Exposure to PM2.5 in utero causes rat offspring pulmonary injury as revealed by micro-CT

Rat offspring pulmonary injury was assessed using the CT slice images from representative rats. Various attributes associated with CT images and lung morphologies are shown in Fig. 1a–e. Focal patchy consolidation was predominantly in the lower lung lobes with the presence of subpleural sparing in maternal PM2.5-exposure rats, and ground-glass opacity was observed with dose increases.

To understand the morphological and spatial significance of various densities, a threshold of −350 HU was used to distinguish the normal, air-filled volumes and volumes associated with inflammation as well as structural and functional components, as shown in the two-color density maps (Fig. 1f–j). High-density volumes, i.e., those volumes associated with inflammation, were largely increased in the PM-2.5 and PM-7.5 groups. The density changes due to PM2.5 exposure appeared near the pleura, enveloping nearby airways and forming an increasing gradient towards the lung periphery, which indicates that the inflammation was primarily associated with the major airways and vessels.

3.2. Exposure to PM2.5 in utero mediates rat offspring pulmonary inflammatory response

To evaluate PM2.5-induced pulmonary inflammation in rats, key inflammatory markers including IL-1, IL-6 and TNF-α in rat offspring lungs were analyzed. A PM2.5 dose-dependent increase of the 3 cytokines was observed on PND1 after maternal PM2.5 exposure (Fig. 2). Notably, the concentrations of IL-1, IL-6 and TNF-α in lung tissues were significantly increased by 2.36-, 3.91- and 4.36-fold, respectively, in the rats of the PM-7.5 group compared with the rats in the control group ($p < 0.001$).

3.3. Exposure to PM2.5 in utero induces the translocation of nuclear HMGB1 to the cytoplasm

HMGB1 is widely expressed in the nucleus of nearly all eukaryotic cells [20]. HMGB1 also contributes to the pathogenesis of various inflammatory diseases via release into the extracellular milieu [9,21]. To determine whether maternal PM2.5-induced HMGB1 release into the airways is a result of the translocation of HMGB1 from the nuclei to the cytoplasm, immunohistochemical analysis was performed in lung tissue sections of newborn pups. HMGB1 was predominantly found in the nuclei of most lung cells in rat offspring from the control group (Fig. 3). However, HMGB1 was primarily localized in the cytoplasm of many lung cells in the maternal PM2.5 exposure groups. The number of HMGB1-positive cells was substantially higher in the PM-2.5 and PM-7.5 groups than in the control group, suggesting that a high dose of maternal PM2.5 exposure resulted in HMGB1 translocation from the nuclei to the cytoplasm of the lung cells in rat the offspring.

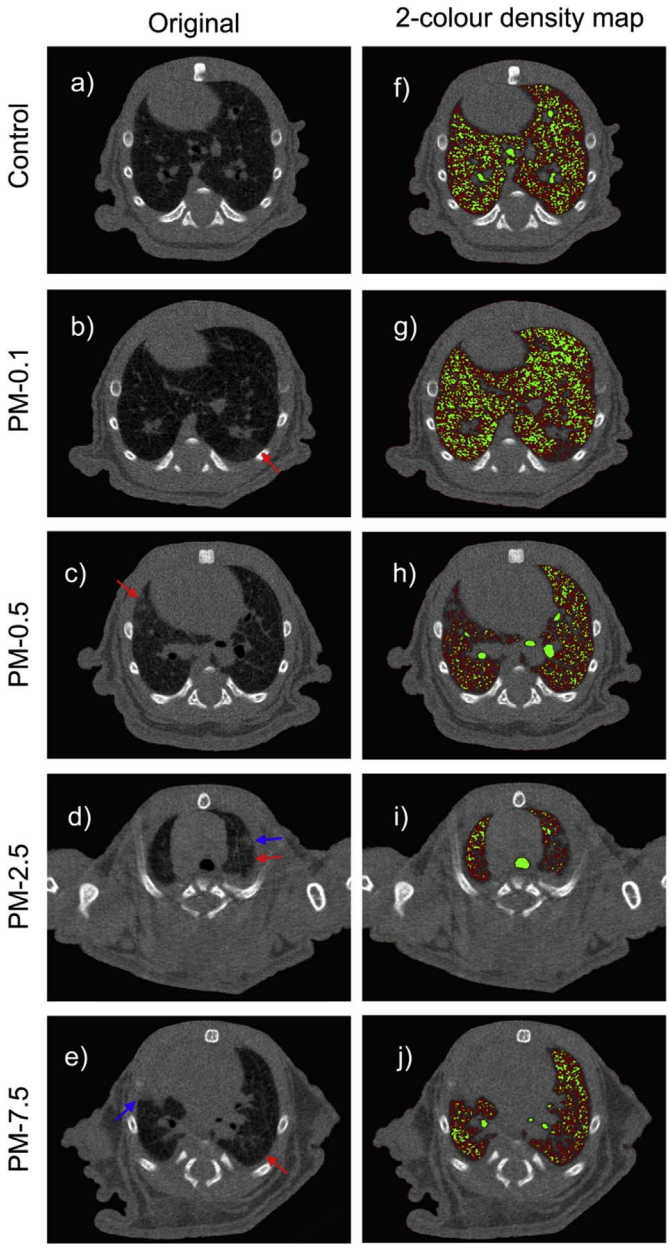

Fig. 1. Representative axial computed tomography (CT) slices with color-mapped duplicate in control and maternal PM 2.5-exposed groups. A-e) original, and f-j) two thresholds. a and f) control; b and g) 0.1 mg/kg PM2.5; c and h) 0.5 mg/kg PM2.5; d and i) 2.5 mg/kg PM2.5; e and j) 7.5 mg/kg PM2.5. Red arrows indicate lung tissue hyperplasia. Blue arrows indicate the consolidation of lung tissue. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

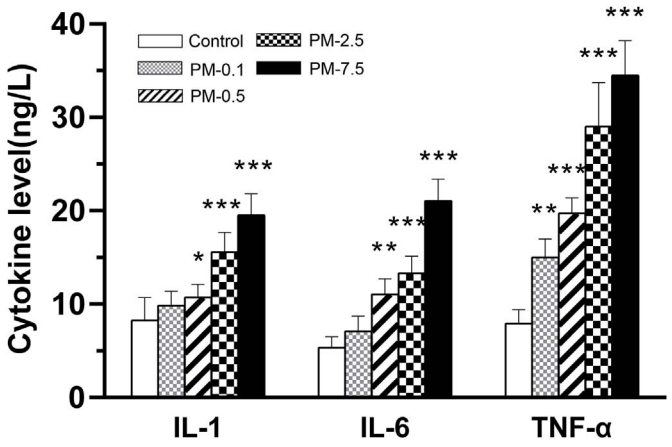

Fig. 2. IL-1, IL-6 or TNF-α concentration in rat offspring lung tissue after maternal exposure to PM2.5. Levels of IL-1(A), IL-6 (B) and TNF-α (C) were determined by ELISA in the lung homogenates. Data are presented as the means ± SD (n = 10). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared to the control group.

3.4. HMGB1 plays a role in rat offspring lung injury

To evaluate the potential role of HMGB1 in rat offspring lung injury, HMGB1 were assessed by Western blot and qRT-PCR analysis. HMGB1 protein concentrations were higher in the rats of all maternal PM2.5-exposure groups compared to the control group (Fig. 4). The HMGB1 concentration was the highest in rats of the PM-7.5 group. Comparing the control and maternal PM2.5-exposure groups, significant differences were observed in the mRNA expression of HMGB1. The expression of HMGB1 mRNA was 2.7-fold higher in the rats of the PM-7.5 group than the control group ($p < 0.001$) (see Fig. 5).

4. Discussion

The present study demonstrated the effects of maternal exposure to PM2.5 during pregnancy on postnatal pulmonary injury in rat offspring and HMGB1 of PM2.5-induced pulmonary inflammation. The animal model and the doses of PM2.5 used in this study were reported in our

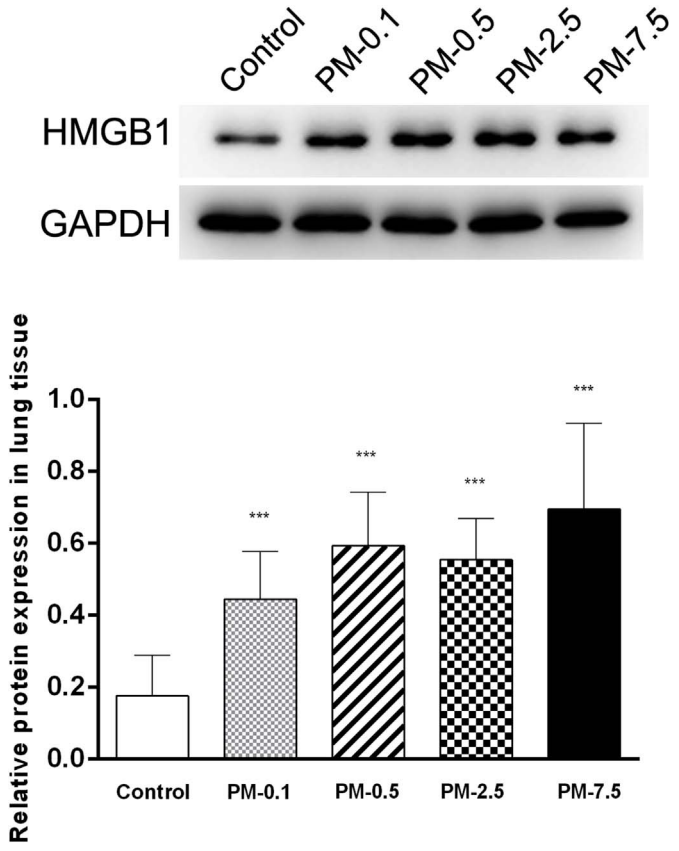

Fig. 4. Protein expression of HMGB1 in lung tissues in control pups and maternally PM2.5-exposed pups, determined by Western blot analysis. GAPDH was used as loading control. The graphs show the ratios of HMGB1/GAPDH as calculated from the density of the Western blot bands. Data are presented as the means ± SE. ***$p < 0.001$ compared to the control group.

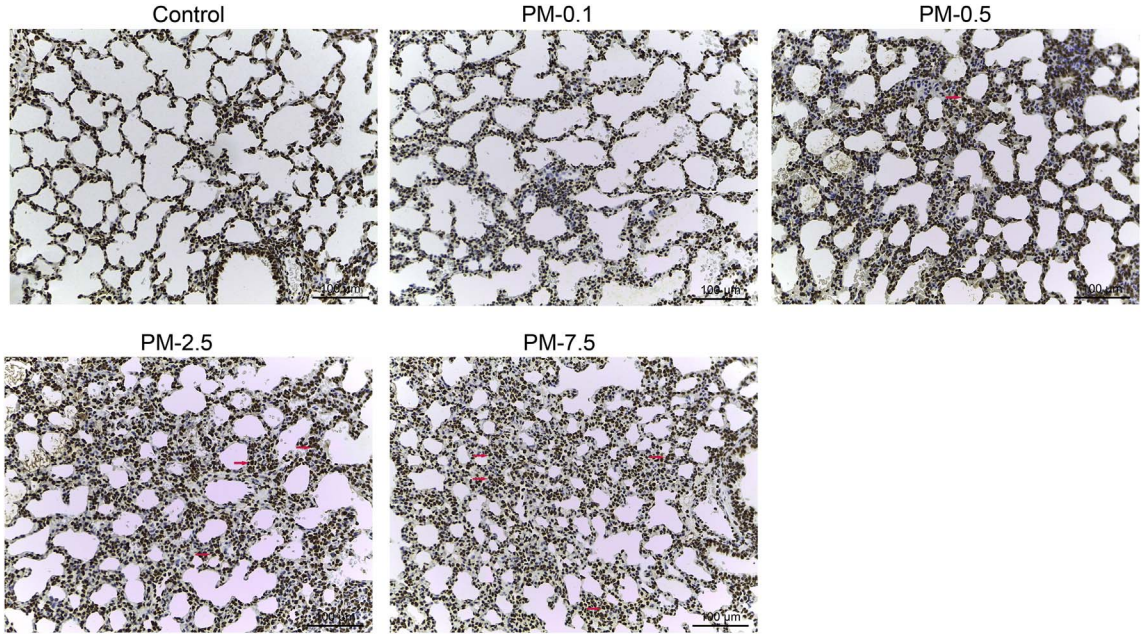

Fig. 3. Representative immunohistochemical images of HMGB1 staining in lung tissues of the control pups and maternally PM2.5-exposed pups (control, PM-0.1, PM-0.5, PM-2.5, and PM-7.5 group). The translocation of HMGB1 from the nuclei to the cytoplasm (red arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

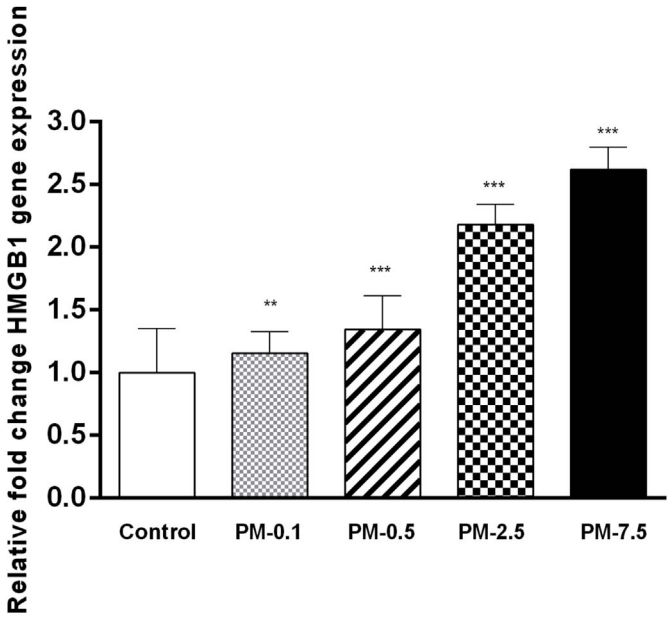

Fig. 5. Quantitative RT-PCR analysis of HMGB1 expression in lung tissues in control pups and maternally PM2.5-exposed pups. Relative mRNA level of the HMGB1 gene was calculated. GAPDH was used as a control gene (n = 7–9 rats/group). Data are presented as the means ± SE. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the control group.

previous study [16]. The ground-glass opacity and high-density volumes in CT slice images of maternal PM2.5-exposure groups were observed. Maternally PM2.5-exposed offspring showed an increased expression of HMGB1 and secretion of inflammatory factors such as IL-1, IL-6 and TNF-α, thereby resulting in pulmonary injury.

Micro-CT is a relatively new technique that can provide a unique means for accurately studying the anatomy in situ and can yield direct visual evidence of structural alterations in rats. The CT value is familiar to those involved in the clinical practice of pulmonary medicine. Micro-CT can also be used to longitudinally monitor lung diseases in mice, including chronic inflammation, emphysema, and cancer [22–27]. Visually, the results of micro-CT scans in this study showed increased ground-glass opacity in consolidation in a dose-dependent manner, which were well correlated with the pathologic features. Interestingly, the pulmonary pathological changes appeared in various parts of the lung; however, the higher the PM2.5 concentration, the more serious the changes. Maternal exposure to PM2.5 during pregnancy on postnatal pulmonary injury was characterized by the combination of bronchiolar destruction with mild to severe bronchiolar obliteration, centrilobular distribution of alveolar damage and remodeling by inflammatory and fibroblastic proliferation, as shown in our previous study [16].

Materials with the same density should show a consistent density in CT scan images, for example air has a density of −1000 HU and water has a density of 0 HU. This property enables the use of a fixed threshold to separate different tissue types, including lung parenchyma and soft tissue. We observed high-density volumes with patchy consolidation in maternally PM2.5-exposed offspring in two-color density maps, which is similar to those of the acute and severe hypersensitivity pneumonitis [28]. Further studies on the developing patterns of occurrence and/or severity of lung injury in individual subject are required; therefore, we cannot determine the most sensitive pregnancy time. If possible, we may explore the newborn in high PM exposure area by imaging studies, so that we would better to evaluate the risk of this developmental pulmonary hazard.

An inflammatory reaction is a common pathological process of many diseases. IL-1, IL-6 and TNF-α are proinflammatory cytokines that lead to pathological changes in acute lung injury. As we would expect, maternal exposure to PM2.5 evoked an obvious cytokine influx (IL-1, IL-6 and TNF-α) into the lung. The placenta is crucial for the growth and development of the fetus, as maternal factors can cross the placenta. A study reported that maternal PM instillation induced maternal pulmonary inflammation and fetal placental inflammatory cytokine expression [7]. A worrying result of endothelial placental dysfunction, which is caused by increased maternal circulation of inflammatory factors resulting from PM exposure, is the promotion of offspring lung injury. Additionally, poisonous components in PM2.5, such as polycyclic aromatic hydrocarbons (PAH) and heavy metals, may readily cross the placenta into the offspring to directly release inflammatory cytokines.

HMGB1 is one of the best-characterized damage-associated molecular patterns (DAMPs) and a proinflammatory factor that can trigger inflammatory responses when released into the extracellular space in response to stress. HMGB1 is actively secreted from immunocompetent cells and passively released by damaged cells [29–31]. Once in the extracellular milieu, HMGB1 assists tissue adaption to stress by acting on surrounding cells. Adaptation often provides short-term benefits. However, if stresses are sustained or excessive, they can become maladaptive and possibly harmful [32]. Human neutrophils have been reported to contain high levels of HMGB1, with an increased release upon cigarette smoke exposure [33]. Our results showed a modest increase of HMGB1 in the maternal PM2.5 exposure groups according to the immunohistochemical analysis. Furthermore, the active secretion of HMGB1 was also supported by upregulated mRNA and protein levels of HMGB1.

Extracellular HMGB1 induces complex cascades of signaling via binding to TLR4, as well as trigger inflammation mediated by NF-κB [29,34]. Increasing studies have demonstrated that HMGB1 can activate alveolar macrophages to produce proinflammatory cytokines (such as IL-1 and TNF-α) and induce acute lung injury via TLR4 [35]. This may be one of the mechanisms of HMGB1 leading to offspring lung injury. Our previous work indicated that EMT up-regulation mediated by the TGF-β/Smad3 pathway plays a role in postnatal pulmonary dysfunction associated with maternal exposure to PM2.5. HMGB1 has been reported to be upregulated in TGF-β-stimulated A549 cells, leading to EMT [36]. The extracellular HMGB1 activated by the TGF-β/Smad3 pathway may also contribute to PM2.5-induced offspring lung injury.

Whether the doses employed in our study can be reachable in physiological situations or not is going to be a controversial issue. The physiological route is inhalation and it is clear that, despite the low size of the PM, the systemic dose (the absorbed PM that reaches the blood) will never be 100% because of a lot of complicated factors. Although the exposure model is frequently used in similar studies [37] due to its ease of manipulation and reproducibility, the route of intraperitoneal injection is not equivalent to inhalation. We believe that applying PM2.5 doses directly into the bloodstream for stability may better reflect the compounds'dose-dependency, which is relevant for the study of children's health. As our previous study [16], the doses used in the study are appropriate and relevant to identify the hazards derived from the exposure to PM. However, it is not physiologically relevant in the sense that it is extremely unlikely that can be reached in vivo even in conditions of heavy PM pollution. For another, the PM in most parts of the world reaches the WHO standards. Since the probability to reach these levels of exposure is very low then the risk of development of the effects described in our results is also very low. Thus, we can be concluded that it is not expectable to see these effects in population exposed to PM at concentrations allowed in international regulations. Despite all this, we must maintain keen vigilance about the dangers of PM. Extremely concentration of PM in the environment may lead to the results of this paper.

There were some limitations to our present study. First, the particles used in the present study were collected in China; however, the properties are different in other ambient particles such as diesel exhaust

particles [38]. Nevertheless, PM2.5 samples would best represent the impact on the residents who live nearby. In addition, the doses used were relevant to how much PM2.5 pollution has been found in the air in China. Secondly, PM2.5 are a heterogeneous mixture of solid and liquid particles emitted from a variety of sources. We could not ensure which component in PM2.5 caused offspring injury in our present study. It is necessary to extract some elements in PM2.5 for further study. Finally, there is a lack of evidence regarding the mechanisms by which PM2.5 exposure ultimately induces inflammatory responses. It is important to confirm these findings using embryonic stem cells through the over-expression of HMGB1 or by blocking its inflammatory pathways after exposure to PM2.5.

5. Conclusion

In conclusion, our study revealed that PM2.5 exposure during pregnancy results in the development of lung inflammation in offspring by mechanisms mediated by increased HMGB1 expression to ultimately promote IL-1, IL-6 and TNF-α secretion. Thus, the findings of this study may contribute to understanding the programming mechanisms of pollutants during pregnancy and its repercussions for the development of lung diseases in later life, which remain to be further elucidated.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgment

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Transparency document

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