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# Application of a real-ambient fine particulate matter exposure system on different animal models

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ABSTRACT

Simulation of fine particulate matter ($PM_{2.5}$) exposure is essential for evaluating adverse health effects. In this work, an ambient exposure system that mimicked real atmospheric conditions was installed in Taiyuan, China to study impacts of chronic $PM_{2.5}$ exposure on adult and aged mice as well as Sirtuin3 knockout (Sirt3 KO) mice and wild-type (WT) mice. The real-ambient exposure system eliminated the possible artificial effects caused from exposure experiments and maintained the physiochemical characteristics of $PM_{2.5}$. The case studies indicated that aged mice exhibited apparent heart dysfunction involving increased heart rate and decreased blood pressure after 17-week of real-ambient $PM_{2.5}$ exposure. Meanwhile, 15-week of real-ambient $PM_{2.5}$ exposure decreased the heart rate and amounts of associated catecholamines to induce heart failure in Sirt3 KO mice. Additionally, the increased pro-inflammatory cytokines and decreased platelet related indices suggested that inflammation occurred. The changes of biomarkers detected by targeted metabolomics confirmed metabolic disorder in WT and Sirt3 KO mice after exposed to real-ambient $PM_{2.5}$. These results indicated that the real-ambient $PM_{2.5}$ exposure system could evaluate the risks of certain diseases associated with air pollution and have great potential for supporting the investigations of $PM_{2.5}$ effects on other types of rodent models.



## Introduction

The rapid development of the economy has caused serious air pollution in China. The World Health Organization (WHO) reported that air pollution might account for 4.2 million death worldwide in 2016 (Cheng et al., 2020; Rohde and Muller, 2015). Particulate matter, especially with an aerodynamic diameter less than 2.5 $\mu$m ($PM_{2.5}$), is closely associated with many diseases, such as respiratory diseases (Chi et al., 2019; Hu et al., 2014), cardiovascular diseases (Brook et al., 2010), and diabetes (Bhatnagar, 2009).

To better evaluate the health risk of airborne $PM_{2.5}$, different kinds of animal models have been used in toxicological studies with multiple techniques developed. The previ-

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ously applied exposure approaches, such as intratracheal instillation (Wang et al., 2017) and nose/mouth $PM_{2.5}$ inhalation system (Xu et al., 2016), have advantages and limitations. As the most popular method, intratracheal instillation was widely employed in toxicological studies on $PM_{2.5}$ exposure (Wang et al., 2017; Zhang et al., 2017) as the dosage of $PM_{2.5}$ could be easily controlled. However, the procedure requires anesthesia of the tested animals, which may cause organ damage or serious physiological variation (Xie et al., 2019). More importantly, the exposure pattern of intratracheal instillation may be significantly different from the deposition of ambient $PM_{2.5}$ in the lung. Although the nose/mouth inhalation system could relatively mimic real $PM_{2.5}$ exposure, this approach involves the use of a large amount of $PM_{2.5}$ samples when generating aerosol and may cause serious discomfort of animals by hindering their movement (Fröhlich and Salar-Behzadi, 2014). A whole-body ambient exposure system was developed recently (Sun et al., 2009), which can simulate the real exposure to the maximum extent (Li et al., 2019). However, the system allows crude PM including $PM_{2.5}$, $PM_{10}$ and dust to enter the animal chambers, causing not just $PM_{2.5}$ exposed to the experimental animals.

The present study established a real-ambient $PM_{2.5}$ system to perform the whole-body $PM_{2.5}$ exposure studies on mice and rats in Taiyuan, China where air pollution was very serious in winter (Li et al., 2014). The system utilized filters of different sizes to supply fresh air and that with $PM_{2.5}$ in the control and exposure groups, respectively. Aged mice model and Sirt3 KO mice model were used as cases in this study. Populations like elderly were potentially more vulnerable than general population to $PM_{2.5}$-induced adverse effects according to epidemiological studies (Peel et al., 2005). Aged mice model exposed to $PM_{2.5}$ through the real-ambient $PM_{2.5}$ exposure system could be used to verify epidemiological studies and explore the related mechanism. Sirt3, a major mitochondria nicotinamide adenine dinucleotide ($NAD^+$)-dependent deacetylase, played important roles in regulating redox homeostasis (Qiu et al., 2010) and mitochondrial functions (Dittenhafer-Reed et al., 2015). Given that $PM_{2.5}$ exposure could cause oxidative stress (Michael et al., 2013) and mitochondrial dysfunction (Li et al., 2015), Sirt3 might contribute greatly to $PM_{2.5}$-induced adverse effects. Therefore, Sirt3 KO mice model exposed to $PM_{2.5}$ using real-ambient $PM_{2.5}$ exposure system could be applied to explore new mechanisms of $PM_{2.5}$ toxicity. The obtained results from these two case studies indicated that $PM_{2.5}$ induced changes of heart rate, blood pressure, catecholamines and metabolites in Sirt3 KO and aged mice models. Additional effects of real $PM_{2.5}$ exposure were identified, for the first time. The general $PM_{2.5}$-induced effects found in the present study agreed with those from previous cohort studies.

## 1. Materials and methods

### 1.1. *Construction of the real-ambient $PM_{2.5}$ exposure system*

The whole-body inhalation exposure of $PM_{2.5}$ was performed to mice models using a real-ambient $PM_{2.5}$ exposure system with improvement over the previously reported system (Li et al., 2019). Briefly, the exposure system consisted of two remodeled conventional individual ventilated cage (IVC) systems, one of which was equipped with three layers of high-efficiency particulate air (HEPA) filters to supply fresh air (FA control), and another with a modified low-efficiency filter which allows only particulate matter less than 2.5 $\mu$m entering the animal chambers ($PM_{2.5}$ exposure). Both high-efficiency filters and low-efficiency filter were bought from Junsheng Laboratory Animal Equipment Co. Ltd. (Suzhou, China). The air supply and temperature control system were installed to keep a relatively constant temperature, humidity, and pressure. The instrument was installed at Shanxi University in Taiyuan, China. The concentrations of $PM_{2.5}$ outdoor and in the chambers during exposure period were measured by DustTrak TM II aerosol monitor (TSI Inc., USA).

### 1.2. *Exposure protocol*

Two animal models were used as cases to test the feasibility of the installed real-ambient $PM_{2.5}$ exposure system and explore the effects of $PM_{2.5}$ on different animal models. The first one was aged mice model. The adult (12 months) male C57BL/6 mice were purchased from the experimental animal center of Guangzhou University of Chinese Medicine (Guangzhou, China), while the aged ones (17 months) were from the Shanghai Model Organisms Center, Inc. (Shanghai, China). After one week's acclimation, the adult and aged mice were continuously exposed to FA or $PM_{2.5}$ in the exposure chambers for 17 weeks from November 1, 2018, to February 28, 2019. Four groups of mice (Six in each group) were housed in four chambers respectively.

The second animal model was Sirt3 KO mice model. Sirt3 KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME). In order to obtain mice of the same age and to eliminate the unnecessary production of heterozygous animals, a knockout-to-knockout breeding strategy was employed (Ali et al., 2019). Three-week-old wild-type (WT) C57BL/6 J male mice were bought from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) as control. After one week's acclimation, the 4-week-old male mice, both WT and Sirt3 KO, were continuously (i.e., 24 hr/day and 7 days/week) exposed to FA or $PM_{2.5}$ in the exposure chambers for 15 weeks from January 21, 2019 to May 5, 2019. Four groups of mice (Six in each group) were housed in four chambers, respectively.

All animals got free access to diet and water during the entire period of exposure experiment. Before and after exposure, animal body weights were recorded, and the blood pressure and heart rate were monitored and recorded with MiniTR non-invasive blood pressure analyzer (Nanjing kaerwen Biotechnology Co., Ltd, China). After exposure, all animals were sacrificed by isoflurane. All tissues and serum were collected for the subsequent studies. The above animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Shanxi University.

### 1.3. *LC-MS based targeted-metabolomics*

For the targeted metabolomic analysis, 50 $\mu$L serum was collected from each of the control and exposed Sirt3 KO

mice. Targeted metabolomics was performed on an Ultimate 3000 ultrahigh-performance liquid chromatography coupled with TSQ Quantiva™ triple-quadrupole MS (QqQ, Thermos fisher scientific, USA) with an Xbridge BEH Amide column (2.1 mm × 100 mm, 1.7 μm, Waters Corporation, Manchester, UK) in both positive and negative ionization modes. The detailed information of sample preparation, equipment conditions and data analysis were presented in the supplementary material.

### 1.4. *Determination of pro-inflammatory cytokines and catecholamines in the serum of Sirt3 KO mice model*

The levels of dopamine, epinephrine, norepinephrine, interleukin-6 (IL-6), interleukin-1$\beta$ (IL-1$\beta$) and tumor necrosis factor $\alpha$ (TNF-$\alpha$) in the serum of Sirt3 KO mice model were measured by mouse dopamine, epinephrine, norepinephrine, IL-6, IL-1$\beta$ and TNF-$\alpha$ ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd, Shanghai, China) according to the manufacturer's instructions.

### 1.5. *Statistical analysis*

Statistical analyses were performed using SPSS version 16.0 (IBM company, Chicago, IL, USA) and Microsoft Excel 2016. A $p$-value of less than 0.05 was considered significant. The results were presented as mean ± SEM.

## 2. Results and discussion

### 2.1. *Installation of the real-ambient $PM_{2.5}$ exposure system*

In order to evaluate the health risk of the regional $PM_{2.5}$ pollution more accurately, the real-ambient $PM_{2.5}$ exposure system was installed in Taiyuan, a city located in North China since November 2018. Because of the intensive coal industrial activities, the large coal combustion and unique geographical structure, Taiyuan has suffered from serious $PM_{2.5}$ pollution for years (Li et al., 2014). The average concentrations of $PM_{2.5}$ in Taiyuan winter was 209.0 $\mu g/m^3$ during 2009–2013 (Li et al., 2016), which was much higher than the recommended level in the WHO air quality guidelines (10 $\mu g/m^3$) (WHO, 2006). Previous toxicological studies using intratracheal instillation of $PM_{2.5}$ collected from the same area have shown severe adverse effects in both mice and rats (Gao et al., 2017; Li et al., 2015; Xie et al., 2019).

To mimic the real-ambient $PM_{2.5}$ exposure, two identical whole-body inhalation systems were installed, as shown in Fig. 1a. Except for air supply with distinct filters, all other physical conditions such as temperature (22–27 °C), humidity (30%−40%), and pressure (1015 Pa) were maintained as the same in both instruments. The systems used high- and low-efficiency filters, through which the main engines pumped fresh air and air with $PM_{2.5}$, into the chambers, respectively. The animals in the chambers were free to access diet and water to maintain relatively natural living conditions.

The total exposure period lasted from November 1, 2018 to May 5, 2019. The daily mean concentration of outdoor $PM_{2.5}$ during each experiment period was 73.2 $\mu g/m^3$ (21/01/2019–05/05/2019) and 76.8 $\mu g/m^3$ (01/11/2018–28/02/2019), respectively. Linear regression results illustrated that $PM_{2.5}$ concentration in the exposure chambers was equivalent to 61.6% (95% confidence interval: [56.0%, 64.6%]) of outdoor $PM_{2.5}$ (Fig. 1b), i.e. the concentration of $PM_{2.5}$ in the exposure chambers was about 42.6 $\mu g/m^3$ (Fig. 1c) and 47.31 $\mu g/m^3$ (Fig. S1) in the two experimental periods, respectively. In the meantime, $PM_{2.5}$ was undetectable in the FA control chambers (Fig. 1c), suggesting that the high-efficiency three-layer filters blocked most of the particulate matter to a level below the detection limit and the efficiency of the $PM_{2.5}$ exposure system was 61.6%. However, gaseous phase like $SO_2$, $NO_2$ and $O_3$ could not be interfered by the filters. The concentrations of $SO_2$, $NO_2$ and $O_3$ in outdoors during experiment periods were listed in the Fig. S1.

There have been various exposure protocols applied to different animal models for the investigations of $PM_{2.5}$ toxicity. Compared to the other methods, including intratracheal instillation method (Wang et al., 2017) and nose/mouth type inhalation exposure system (Xu et al., 2016), the current system not only avoided anesthesia which would be toxic to animals but also greatly reduced the pain of mice caused by restraint (Jackson et al., 2011). The system also eliminated the possible artificial effects, such as stress, mechanical damage and lung inflammation, caused by daily exposure operations (Fröhlich and Salar-Behzadi, 2014; Jackson et al., 2011). The physiochemical characteristics of $PM_{2.5}$ were maintained in the chambers with no need for sample collection and preparation. Compared to the versatile aerosol concentration enrichment system, which could result in more observable responses in animals (Sun et al., 2009), the real-ambient $PM_{2.5}$ exposure system provided the experimental conditions closer to the real exposure scenarios. The responses in animal models to this exposure system are likely to be of higher value in understanding the impacts of $PM_{2.5}$ exposure on human health.

### 2.2. *Application of the real-ambient $PM_{2.5}$ exposure system in aged mouse model*

The exposure system was firstly applied for the investigation of the effects of real $PM_{2.5}$ exposure on aged mice. Previous cohort and toxicological studies reported that old individuals were potentially more vulnerable than the adult individuals to $PM_{2.5}$-induced adverse effects (Sacks et al., 2011; Yan et al., 2020). Epidemiological evidence also indicated the increased risk of cardiovascular morbidity with particulate matter exposure in elderly (Pope et al., 2008). To compare the effects of $PM_{2.5}$ at different ages, adult and aged mice were housed in the exposure system for 17 weeks. The results showed that 17-week of $PM_{2.5}$ exposure in Taiyuan significantly affected the aged mice, but not the adult ones. $PM_{2.5}$ exposure resulted in the gain of body weight (Fig. 2a), rise of heart rate (Fig. 2b), and drop of blood pressure (Fig. 2c) only in the aged mice. As the essential indicators of heart function, the changes in heart rate and blood pressure indicated the cardiac functional injury (Fiordelisi et al., 2017). The results agreed with the previous longitudinal study that $PM_{2.5}$ exposure increased heart rate in elderly (Lim et al., 2017). Consistent with the epidemiologi-

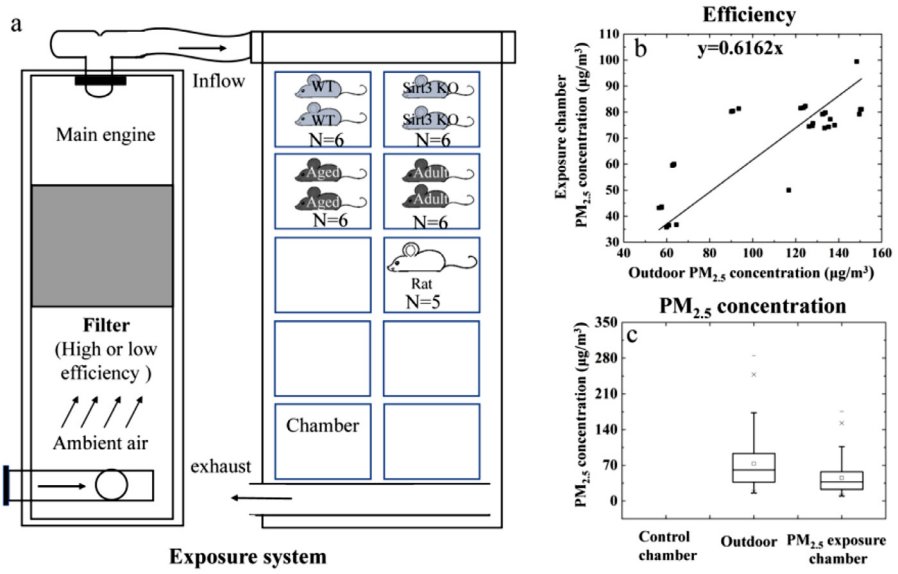


**Fig. 1. – Real-ambient $PM_{2.5}$ exposure system. (a) Schematic diagram of the exposure system; (b) linear regression of $PM_{2.5}$ concentrations in and out of the exposure chambers; (c) average $PM_{2.5}$ concentration in the control and exposure chambers during the exposure period (21/01/2019–05/05/2019).**

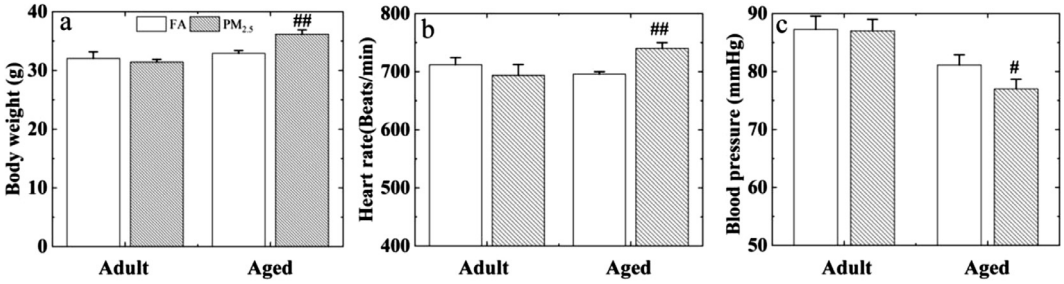


**Fig. 2 – Physiological indices changes in aged mice model. (a) Body weight; (b) heart rate; (c) blood pressure. # vs. FA; #, $P < 0.05$; ##, $P < 0.01$.**

cal results, the elderly showed high susceptibility in the real-ambient $PM_{2.5}$ exposure system, providing data for the further study on age-dependent $PM_{2.5}$ toxicity.

### 2.3. *Application of the real-ambient $PM_{2.5}$ exposure system in Sirt3 KO mice model*

Sirt3, a mitochondrial protein belonging to the family of $NAD^+$-dependent protein deacetylases (Hirschey et al., 2011), regulates many critical cellular processes, including fatty acid oxidation (Hirschey et al., 2010), urea cycle (Hallows et al., 2011), oxidative phosphorylation (Overmyer et al., 2015) and antioxidative system (Dittenhafer-Reed et al., 2015), which have been proved to involve in $PM_{2.5}$-disturbed pathways (Song et al., 2019, 2020). $PM_{2.5}$ exposure has been found to decrease the expression of Sirt3 in bronchial cells (Chen et al., 2016) and heart and lung of the normal mice (Fig. S2). To elucidate the potential roles of Sirt3 in $PM_{2.5}$-induced damage, both WT and Sirt3 KO mice were exposed for 15 weeks using the real-ambient $PM_{2.5}$ exposure system. After 15 weeks of $PM_{2.5}$ exposure, four groups of mice showed no changes in body weight (data not shown), but the heart rate decreased significantly in $PM_{2.5}$-exposed Sirt3 KO mice (Fig. 3a). Furthermore, the levels of some catecholamines, including dopamine, epinephrine, and norepinephrine, which participated in the regulation of the central nervous system on heart function (Ying et al., 2014), were also significantly decreased in and only in the $PM_{2.5}$-exposed Sirt3 KO mice (Fig. 3b), suggesting that $PM_{2.5}$ may induce heart failure in Sirt3 KO mice (Brook et al., 2010; Li et al., 2018). Because it has been reported that $PM_{2.5}$ exposure would cause inflammation in humans (Nikasinovic et al., 2006; Zhao et al., 2013), pro-inflammatory cytokines were also determined in the mouse serum. Among the three pro-inflammatory cytokines detected, 15 weeks of exposure increased the levels of both IL-1$\beta$ and IL-6 in Sirt3 KO mice significantly but only the level of IL-6 in WT mice (Fig. 3c), indicating that the loss of Sirt3 resulted in more susceptibility to $PM_{2.5}$ exposure in mice. While the results of blood routine examination showed that platelet-related indices such as blood platelet, platelet distribution width, and thrombocytocrit (Fig. S3) decreased in the serum of Sirt3 KO mice, espe-

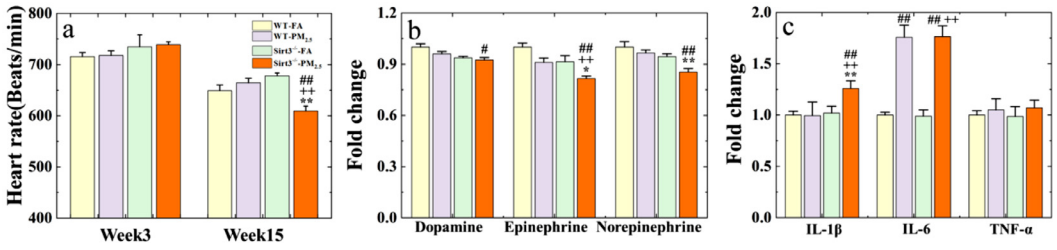


**Fig. 3 – Physiological indices changes in Sirt3 KO mice model. (a) Heart rate; (b) catecholamines, (c) pro-inflammatory cytokines. Note: # vs. WT-FA; + vs. WT-PM$_{2.5}$; * vs. Sirt3 KO-FA; #, +, * $P < 0.05$; ##, ++, ** $P < 0.01$.**

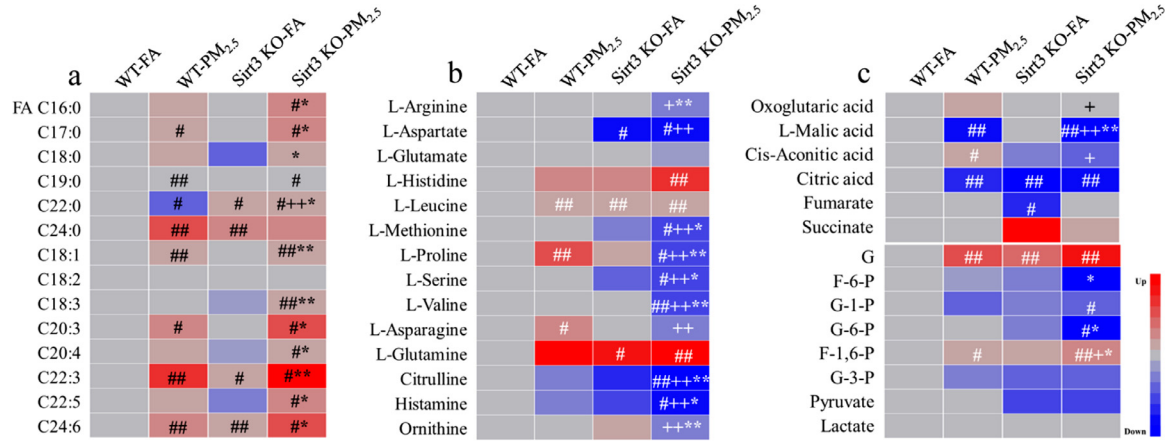


**Fig. 4 – Heat maps of Sirt3-regulated metabolites in Sirt3 KO mice model. (a) Long-chain free fatty acids; (b) amide acids; (c) glycolysis and TCA cycle related metabolites. Note: # vs. WT-FA; + vs. WT-PM$_{2.5}$; * vs. Sirt3 KO-FA; #, +, * $P < 0.05$; ##, ++, ** $P < 0.01$.**

cially after the exposure of $PM_{2.5}$. The previous studies have reported that platelet was closely related to immune system (Klinger and Jelkmann, 2002; Weyrich et al., 2003), therefore, the decreases of platelet related indices may also indicate the occurrence of inflammation (Nurden, 2011).

To further verify the effects of real-ambient $PM_{2.5}$ exposure system at molecule levels, the metabolites related to Sirt3-regulated pathways, including urea cycle, TCA cycle, fatty acid oxidation and glycolysis, were detected by targeted metabolomics. The obtained heat maps indicated that exposure to $PM_{2.5}$ resulted in the increase of free fatty acids (Fig. 4a) and decrease of the intermediates of the TCA cycle (Fig. 4c) in both WT and Sirt3 KO mice compared to FA groups, a result which agreed with the previous studies that $PM_{2.5}$ exposure disturbed energy metabolism including fatty acid oxidation (Xu et al., 2019) and oxidative phosphorylation (Song et al., 2019). Different from the results above, the metabolites in urea cycle (Fig. 4b) and glycolysis (Fig. 4c) were only altered in $PM_{2.5}$-exposed Sirt3 KO mice. Because urea cycle in arginine and proline metabolism pathway has been proved to be associated with inflammation (Song et al., 2019), the changes of the detected metabolites might also suggest the inflammation occurred in Sirt3 KO mice, which consistent with the rise of pro-inflammatory cytokines (Fig. 3c).

Those results indicated that chronic $PM_{2.5}$ exposure induced heart failure, inflammation and metabolic disorders in Sirt3 KO mice. With the help of the real-ambient $PM_{2.5}$ exposure system, the mice showed the physiological responses similar to previous observations from cohorts and toxicological studies (Hsieh et al., 2013; Nikasinovic et al., 2006; Song et al., 2019). Furthermore, the more severe results from the knockout mice suggested the potential function of Sirt3 in the $PM_{2.5}$ toxicity and possibility of the real-ambient $PM_{2.5}$ exposure system for biological mechanism research.

This exposure system has also been successfully applied to study the effects of $PM_{2.5}$ exposure on rat models (Bai et al., 2020). Our previous study showed that the relative weights of the liver, spleen and kidney significantly increased after one-month of $PM_{2.5}$ exposure in rats, indicating that the liver, kidney, and spleen might also be the target organs for $PM_{2.5}$ (Fig. S4). The HE staining data exhibited that one-month of $PM_{2.5}$ exposure in rats induced structural disorder in the liver, spleen, kidney and stomach (Bai et al., 2020).

## 3. Conclusions

The real-ambient $PM_{2.5}$ exposure system was designed to simulate experimental conditions similar to the daily $PM_{2.5}$ exposure scenario of human life. The results, such as the rising pro-inflammatory cytokines and the higher susceptibility of aged mice, were consistent with the epidemiological observations, demonstrating that the system is effective in establishing animal models for $PM_{2.5}$ toxicity studies. Beyond that, additional

effects from the real $PM_{2.5}$ exposure were identified, for the first time, such as the decreased heart rate in Sirt3 KO mice, which means that the real-ambient $PM_{2.5}$ exposure system has great potential to help more deeply explore novel molecular mechanisms of $PM_{2.5}$-induced adverse biological effects.

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## Appendix A. Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.12.007.

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