



Short-term PM_{2.5} exposure induces sustained pulmonary fibrosis development during post-exposure period in rats

Baiyang Sun^{a,b,1}, Yanfeng Shi^{a,b,1}, Yang Li^{a,b}, Jinjin Jiang^{a,b}, Shuang Liang^{a,b}, Junchao Duan^{a,b,*}, Zhiwei Sun^{a,b,*}

^a Department of Toxicology and Sanitary Chemistry, Capital Medical University, Beijing, 100069, PR China

^b Beijing Key Laboratory of Environmental Toxicology, Capital Medical University, Beijing, 100069, PR China

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ABSTRACT

Up to now, while some toxicological studies have identified pulmonary fibrosis immediately induced by long-term PM_{2.5} exposure, there has been no evidence indicating, whether short-term exposure can lead to post-exposure development of pulmonary fibrosis. Here, we treated rats with PM_{2.5} for 1 month (10 times), followed by normal feeding for 18 months. ¹⁸F-FDG intake, which is linked with the initiation and development of pulmonary fibrosis in living bodies, was found to gradually increase in lung following exposure through micro PET/CT imaging. Histopathological examination revealed continuous deterioration of pulmonary injury post-exposure. Collagen deposition and hydroxyproline content continued to increase all along in the post-exposure duration, indicating pulmonary fibrosis development. Chronic and persistent induction of pulmonary inflammatory gene expression (Tnf, Il1b, Il6, Ccl2, and Icam1), epithelial mesenchymal transition (EMT, reduction of E-cadherin and elevation of fibronectin) and RelA/p65 upregulation, as well as serum inflammatory cytokine production, were also found in PM_{2.5}-treated rats. Pulmonary oxidative stress, manifested by increase of MDA and decrease of GSH and SOD, was induced during exposure but disappeared in later post-exposure duration. These results suggested that short-term PM_{2.5} exposure could lead to sustained post-exposure pulmonary fibrosis development, which was mediated by oxidative-stress-initiated NF-κB/inflammation/EMT pathway.

1. Introduction

PM_{2.5}, airborne fine particulate matter with an aerodynamic diameter less than 2.5 μm, is one of the most hazardous pollutants in the atmosphere. According to the Global Burden of Disease Study 2017, the number of deaths attributable to ambient particulate matter pollution has risen to 2.94 million, with the percentage increase of more than 20% from the year of 2007 to 2017 (GBD Risk Factor Collaborators, 2018). Inhaled, PM_{2.5} can get into bronchi, reach and deposit in alveoli, and even penetrate into blood circulation, thus exerting detrimental effects on multiple organ systems (Manigrasso et al., 2017; Nemmar et al., 2013; Schulze et al., 2017). Among them, the lung is the primary target of PM_{2.5}, and nowadays the influences of PM_{2.5} on respiratory system have become one of the research hotspots in the field of environmental health sciences. Evidences are accumulating to demonstrate that PM_{2.5} exposure is associated with morbidity and mortality of various respiratory diseases, especially the chronic ones such as asthma, chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis,

which have brought great health burden to human beings (Xing et al., 2016; Sese et al., 2018; Kelly and Fussell, 2011).

Pulmonary fibrosis is a chronic and progressive disease with high mortality and limited therapeutic options (Kolahian et al., 2016). It is characterized by excessive deposition of collagen fibers in the pulmonary mesenchyme as well as scarring of the lung, finally leading to structure destruction of respiratory system and reduction of respiratory capacity (Dong et al., 2016). Toxicological models have been used to investigate the pro-fibrogenic effect of PM_{2.5}; however, most studies have only explored the immediate alterations which developed synchronously with continuous exposure. For instance, Hu et al. used a murine bleomycin-induced idiopathic pulmonary fibrosis (IPF) model to demonstrate that PM_{2.5} from straw burning could aggravate pulmonary inflammation and fibrosis (Hu et al., 2017). In the study by Xu et al., deteriorated signatures of pulmonary fibrosis were identified in mice exposed to PM_{2.5} consecutively for 8 weeks, indicating the direct pro-fibrogenic effect of PM_{2.5} (Xu et al., 2019). Yet, the development of fibrosis post-exposure (ie after cessation of PM_{2.5} exposure) has

* Corresponding authors at: Department of Toxicology and Sanitary Chemistry, School of Public Health, Capital Medical University, Beijing, 100069, PR China. E-mail addresses: jcduan@ccmu.edu.cn (J. Duan), zwsun@ccmu.edu.cn (Z. Sun).

¹ These two authors contribute equally to this work.

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received surprisingly little attention, especially given that repeated exposure to some kinds of particulates such as nanotubes can initiate non-resolving inflammation (Nathan and Ding, 2010; van den Brule et al., 2014). In the pathogenesis of organ fibrosis, chronic inflammation acts as a relentless driver of fibrogenesis after injury, leading to a vicious cycle with tissue damage and fibrosis (Liu, 2011). Therefore, if PM_{2.5} exposure could be capable of triggering chronic inflammation and other relevant nonresolving biological effects, long-term development of fibrosis would potentially be initiated in post-exposure duration. In this case, the impact of PM_{2.5} on respiratory system would be more than what the existing studies have indicated. However, to the best of our knowledge, there has been no relevant evidence to date to support this viewpoint.

Multiple mechanisms are involved in the initiation and development of pulmonary fibrosis, with inflammation and epithelial-mesenchymal transition (EMT) playing key roles. Inflammation has a pivotal effect in most interstitial lung diseases and, if chronic, leads to fibrosis (King et al., 2011). There are a host of immune cells and inflammatory mediators promoting fibrotic progression (Mack, 2018). EMT is a vital source of fibroblasts and myofibroblasts which are integral in the development of fibrosis in the lung (Willis et al., 2006). Additionally, oxidative stress, defined by the imbalance between increased levels of oxidants and low activity of antioxidant mechanisms, is also linked to the initiation of pulmonary fibrosis (Kinnula, 2008). As to the relationships between these mechanisms and PM_{2.5}, inflammation and oxidative stress have been widely accepted as two fundamental mechanisms underlying PM toxicity (van Berlo et al., 2012); EMT can occur in respiratory system following PM_{2.5} exposure (Chi et al., 2018). Our previous work of bioinformatics suggested that, in BEAS-2B cells, the differentially expressed genes with the highest significance were functionally annotated to the NF- κ B signaling pathway following PM_{2.5} exposure, highlighting the central role of NF- κ B under PM_{2.5}-associated pulmonary toxicity (Shi et al., 2019). NF- κ B is also implicated in the pathogenesis of pulmonary fibrosis (Christman et al., 2000). At present, though, it remains unknown whether these biological processes could be triggered in post-exposure duration and play a role in the development of PM_{2.5}-initiated pulmonary fibrosis.

Therefore, we hypothesized that short-term PM_{2.5} exposure could initiate long-term pulmonary fibrosis development in post-exposure duration. According to the roles of the above potential mechanisms and their regulatory relationships, we further hypothesized the fibrosis was mediated by oxidative stress/NF- κ B/inflammation/EMT pathway. To verify our conjectures, in the present study, we adopted Sprague-Dawley rats to establish an animal model successively experiencing short-term PM_{2.5} exposure and long-term post-exposure normal feeding. We firstly utilized micro positron emission tomography-computed tomography (PET/CT) to monitor the post-exposure alteration of ¹⁸F-FDG uptake in lung, which is associated with the development of pulmonary fibrosis in living bodies. After the rats were sacrificed successively, the development of lung injury and fibrosis in post-exposure duration was assessed. Further, we explored pulmonary inflammatory gene expression, EMT marker changes, RelA/p65 expression, levels of oxidative-stress indicators (MDA, SOD and GSH), and serum inflammatory cytokine levels, to provide potential mechanisms that were responsible for the pulmonary fibrosis development. This study will provide evidences of post-exposure toxicity induced by PM_{2.5} and help clarify the contribution of PM_{2.5} in initiation and development of pulmonary fibrosis.

2. Materials and methods

2.1. Collection of PM_{2.5}

The preparation procedure of PM_{2.5} samples conducted in this study has been detailedly described in our previous study (Zhang et al., 2017). Briefly, PM_{2.5} was collected on quartz fiber filters by the sampler

(TH-1000CII, Wuhan Tianhong, China) and stored at -80°C until extraction. Sampled filters were sonicated in ultrapure water and the suspension was filtered through eight layers of gauze. The eluate was distributed in sterile tubes and then freeze-dried in vacuum to acquire the particulates (PM_{2.5}). After irradiated with ultraviolet radiation, PM_{2.5} was suspended in normal saline to designated concentration for later use. The characterization of PM_{2.5} has been processed and described in our previous study, including detection of organic and inorganic elements and analysis of polycyclic aromatic hydrocarbons (Zhang et al., 2017).

2.2. Experimental animals and exposure scenario

Male Sprague-Dawley rats (seven weeks old) were obtained from Experimental Laboratory Animal Technology Co., Ltd. (Vital River, Beijing, China). Animal experimental procedures were approved by the Experimental Animal Welfare Committee (Capital Medical University: AEEI-2016-076). The establishment of animal model was composed of two main procedures, including short-term exposure to PM_{2.5} and post-exposure normal feeding for an 18-month duration. In detail, three groups of rats were respectively treated with PM_{2.5} with the dose of 1.8, 5.4, 16.2 mg/kg body weight (bw) via intratracheal instillation under anesthesia (5% chloralhydrate, 7 ml/kg bw) every 3 days and for 10 times in total, with another one group treated with normal saline as the negative control. Then all of the rats of 1.8 and 16.2 mg/kg bw groups, and a part of the rats of 5.4 mg/kg bw and control groups, were sacrificed after the final exposure. The left rats of the two groups were preserved and sacrificed at the 6-, 12- and 18-month time point successively after the cessation of PM_{2.5} treatment. The entire workflow of the animal experiment is shown in Fig. S1 in the supplementary material.

The exposure dose of PM_{2.5} was designed according to both physiological parameters of rats and the World Health Organization (WHO) air quality guidelines. The respiratory volume and breath rate of an adult rat weighing 200 g is 0.86 ml per breath and 85 times per minute, respectively. Combined with the annual mean concentrations of interim target-1 of PM_{2.5} (35 $\mu\text{g}/\text{m}^3$) recommended by the WHO, we obtained the total mass of PM_{2.5} for one-day exposure, which was 3.684 μg . After the application of a 100-fold uncertainty factor, the exposure concentration every day for rats was determined to be 1.8 mg/kg bw, which was adopted as the low exposure dose. Then, 3-fold (5.4 mg/kg bw) and 9-fold (16.2 mg/kg bw) of the dose were used as the medium and high ones, respectively (Zhang et al., 2017).

2.3. Micro PET/CT scanning

In human study, increase of ¹⁸F-FDG uptake in the lung with fibrosis have been found in some researches, and some PET/CT parameters such as standardized uptake value (SUV) possess some prognostic value in IPF patients (Groves et al., 2009; Win et al., 2018; Lee et al., 2014). Also, pulmonary ¹⁸F-FDG uptake value has been found correlated with pulmonary fibrosis in animal models, demonstrating the implications of micro PET/CT imaging (Bondue et al., 2015). Micro PET/CT scanning was performed on the rats of exposure and control groups from 0 to 18 months after exposure using Inveon micro PET/CT scanner (Siemens, Germany) to evaluate ¹⁸F-FDG uptake in rat lung, thus reflecting the development of fibrosis. In brief, after experiencing fasting for at least 12 h, rats were injected with ¹⁸F-FDG (0.2 mCi/kg bw) through the tail vein following anesthesia by isoflurane. They were allowed to experience a period of 60 min for the uptake of ¹⁸F-FDG while awake. Then the rats were placed on the examine bed and underwent scanning procedure during continuous isoflurane anesthesia. All images were reconstructed using a two-dimensional ordered-subset expectation maximum algorithm and further analyzed by Inveon Research Workplace 1.5 software (Siemens, Germany). Experimenter who was blinded to the study design manually selected regions of interest on the fusion

images. SUV was presented as the regional radioactivity concentration normalized by the ^{18}F -FDG dose and body weight of every rat.

2.4. Blood and lung tissue sampling

Rats were anesthetized with chloral hydrate and then blood samples were drawn from abdominal aorta into serum separator tubes, allowed to clot at room temperature for 30 min, and centrifuged at 3000 rpm for 15 min. Serum was then extracted and stored at -80°C for later use. For every rat, the left lung was harvested and then processed or kept in required environment immediately following the sampling procedure of blood. In detail, the left lung apex was sliced for histopathological examination, while the rest of the left lung was separated into two parts. One part was immediately frozen in liquid nitrogen, and then stored at -80°C for the extraction of RNA and protein; the other was homogenized in ice-cold normal saline with glass-homogenizer for biochemical analysis.

2.5. Evaluation of lung histopathology and collagen distribution

Lung samples were fixed with 4% paraformaldehyde for 24 h, rinsed in distilled water, dehydrated in an ethanol series from 70% to 100%, and put in bio-plast tissue embedding medium. Serial 7.0 μm -thick sections of lung samples were acquired by a rotary microtome and mounted on slides. The sample slides were processed through haemalaun and eosin (H&E), and Masson's trichrome staining, to respectively examine the histopathology and collagen distribution of rat lung tissue. Representative images were obtained via scanning of the stained slides with blind method by Panoramic Digital Slide Scanner (3DHISTECH, Hungary) according to the standard techniques.

2.6. Hydroxyproline assay

Hydroxyproline is considered as an indicator of collagen content in the lung tissue, which can provide strong evidence for the occurrence of lung fibrosis. Hydroxyproline content was determined through Hydroxyproline Assay Kit (Nanjing Jiancheng, China) according to the manufacturer's instructions, with the detection of absorbance at the wavelength of 550 nm by the microplate spectrophotometer.

2.7. Detection of redox status in rat lung tissue

As is mentioned above, part of left lung was homogenized after being harvested. The homogenate mixture, with the final concentration of 10%, was centrifuged at 3000 rpm for 10 min at 4°C . Supernatant was collected and stored at -80°C for subsequent examination. Redox status of rat lung tissue, reflected by the contents of GSH, SOD and MDA in homogenate, was measured using the commercial colorimetric assay kit (Nanjing Jiancheng, China), with the signal data obtained by the microplate spectrophotometer (BioTek, USA).

2.8. Quantification of inflammation cytokines in serum

The levels of inflammatory cytokines in rat serum, including TNF- α , IFN- γ , IL-1 β , IL-6, MCP-1, and ICAM-1, were determined with a quantitative rat cytokine antibody array (Quantibody® Rat Cytokine Array 2; RayBiotech Inc., Norcross, GA, USA). Data of the signals (Cy3, 555 nm excitation) were obtained through using the laser scanner equipped with InnoScan 300 Microarray Scanner (Innopsys, France) and analyzed by GenePix according to the manufacturer's instructions.

2.9. RNA isolation and quantitative real-time PCR

TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA) was applied to extract the total RNA from lung tissue, followed by purification with RNeasy kit (Qiagen, Germany) according to the

manufacturer's protocol. After the quality and quantity of RNA samples were determined by Nano Drop 2000 (Thermo Fisher Scientific, USA), the total RNA was reversely transcribed into first-strand cDNA using the cDNA Synthesis Kit (Promega, USA). Quantitative RT-PCR reaction was performed with SYBR Premix Ex TaqII (TliRNaseH Plus) (TAKARA, Japan) and monitored by Realplex2 (Eppendorf, Germany). The expression levels of target genes were normalized to GAPDH and the fold changes between exposure groups and their controls were calculated using $2^{-\Delta\Delta\text{Ct}}$ method. The primers of selected genes are listed in Table 1 in the supplementary materials.

2.10. Western blotting

Protein extracts of rat lung tissue were prepared using Whole Cell Lysis Assay Kit (Keygen Biotech, China), followed by measurement of concentration by BCA Protein Quantitation Kit (Dingguo Changsheng Biotech, China). Equal amounts of protein were loaded, separated in 8%–12% SDS-PAGE gels and transferred to nitrocellulose membrane (Pall Corporation, USA). After blocked with 5% skim milk in Tris-buffered saline, the membrane was incubated with primary antibodies overnight at 4°C including NF- κB p65 (CST, USA), E-cadherin (CST, USA), Fibronectin (Abcam, UK) and GAPDH (CST, USA). In the following day, the membrane was incubated with anti-mouse/rabbit IgG (DyLight™ 680 Conjugate) secondary antibody (CST, USA). Signals of the specific protein bands were detected by the LI-COR Odyssey® CLx Infrared Imaging System (LI-COR Biosciences, USA) and visualized by Image Studio™ Software (LI-COR Biosciences, USA). Densitometric analysis of the detected protein bands was performed using Image J software (NIH, Bethesda, MD). The expression of each target protein was normalized to GAPDH.

2.11. Statistical analysis

The SPSS 18.0 software was used for statistical analysis. Comparisons among groups of different doses were performed with one-way analysis of variance (ANOVA), followed by subsequent multiple comparisons through Dunnett test. For the comparisons between single exposure group and its concurrent control, independent-sample *T*-test or Mann-Whitney *U* test (non-normal distribution of data) was conducted to determine the significance levels of difference. Data were expressed as mean $\pm$ standard deviation, or median and interquartile range ($Q_{0.25}/Q_{0.75}$). Additionally, for the data of pulmonary ^{18}F -FDG uptake, hydroxyproline content, gene expression of Ccl2 and Icam1, and protein expression of RelA/p65 during the whole post-exposure period, two-way ANOVA was used to examine the effects of interaction between PM_{2.5} and post-exposure duration (PM_{2.5} $\times$ post-exposure duration). Data of interaction were presented using plots where two parallel lines indicated no interaction while nonparallel lines indicated an interaction between the two factors. The raw data were transformed to log values before conducting two-way ANOVA if the variances were not homogeneous. The *p* value less than 0.05 was considered as statistical significance.

3. Results

3.1. PM_{2.5} exposure led to higher pulmonary ^{18}F -FDG uptake in post-exposure period

Here, ^{18}F -FDG uptake in rat lung was assessed by PET / CT imaging technique to determine development of pulmonary fibrosis in post-exposure duration. As is shown in Fig. 1A, at the post-exposure 0-month and 6-month time point, there was no obvious region of high ^{18}F -FDG uptake in both exposure and control groups. However, at the 12-month time point, some sporadic areas of high uptake appeared, and at the 18th month high uptake area turned to be diffuse in the lung of the exposed rats. In the control groups, there was no high uptake area

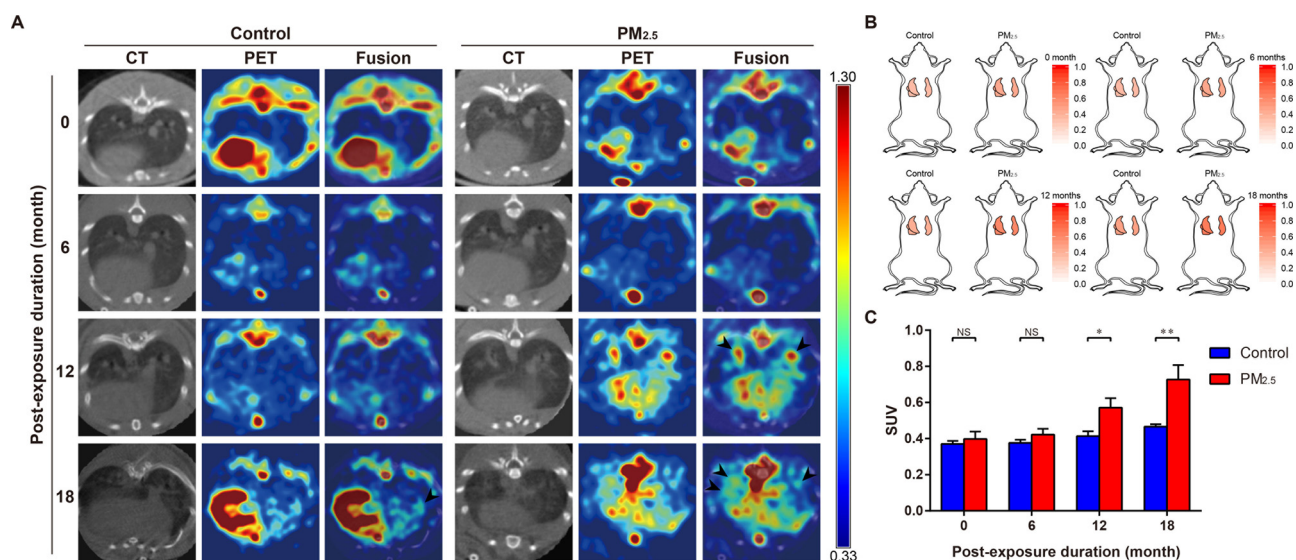


Fig. 1. Post-exposure gradual increase of pulmonary ¹⁸F-FDG uptake triggered by PM_{2.5}. (A) Representative images of micro PET/CT scanning. (B) Graphical representation of ¹⁸F-FDG standardized uptake value. (C) Statistical graph of standardized uptake value of ¹⁸F-FDG. **Black arrows** point to the areas of high ¹⁸F-FDG uptake. N = 3. **p* < 0.05, ***p* < 0.01 compared with the concurrent control group; NS: not significant.

appearing at the 12-month time points, but at the 18-month time point, very few high ¹⁸F-FDG uptake regions appeared but were still very slight compared to the concurrent rats of exposure group. Correspondingly, SUV of the exposure groups showed slight and insignificant increases at the first two points, while there were remarkable increases at the following two time points in exposed rats compared to the concurrent controls (*p* < 0.05, Fig. 1B and C). Also, the difference between the exposure group and its control increased with the post-exposure duration going on. In addition, there was a significant interaction between PM_{2.5} exposure and post-exposure duration, which further indicated the time-dependent increase of SUV of exposure group in comparison with the control (*p* < 0.01, Fig. S2). These results provided evidence of sustained pulmonary fibrosis development after short-term PM_{2.5} exposure in living rats.

3.2. PM_{2.5} exposure caused deteriorating lung injury in post-exposure duration

To evaluate the histological alteration in post-exposure duration, H & E staining was conducted on the lung sections at each time point. At the first time point immediately after treatment (Fig. 2A), we observed a dose-dependent increase of lung injury characterized by extensive infiltration of inflammatory cells and remarkable thickening of alveolar wall in the exposed rats, compared with the control group. In addition, alveolar capillary congestion and bronchiolar epithelial hyperplasia were aggravated with the increase of PM_{2.5} dose. In the later post-exposure duration (Fig. 2B), lung damage of the exposed rats had been persisting and even becoming more severe with the time going on, reflected by the pathological changes including: sustained inflammatory cell infiltration, hyperplasia and necrosis of bronchiolar and alveolar epithelial cells, uneven bronchiolar wall thickening, bronchiolar destruction with scarring and the remodeling of alveoli. There was no pathological alteration in the control groups of the first three time points, while only a slight disorder of alveolar structure was observed at the 18-month time point. Collectively, lung injury was induced in a dose-dependent manner during PM_{2.5} exposure and deteriorating with the post-exposure period going on.

3.3. PM_{2.5} exposure induced sustained post-exposure development of pulmonary fibrosis

The results of collagen distribution and content have been shown in Fig. 3. Masson's trichrome staining showed no significant change of collagen fiber formation in lung tissue among the PM_{2.5}-treated groups and the control at the time point immediately after exposure (Fig. 3A). In accordance with this result, hydroxyproline content was not altered among groups (Fig. 3C). Still, there was a slight dose-dependent increase of collagen formation and deposition around the bronchi in the exposure groups. At the following time points (Fig. 3B), increased collagen fibers were found diffusely distributed in lung parenchyma in PM_{2.5}-treated rats, which was deteriorating with the post-exposure period going on. As to the control groups, collagen fibers didn't change at the time point of 6 and 12 month, but there was an increase of collagen distribution at the last time point which was relative slight in comparison to that of the exposure group. Correspondingly, increased levels of hydroxyproline in PM_{2.5}-treated rats were observed at the 6-, 12-, and 18-month time points compared with the concurrent controls (Fig. 3D). In particular, the difference between the exposure and control groups reached significant level at the last time point (*p* < 0.05). Although the effect of interaction between PM_{2.5} and post-exposure duration was insignificant (*p* > 0.05, Fig. S2), the difference between the two groups enlarged with the time going on. To sum up, pulmonary fibrosis was consecutively developing in post-exposure duration.

3.4. PM_{2.5} exposure triggered sustained pulmonary and systemic inflammation during post-exposure period

We previously identified that, there was a remarkable induction of cytotoxicity biomarker and inflammation cytokines including total protein, LDH, TNF-α, IL-1β, and IL-6 in bronchoalveolar lavage fluid (BALF), as well as mRNA expression of Il6, Il1b and Tnf at the first time point, hinting the toxic effects including inflammatory response directly induced by PM_{2.5} (Shi et al., 2019). Here, the expression of inflammatory genes was detected in rat lung tissue at different time points after PM_{2.5} exposure, including Ccl2 (encoding MCP-1) and Icam-1 (all the post-exposure time points), as well as Tnf, Il1b and Il6 (post-exposure 6- to 18-month time points). According to Fig. 4A and B, at the first time point, the expression of Icam1 and Ccl2 was upregulated in PM_{2.5}-exposed groups in a dose-dependent manner. In detail,

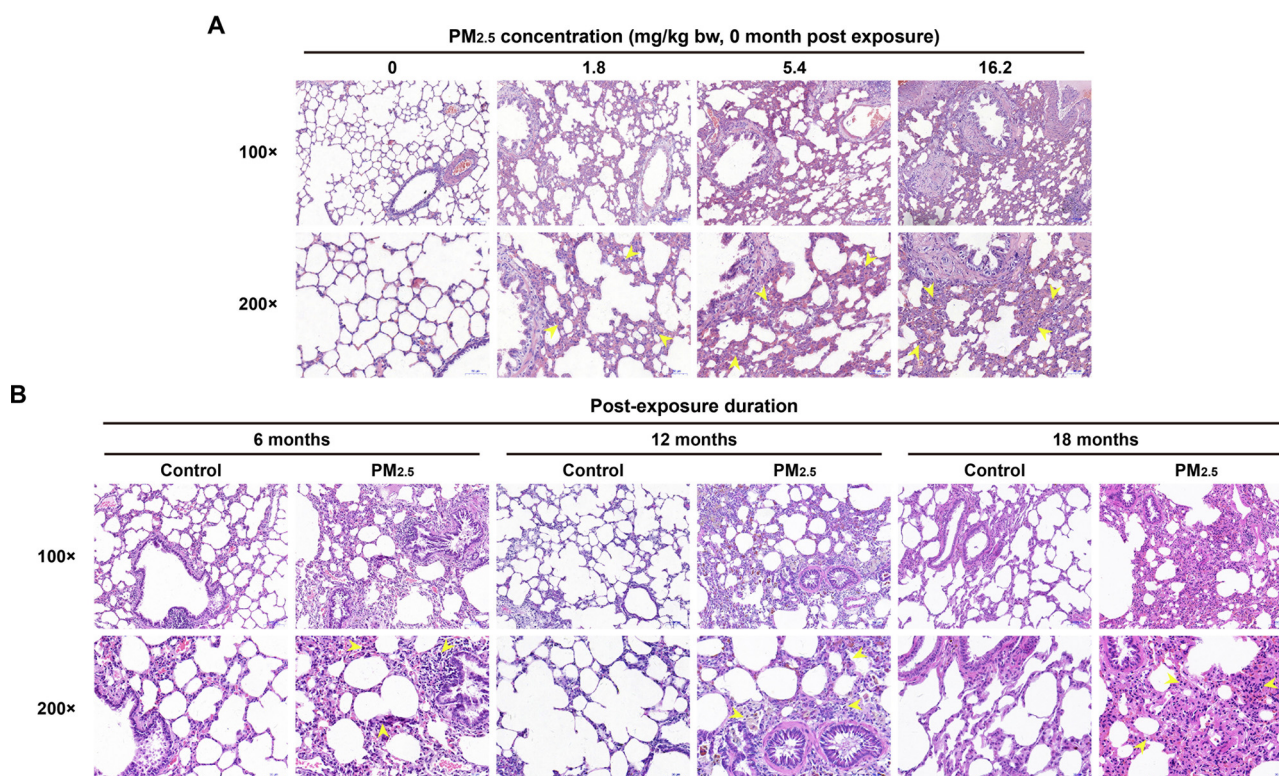


Fig. 2. Deteriorating injury of rat lung in post-exposure duration induced by PM_{2.5}. Representative histological sections of lung tissue processed through H&E staining. (A) Immediately after PM_{2.5} exposure. (B) Post-exposure 6-, 12- and 18-month time points. Yellow arrows point to inflammatory cell infiltration. Scale bars of 100 and 50 μ m represent the magnification of 100 $\times$ and 200 $\times$, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the expression levels of Icam1 in medium and high group and Ccl2 in all exposure groups were significantly elevated in comparison to the control group ($p < 0.05$). As to the following post-exposure duration, there were significant expression increases of all the detected genes in the lung of exposed rats at every time points compared to the concurrent controls ($p < 0.05$, Fig. 4C–G). In addition, the effects of interaction between PM_{2.5} and post-exposure duration on the expression of Ccl2 and Icam1 were both insignificant ($p > 0.05$, Fig. S2), further demonstrating the sustained pro-inflammatory effect of PM_{2.5} which was independent of time.

We previously found the occurrence of systemic inflammation reflected by a significant increase of IL-6, IL-1 β , CRP, ICAM-1 and VCAM-1 in rat serum, indicating the occurrence of systemic inflammation immediately after the exposure (Liang et al., 2019). In the present study, to determine whether the systemic inflammation persisted in the whole post-exposure period, we detected the levels of inflammatory cytokines in serum at following post-exposure time points, including TNF- α , IFN- γ , IL-1 β , IL-6, MCP-1, and ICAM-1 (Fig. S3). The result suggested a trend of persistent increase of serum levels of all the detected cytokines in exposure groups during post-exposure period, compared with the concurrent controls. Specifically, the serum levels of MCP-1 at the 6th month, IFN- γ and IL-1 β at the 12th month, and TNF- α and ICAM-1 at the 18th month were significantly elevated in the exposure groups compared with the concurrent controls ($p < 0.05$). IL-6 level in serum was not significantly changed at those time points but still showed a trend of continuous increase in the exposure groups. In sum, there was chronic pulmonary and systemic inflammation in rats during post-exposure duration.

3.5. PM_{2.5} exposure induced sustained EMT and RelA/p65 upregulation in lung during post-exposure period

To find out the involved mechanisms of PM_{2.5}-induced sustained

pulmonary inflammation and fibrotic development, the present study detected the protein expression levels of E-cadherin and fibronectin, the markers of EMT, as well as the expression of RelA/p65, a vital member of NF- κ B family which is an upstream regulator of inflammation, EMT and fibrosis. The expression of E-cadherin declined slightly in the exposure groups at the first time point, but had all along showed a trend of decrease in PM_{2.5}-treated groups in the following post-exposure duration compared to the concurrent controls, with the significant difference occurring at the 18-month time point ($p < 0.05$; Fig. 5A, C and F). Fibronectin expression showed an inverse trend at all the time points in post-exposure duration in comparison to E-cadherin, with the difference reaching significance level likewise at the final time point ($p < 0.05$; Fig. 5A, D and G). Therefore, it can be seen that EMT was initiated during exposure and had consecutively developed in post-exposure duration. RelA/p65 was significantly upregulated at the first time point in a dose-dependent manner ($p < 0.05$; Fig. 5A and B). At the later time points, although the difference of RelA/p65 expression between the exposure groups and the concurrent controls became insignificant, the exposure groups were still higher and the changes were respectively 1.75, 2.52 and 1.56 fold at the 6th, 12th and 18th month (Fig. 5A and E). There was no significant effect of interaction between PM_{2.5} and duration, indicating the sustained upregulating effect of PM_{2.5} on RelA/p65 expression which was not influenced by time ($p > 0.05$, Fig. S2). This result suggested the sustained upregulation of NF- κ B signaling throughout the post-exposure duration, which could play a role in PM_{2.5}-induced toxicity.

3.6. PM_{2.5} exposure induced pulmonary oxidative stress during exposure, but not in post-exposure duration

The contents of SOD, GSH and MDA were assessed to determine the oxidative stress induced by PM_{2.5} in rat lung. At the first time point immediately after exposure (Fig. 6A–C), MDA level was significantly

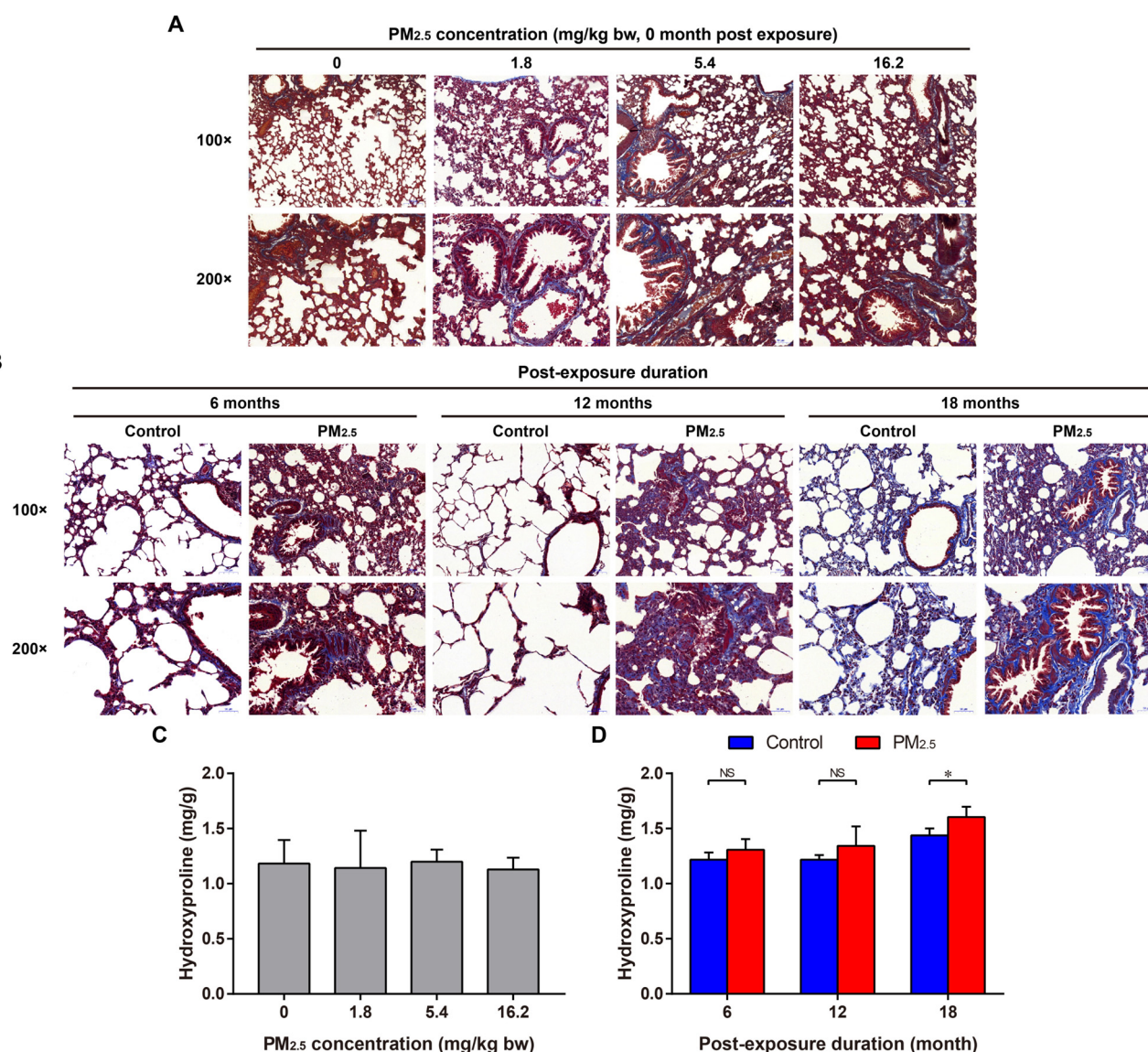


Fig. 3. Sustained post-exposure pulmonary fibrosis development caused by PM_{2.5}. Representative histological sections from lung tissue processed through Masson's trichrome staining. (A) Immediately after the final exposure and (B) Post-exposure 6-, 12- and 18-month time points. Pulmonary hydroxyproline content of the rats (C) immediately after the final exposure and (D) at the post-exposure 6-, 12- and 18-month time points. Scale bars of 100 and 50 μ m represent the magnification of 100 $\times$ and 200 $\times$, respectively. Hydroxyproline assay: N = 4 for the time point immediately after exposure; N = 3–5 for the post-exposure 6-, 12- and 18-month time points. * p < 0.05 compared with the concurrent control group; NS: not significant.

elevated with the increase of PM_{2.5} concentration, while the levels of SOD and GSH were decreased in the exposure groups compared with the control in a dose-dependent manner. In particular, the medium and high doses of PM_{2.5} significantly augmented MDA level and reduced the contents of GSH and SOD in rat lung tissue compared to the control (p < 0.05). At the following time points (Fig. 6D–F), the levels of MDA, GSH and SOD were not changed in the exposure groups compared with the concurrent controls. These results indicated that oxidative stress was triggered during exposure but disappeared in later post-exposure duration.

4. Discussion

PM_{2.5} exposure causes great harm on the health of human respiratory system. Many immediate toxic effects on respiratory system induced by long-term PM_{2.5} exposure have been identified in vivo and in vitro (Wei and Tang, 2018; Peixoto et al., 2017; Wang et al., 2018). However, whether long-term post-exposure toxicity can be triggered by

short-term PM_{2.5} exposure remains unclear. Nonresolving toxic effects such as chronic inflammation could be induced by exogenous stimuli, persist in post-exposure duration and further contribute to the initiation and development of chronic respiratory diseases such as pulmonary fibrosis. To investigate such a post-exposure impact induced by PM_{2.5}, the present study set up a rat model which successively experienced short-term PM_{2.5} exposure and following long-term normal feeding. In accordance with our hypothesis, our results collectively revealed a post-exposure toxicity pattern that, PM_{2.5}-induced chronic pulmonary inflammation and deteriorating lung injury appeared during exposure and persisted in later post-exposure period, followed by excessive repair manifesting as fibrogenesis and extracellular matrix accumulation, ie, the development of pulmonary fibrosis. Also, we identified pulmonary oxidative stress during exposure, as well as post-exposure RelA/p65 upregulation, chronic inflammation and continuous EMT, which acted as the mechanisms underlying the PM_{2.5}-initiated pulmonary fibrosis development. Additionally, there was even a trend of persistence in those post-exposure effects at the end of the experiment. In view of the

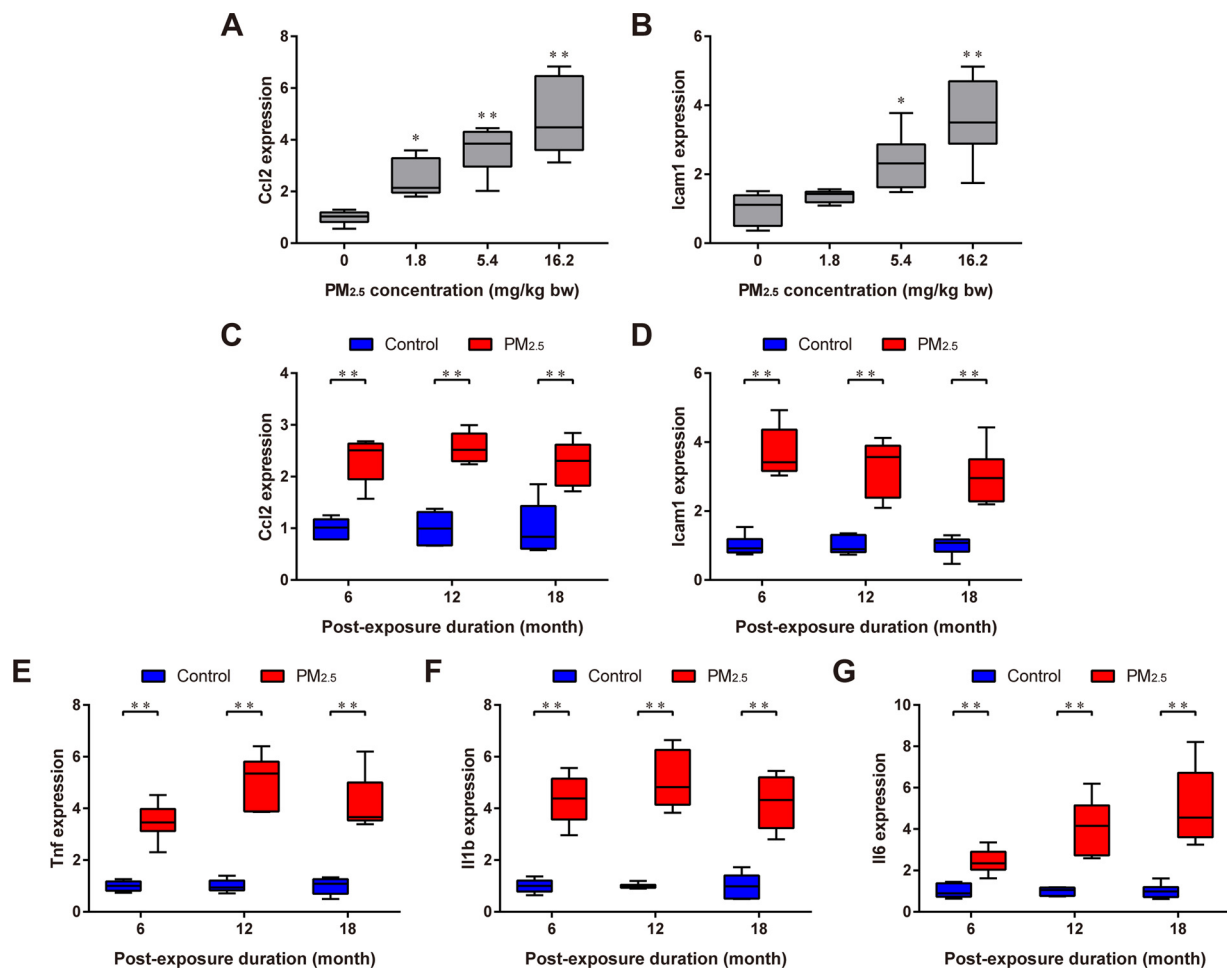


Fig. 4. Sustained pulmonary inflammation in post-exposure duration triggered by PM_{2.5}. The expression levels of inflammatory genes including: (A) Ccl2 and (B) Icam1 immediately after the final exposure and (C) Ccl2, (D) Icam1, (E) Tnf, (F) Il1b and (G) Il6 at the post-exposure 6-, 12- and 18-month time points. N = 6. **p* < 0.05, ***p* < 0.01 compared with the concurrent control group.

average lifetime of rats, the present study hint that once PM_{2.5} exposure may have the capability of exerting continuous impacts on respiratory system in whole later life of individuals.

The sustained pulmonary fibrosis development was characterized by increased content of collagen from only around the bronchi at the time point immediately after exposure to in all pulmonary interstitial area in the exposed group (Fig. 3). This is also highly consistent with our observation of post-exposure pulmonary ¹⁸F-FDG uptake during the establishment of the animal model (Fig. 1). The result at the first time point is comparable with the study by Zheng et al., which indicated increased collagen deposition around small airways of mice induced by PM_{2.5} exposure (Zheng et al., 2018). Here we speculate that bronchi may have more chance of exposure during the treatment and are probably more susceptible to PM_{2.5}. With the time going on, post-exposure effects induced by PM_{2.5} contributed to the later fibrotic development which diffusely involved other interstitial area of the lung. However, there was also a significant fibrotic increase in control group at the 18th month compared with the former control rats. The cause of the increase is unknown. We regard the increase as a consequence of aging of the rats, considering the association of aging with pulmonary fibrosis (Selman et al., 2016). Still, the collagen content of exposed group at that time point exhibited a larger increase compared with the former time points, suggesting the pro-fibrogenic role of PM_{2.5}. As for the real world significance, since there have been limitations preventing the recognition of environmental agents as a direct etiological factor, PM_{2.5} has been only considered as a risk factor for idiopathic pulmonary fibrosis (IPF), the most common type of unknown etiology

(Wuyts et al., 2013; Taskar and Coultas, 2006). The lung features of our rat model resemble those of patients with IPF to some extent, which mainly include mild-to-moderate inflammation with infiltration, injury and hyperplasia of alveolar epithelial cells, enhanced deposition of extracellular matrix, thickened alveolar wall, and temporally heterogeneous fibrotic remodeling of lung structures (Meltzer and Noble, 2008; Raghu et al., 2011). Hence, our study has provided experimental supports on the idea that PM_{2.5} contributes to the initiation and development of IPF in patients who once experienced exposure of the particulates.

Pulmonary inflammation is the central effect contributing to the pathogenesis of pulmonary disorders, including lung injury and fibrotic disorders (Gualtieri et al., 2010). A variety of immune cells including T cells, monocytes/macrophages, lymphoid cells and granulocytes, and their released pro-inflammatory mediators such as IL-6, IL-1β, and TNF-α, participate in inflammation and tissue injury, and further promotes organ fibrosis (Mack, 2018). In the present study, we found sustained upregulation of inflammatory gene expression in rat lung tissue during post-exposure duration, including Tnf, Il1b, Il6, Ccl2, and Icam1 (Fig. 4). The functions of these genes involve pro-inflammatory cell recruitment and adherence promotion, which initiate and amplify inflammatory response (Zhang et al., 2018). Acute inflammation is a beneficial process that promotes healing of damage, while chronic inflammation is capable of evoking immune cells from the bloodstream to amplify the inflammatory response, which can misdirect the process of healing initiation to destroy healthy tissues (Lee et al., 2009; Ward, 2003). From this perspective, the sustained lung damage in our study

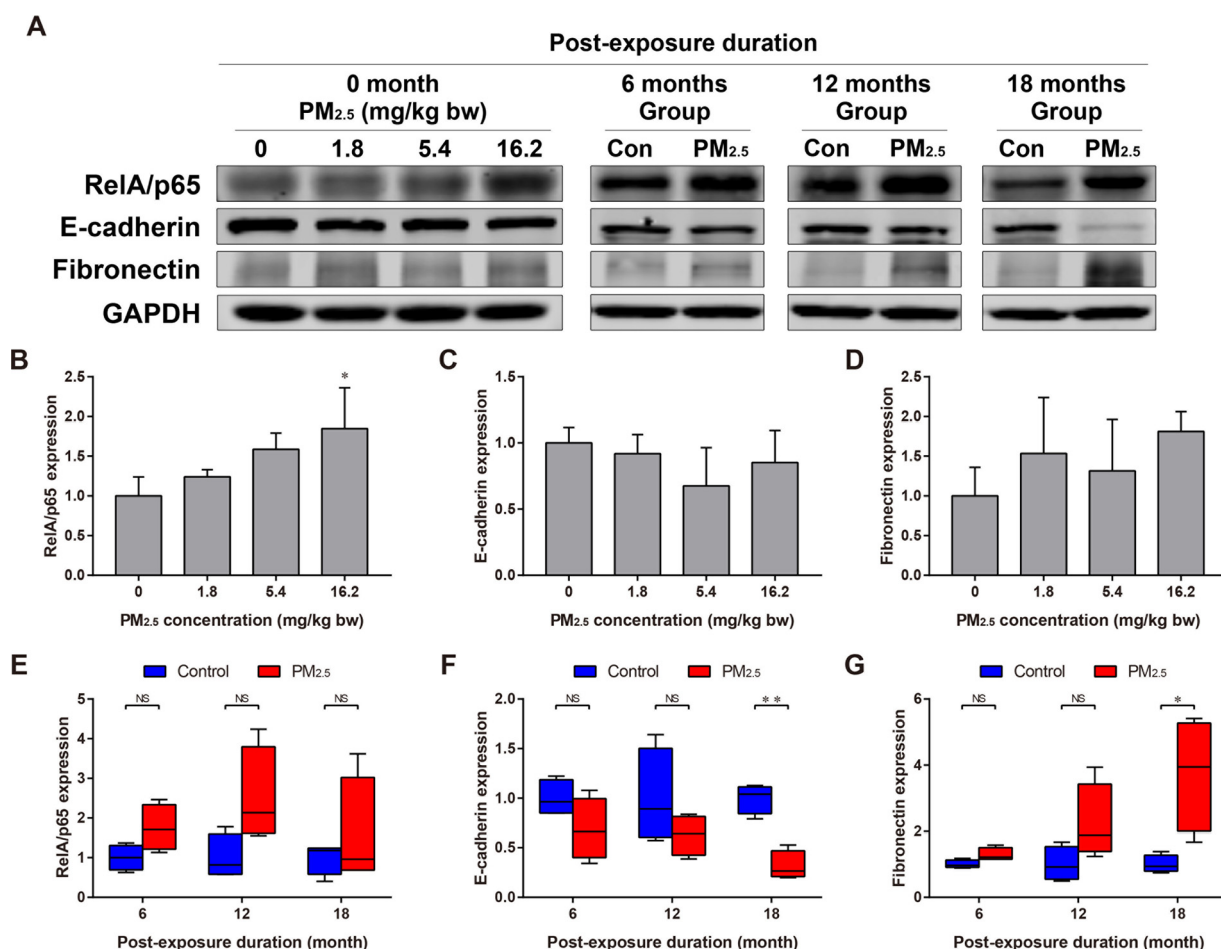


Fig. 5. Sustained epithelial-mesenchymal transition and NF- κ B upregulation caused by PM_{2.5} in post-exposure duration. (A) Representative western blotting bands. Protein expression levels normalized to GAPDH, including: (B) NF- κ B, (C) E-cadherin and (D) fibronectin immediately after the final exposure; (E) NF- κ B, (F) E-cadherin and (G) fibronectin at the post-exposure 6-, 12- and 18-month time points. N = 3 for the time point immediately after exposure; N = 4 for the post-exposure 6-, 12- and 18-month time points. * p < 0.05, ** p < 0.01 compared with the concurrent control group; NS: not significant.

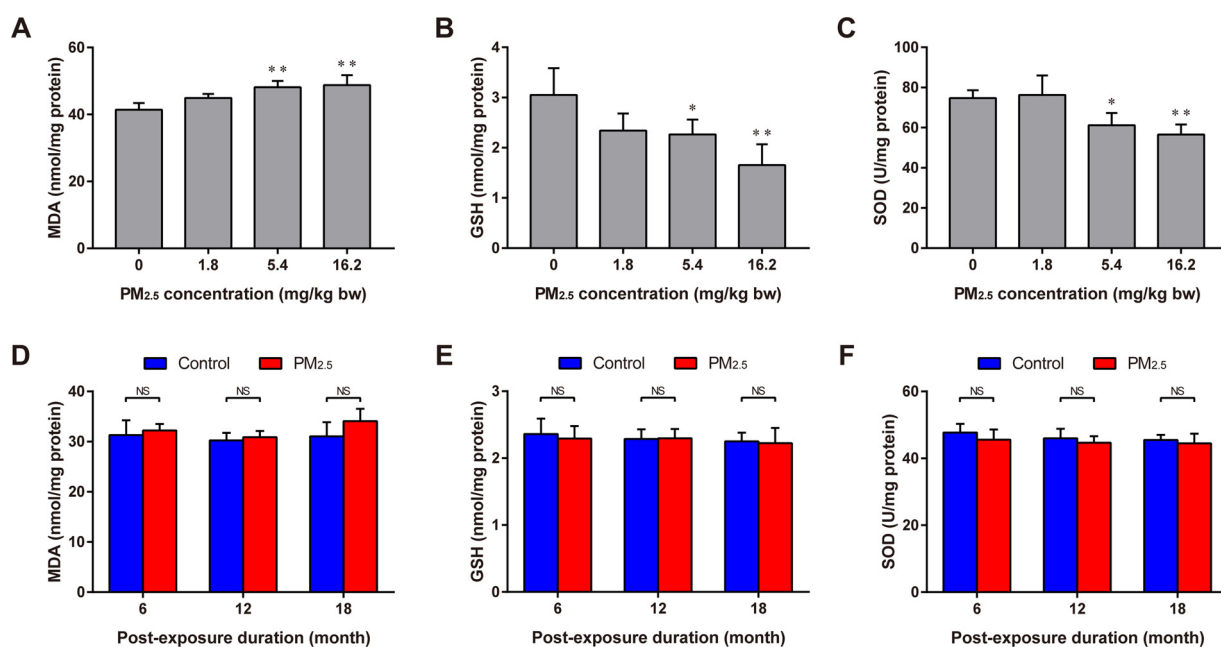


Fig. 6. Pulmonary oxidative stress triggered by PM_{2.5} exposure. The levels of oxidative product and antioxidants including: (A) MDA, (B) GSH and (C) SOD immediately after the final exposure; (D) MDA, (E) GSH and (F) SOD at the post-exposure 6-, 12- and 18-month time points. N = 4. * p < 0.05, ** p < 0.01 compared with the concurrent control group; NS: not significant.

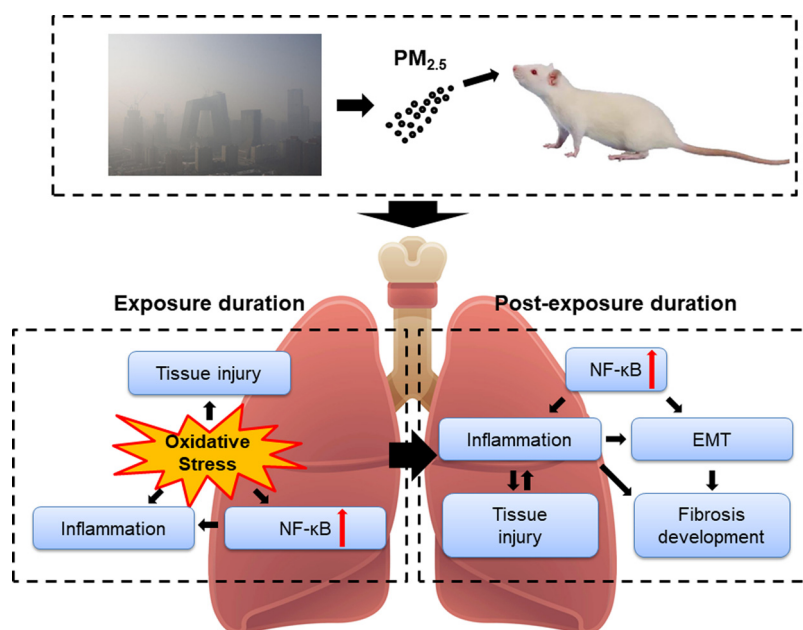


Fig. 7. Schematic of PM_{2.5}-induced toxicity and related mechanisms both during exposure and in post-exposure duration.

was, at least partly, attributable to chronic inflammation induced by PM_{2.5} exposure (Fig. 2). Tissue damage can further help perpetuate inflammation to some extent, thus forming a loop to promote the pulmonary fibrosis (Nathan and Ding, 2010). In addition, pro-inflammatory cytokines detected above, such as IL-1 β , can promote some pro-fibrogenic mediators (e.g., TGF- β), and may further contribute to pulmonary fibrosis caused by PM_{2.5} (Wynn, 2011; Bringardner et al., 2008). Systemic inflammation, reflected by upregulation of serum inflammatory cytokines, was also found existing throughout the post-exposure duration (Fig. S3). In our previous studies, we identified significant increases of inflammatory cytokines in BALF (an index which indicates pulmonary inflammation) and serum in rats after PM_{2.5} exposure (Shi et al., 2019; Liang et al., 2019). Many studies have proved that the levels of inflammatory cytokines in BALF and serum can be always elevated simultaneously by PM exposure, hinting their potential relevance (Jiang et al., 2018; Farina et al., 2013). A study by Kido et al. demonstrated that PM exposure could increase pulmonary inflammatory mediators that translocate to the circulation, thus contributing to systemic inflammation (Kido et al., 2011). Hence, persistent systemic inflammation could in turn help demonstrate the existence of continuous pulmonary inflammation in post-exposure duration. In addition, systemic inflammation may also lead to pulmonary vascular disorders, thus contributing to the pathogenesis of pulmonary fibrosis following PM_{2.5} exposure (Jagadapillai et al., 2016).

Another important event linking to pulmonary fibrosis is EMT induced by short-term PM_{2.5} exposure (Fig. 5). EMT is a process in which fully differentiated epithelial cells are transformed into a mesenchymal phenotype, with the loss of epithelial markers, acquisition of mesenchymal property, reassembly of cytoskeleton, enhanced capability of migration and interstitial matrix synthesis (King et al., 2011). EMT functions as one of the vital driving forces of lung fibrosis, taking part in the expansion of two main pro-fibrogenic types of cells, fibroblasts and myofibroblasts (King et al., 2011; Rout-Pitt et al., 2018). These cells can lead to deteriorated deposition of extracellular matrix, the hallmark of the scarring process that causes the ruin of the lung structure. In mice experimental model with lung fibrosis induced by bleomycin, fibroblasts originated from EMT accounted for about 33%, demonstrating the pro-fibrogenic role of this process (Tanjore et al., 2009). The occurrence of EMT in the present study was reflected by loss of epithelial marker E-cadherin, the hallmark of EMT occurrence that are

responsible for tight junctions, and gain of fibronectin, a mesenchymal marker helping form the extracellular matrix (Fig. 5). A study by Lin et al. indicated the occurrence of EMT through increase of E-cadherin and decrease of fibronectin, and further manifested EMT had a mediating effect on lung fibrosis, which is consistent with our results (Lin et al., 2017). What's more, some pro-inflammatory mediators such as IL-1 β and IL-6 are capable of inducing EMT, suggesting the chronic inflammation could also contribute to pulmonary fibrosis through EMT (Masola et al., 2019; Hamada et al., 2016).

Oxidative stress, caused by the imbalance between oxidants and antioxidants, is one of the fundamental mechanisms underlying PM toxicity. Existing evidences have indicated the existence of increased oxidative stress in fibrosis, although the specific role of oxidative stress in the pathogenesis of fibrosis has not been well clarified (Kurundkar and Thannickal, 2016). It has been well documented that oxidative stress functions as a central factor initiating the pulmonary inflammation and damage induced by particle exposure, which may in turn regulate pulmonary fibrotic development in our study (Zheng et al., 2016; Yang et al., 2018). We identified obvious pulmonary oxidative stress at the time point immediately following PM_{2.5} exposure in this study (Fig. 6). However, the alterations of oxidative-stress indicators disappeared at the later time points (Fig. 6). These results suggested that oxidative stress was induced only during exposure and could be the initiation of PM_{2.5}-induced toxicity including lung injury and inflammation. Additionally, the post-exposure deterioration of lung injury occurred in absence of oxidative stress, further supporting the idea that chronic inflammation could be the main contributor for the pulmonary pathological alterations. RelA/p65, the most important member of NF- κ B family which plays a fundamental role in inflammatory responses, was continuously upregulated in post-exposure duration (Fig. 5). The inflammatory genes detected in this study, including Tnf, Il1b, Il6, Ccl2, and Icam1, have been demonstrated to be the downstream transcription target of RelA/p65 in rats (Cafe-Mendes et al., 2017; Xing et al., 2012; Huang et al., 2014; Chen et al., 2017). Additionally, a study by Zhang et al. indicated that, downregulated E-cadherin expression and upregulated fibronectin expression induced by placental growth factor were counteracted by RelA/p65 inhibitor in primary type II alveolar epithelial cells from rats, demonstrating its regulating effect on EMT (Zhang et al., 2016). Therefore, continuous upregulation of RelA/p65 was, at least partially, responsible for the

chronic inflammation and EMT, and further contributed to the sustained development of pulmonary fibrosis. Collectively, we can conclude that, long-term pulmonary fibrosis development in post-exposure duration triggered by short-term PM_{2.5} exposure was initiated by oxidative stress at first and later induced through a NF- κ B/inflammation/EMT pathway (Fig. 7). However, how these toxic mechanisms were continuously induced in post-exposure duration still remains unclear and needs to be further elucidated.

5. Conclusion

Taken together, we systematically investigated the post-exposure impact of PM_{2.5} in Sprague-Dawley rats and identified that short-term PM_{2.5} exposure could lead to continuous pulmonary fibrosis development in later post-exposure duration, which was induced via oxidative-stress-initiated NF- κ B/inflammation/EMT pathway. While most of the studies have focused on PM_{2.5}-induced immediate toxic effects occurring simultaneously with exposure, our work is the first to report the sustained post-exposure toxicity on respiratory system triggered by PM_{2.5} in later long-term duration after exposure. As for the real-world significance of this study, being once exposed to PM_{2.5} may lead to increased risk of pulmonary fibrosis in nearly whole later lifetime, even if the exposure fades away. From this perspective, the health hazards of PM_{2.5} tend to be long-term, and could be much more than the extent we have thought before. Therefore, there is no time to delay in the abatement of PM_{2.5} pollution, and unflagging efforts are required in order to minimize the immense health impacts that PM_{2.5} could bring.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jhazmat.2019.121566>.

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