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Abstract

Uncertainties for optimized air pollution control remain as the underlying mechanisms of city-specific ambient particulate matter (PM)-induced health effects are unknown. Here, water-soluble extracts of PMs collected from four global cities via automobile air conditioning filters were consecutively injected three times by an amount of 1, 2 and 2 mg into the blood circulation of Wistar rats after filtration by a 0.45 μm pore size membrane. Acute health effects such as immune and inflammatory responses and hemorrhage in alveoli were observed right after the PM extraction injection. Significant differences between cities in biomarker TNF-α and MCP-1 levels were detected following the second and third PM injections. Rats' inflammation responses varied substantially with the injections of city-specific PMs. Repeated PM extract exposure rendered the rats more vulnerable to subsequent challenges; and down-regulations of certain microRNAs were observed in rats. Among the studied miRNAs, miR-125b and miR-21 were most sensitive to the PM exposure, exhibiting a negative dose-response type relationship with source-specific PM (oxidative potential) toxicity (r^2=0.63 and 0.57; p-values<0.05). The results indicated that city-specific PMs could induce different health effects by selectively regulating different miRNAs; and certain microRNAs, e.g., miR-125b and miR-21, may be externally mediated to neutralize PM-related health damages.

Keywords: Particulate Matter; Toxicity; MicroRNA; Inflammation; Biomarker; Catheter-embedded Rat Model

50 **TOC**

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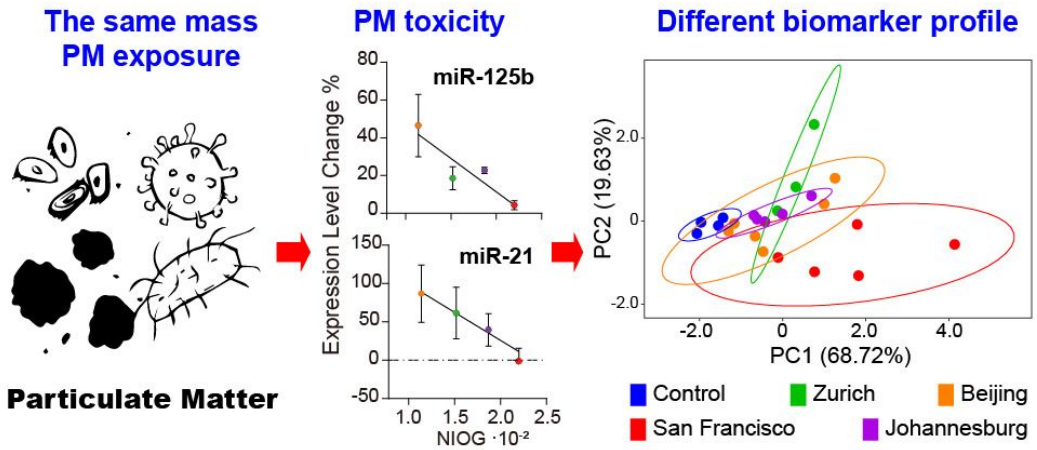

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Introduction

Air pollution, especially the particulate matter, has become a growing global health concern. Exposure to particulate matter with a diameter of no more than 2.5 micrometer ($PM_{2.5}$) is estimated to have resulted in 4.2 million deaths in 2015 worldwide.[1-2] Particularly for Asian countries, there were more severe haze episodes in recent years with much higher average levels of ambient $PM_{2.5}$, which accordingly explains for more air pollution related deaths from these regions.[1-2] For example, China and India together had the largest numbers of attributable deaths to the total 4.2 billion deaths: 1.11 and 1.09 million, respectively; while the United States alone had 0.09 million.[1] Yet, it is interesting to note that the actual investigated mortality rates of these different regions resulting from air pollution tell a different story. For instance, according to an epidemiologic study conducted in 272 Chinese cities, the magnitude of the associations between short-term exposure to $PM_{2.5}$ and increased mortality from various cardiopulmonary diseases in China was lower than those reported in Europe and North America.[3] In addition to its mass level and many others, the observed difference could be also resulting from the different compositions of different sourced PMs, which result in different PM toxicity as previously found for global cities.[4]

To investigate PM toxicity, biomarkers in cultured cells (*in vitro* test) or animal and human samples such as bronchoalveolar lavage fluid (BALF), blood and urine (*in vivo* test) are widely used in both epidemiological and toxicological studies.[5] IL-6 and TNF-α have been chosen as biomarkers along with other cytokines or chemokines to indicate immune and inflammatory responses.[6-7] To some extent, biomarker level changes can signal an inflammatory response to the toxicity of PM. In our previous work, breath-borne IL-6 was online monitored to reflect the inflammatory levels in rats after being injected with extractions of PMs collected from different cities.[8] In addition to these protein markers, microRNA has been increasingly used in studying environmental exposure and health effects.[9] MicroRNAs (miRNAs) are a series of post-

transcriptional regulators of gene expressions. It is believed that microRNAs play important roles in many developmental and cellular processes.[10] In general, microRNA guides RNA-induced silencing complex (RISC) to target mRNA in the 3' untranslated region (UTR), then represses or degrades its translation. Accordingly, most microRNAs are expected to reduce the mRNA levels and protein expression of the target genes.[11] Several microRNAs such as miR-125b, miR-155, miR-146a, and miR-21 were found to be closely involved in the innate immune and inflammatory process and widely investigated in air pollution related studies.[9, 12] For example, in an cohort study of steel plant workers, it was reported that miR-21 responded to the production of reactive oxygen species (ROS) in the blood due to the PM-induced increase in oxidative stress.[13] Besides, it was shown that exposure to ambient particles could cause down-regulations of related microRNAs, such as miR-126, miR-146a, miR-155, miR-21, etc.[14] However, how their regulations vary with different toxicity PMs from different sources is not clear.

Here, this work was carried out to mainly investigate: 1) Whether there are inflammation biomarker differences in repeated exposures of PMs from different cities on an animal's protein biomarker level? 2) What specific miRNAs are expressed when rats are exposed to PMs of different sources? 3) If there is a toxicity dependence for the expressions of specific miRNAs? Different from DTT (Dithiothreitol) assay for PM toxicity analysis, we employed an animal-based $PM_{2.5}$-toxicity protocol developed in our lab which uses the intravenous injection to expose rats to PMs.[15] PM samples collected from world cities via automobile filter method[4, 16] were used in this work. PM water-soluble extracts filtered using 0.45 μm syringe filters were directly injected into the blood circulation of rats in this study. Serum biomarkers (TNF-α, MCP-1 and IL-1α) and blood plasma microRNAs (miR-146a, -125b, -126, -132, -155, -21, -223 and -26a) were measured in the exposure experiments. Histopathological analysis of organs (heart, liver, spleen, lung, kidney) were also performed. Differences in PM toxicity and also the molecular responses induced were analyzed. Results from this

work provide information about dose-response relationship between PMs with different toxicity and biomarker expression as well as miRNA regulations, and further shed new light on the underlying health effect mechanisms of source-specific PM exposure.

Materials and Methods

PM Sampling and Preparation

The PM samples from Beijing, San Francisco, Zurich, and Johannesburg were collected using automobile air conditioning filters, and then the pooled PM (N=5-15 auto filter samples from each city) water-soluble components were extracted by normal saline. The sampling and extracting protocols were described in our previous studies.[4, 16] Extracts of pooled PM samples from each city were prepared in two suspensions using normal saline: 1 mg/mL and 2 mg/mL, and filtered by a 0.45 μm-pore sterile PTFE hydrophilic Syringe filter (Agela Technologies Inc., China) before the injection.

Rat Breeding and PM Injection

Male Wistar rats (n=30) at an age of 10 weeks weighing 200-250 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All rats were performed an operation embedding a flexible sterile catheter into the jugular vein with 1 cm of catheter out of the skin and fixed onto the back of the rat with staples. Extracts injection and blood sampling were performed through the catheters using sterile syringes with 23G flat-end needles. Detailed catheter operation protocol as well as the injection and blood sampling were described in our previous work.[15] All rats were kept inside a house-made cabinet with 24-h ventilation, 24-h video recording, 12:12 light-dark cycle, food and water *ad libitum*. After one week of acclimation, the rats were randomly divided into five groups right before the experiments: Control group, San Francisco group, Zurich group, Johannesburg group and Beijing Group (each group consisted 6 rats). PM water-soluble extracts of different cities after the

143 filtration were injected into the blood circulation of rats from corresponding groups.
144
145 As shown in Figure S1 (Supporting Information), in order to investigate the health
146 effects induced by repeated exposures of PMs, we performed three separate
147 injections of PM extracts at different times. The first injection was on day 0 (the first
148 day) with 1 mL of 1mg/mL extracts for each rat, and the following two injections were
149 carried out on day 3 (three days later) and day 7 (seven days later) respectively with 1
150 mL of 2mg/mL extracts each time. The control group rats were injected with normal
151 saline with the same volume. Here, use of 1 mg or 2 mg PMs was based on
152 approximate 1-year inhalation exposure of rats to ambient average PM levels of 50
153 $\mu g/m^3$ or 100 $\mu g/m^3$, respectively, assuming a body weight ratio of 300 (human vs. rat)
154 and a human breathing rate of 12 L/min. Although the injection does not reflect a true
155 exposure, it represents the worst scenario where all inhaled particles could possibly
156 get into the blood especially after the filtration. After the injection, both control and
157 exposed groups were physically monitored using video camera. On day 14, twenty rats
158 (four rats from each group, randomly selected) were euthanized by using
159 pentobarbital sodium (Beijing Skillsmodel Biotechnology Co., Ltd) and anatomized for
160 the histopathological analysis. The remaining rats from each group were kept for the
161 follow-up observation of chronic health effects as additional evidence until day 473
162 (one year and 108 days), during which the weight of rats was recorded every 7-10 days.
163 Because of lab space limitation and resource constraint, not all rats could be kept for
164 long-term observation. All animal experiments were approved by the Institutional
165 Review Board of Peking University, and the experiments were performed in
166 accordance with ethical standards (approval # LA2017204).
167
168 ***Blood Sampling and Measurement***
169 Blood samples were taken (0.3mL for each time) before the injection and 1h later
170 after the injection. After a 20-min standing at room temperature, blood samples were
171 subjected to centrifugation (5804 R, Eppendorf Inc., Germany) at 3000 rpm for 10 min

to separate serum from plasma. The supernatant serum and plasma were stored separately at -20^{o}C for further analysis. Serum biomarkers including monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) were measured using a Rat Cytokine/Chemokine Magnetic Bead Panel (Merck Milliplex, Cat. #RECYTMAG-65K, RECYMAG65K27PMX, RECYMAG65PMX27BK), according to the manufacturer's instructions. As shown in a previous study, exposure to ambient particles could cause down-regulations of related microRNAs.[14] Thus, the blood plasma microRNAs including miR-146a, miR-125b, miR-126, miR-132, miR-155, miR-21, miR-223 and miR-26a were measured using a qRT-PCR array. The experiments were conducted at Wcgene Biotech, Inc., China. RNA isolation, primers and cycling condition of miRNA qRT-PCR were described in the Supporting Information (Table S1).

Anatomy and Histopathological Analysis of Rats' Organs

After the rats were sacrificed, heart, liver, spleen, lung and kidney from the rats were taken and subsequently washed using normal saline. Then, the organs were steeped in 4% formaldehyde solution provided by Wuhan Servicebio Technology for 72 hours. The fixed organs were subjected to dehydration and paraffin-embedded as the preparation of tissue section. Then the cross sections were mounted onto the glass slides and deparaffinized. All the slides were stained with hematoxylin and eosin (H&E) for histopathological analysis. At least 20 slides were obtained and examined from the samples taken from each organ of each rat. Experiments including dehydration, embedment, cutting, deparaffinization and HE stain were conducted by Wuhan Service Biotechnology Corporation.

Statistical Analysis

The statistical differences in serum MCP-1, TNF-α and IL-1α concentrations among different groups were analyzed via one-way ANOVA (data exhibited a normal distribution) or Kruskal-Wallis one-way ANOVA on Ranks (data did not follow a normal distribution or equal variance test failed). To determine the expressions of microRNAs,

a relative fold value was calculated using the $2^{-\triangle Ct}$ method.[17-18] Differences in microRNAs expression among different groups were also analyzed via one-way ANOVA. All statistical tests were performed with Graph Pad 8.0 prism software. Besides, the software R (x64 3.6.2) with package "ggord" was used to visualize the biomarker profile distance and relatedness of different groups using the principal component analysis (PCA). The software R (x64 3.6.2) with package "vegan" was used to study the relationship between the biomarker profiles, PM toxicity and the microRNAs expression levels among different groups using the Redundancy analysis (RDA). In this study, due to the sampling or testing failure, the effective sample size of each group was shown in Table S2 (Supporting Information). For all the biomarkers and miRNAs studied, we had at least three values from each group. A *p-value* of less than 0.05 indicated a statistically significant difference at a confidence level of 95%.

Results and Discussion

Toxic effects of PM injection to Rats Revealed by Protein Biomarkers and Pathological Observations

The concentration percentage changes of TNF-α, MCP-1 and IL-1α in blood sera of rats from different groups on three consecutive independent injections are shown in Figure 1(A). The concentration percentage changes of biomarkers for each injection were calculated by dividing the biomarker concentration 1h after injection by the concentration before the injection. As shown in the figure, levels of three biomarkers increased after each injection, which indicated an acute inflammatory response induced by PM water-soluble extracts within one hour after the injection. The surveillance video (Figure 1(B) and Supporting Information Video S1) also showed that rats appeared to be drowsy after injected with PM extracts, i.e., staying in the corner of the cage without moving around much after the PM injection; while the rats in the NS (control) and Beijing groups moved around the cage and were relatively sensitive to external interruptions. In a recent study, similar behavioral discrepancies were observed between groups of rats injected with high and low doses of PM[15], suggesting

that the PM injected into the blood circulation indeed caused acute health effects to rats. In future efforts, rat behavior/movement analysis software can be used to quantitatively assess the behavioral differences between different rats. Previously, it was clearly shown using Beijing PM samples that adverse health effects depend on the PM mass injected.[15] Using world cities' PM samples, we further demonstrated here that PM toxicity in addition to mass also played a very important role for PM-related health effect.

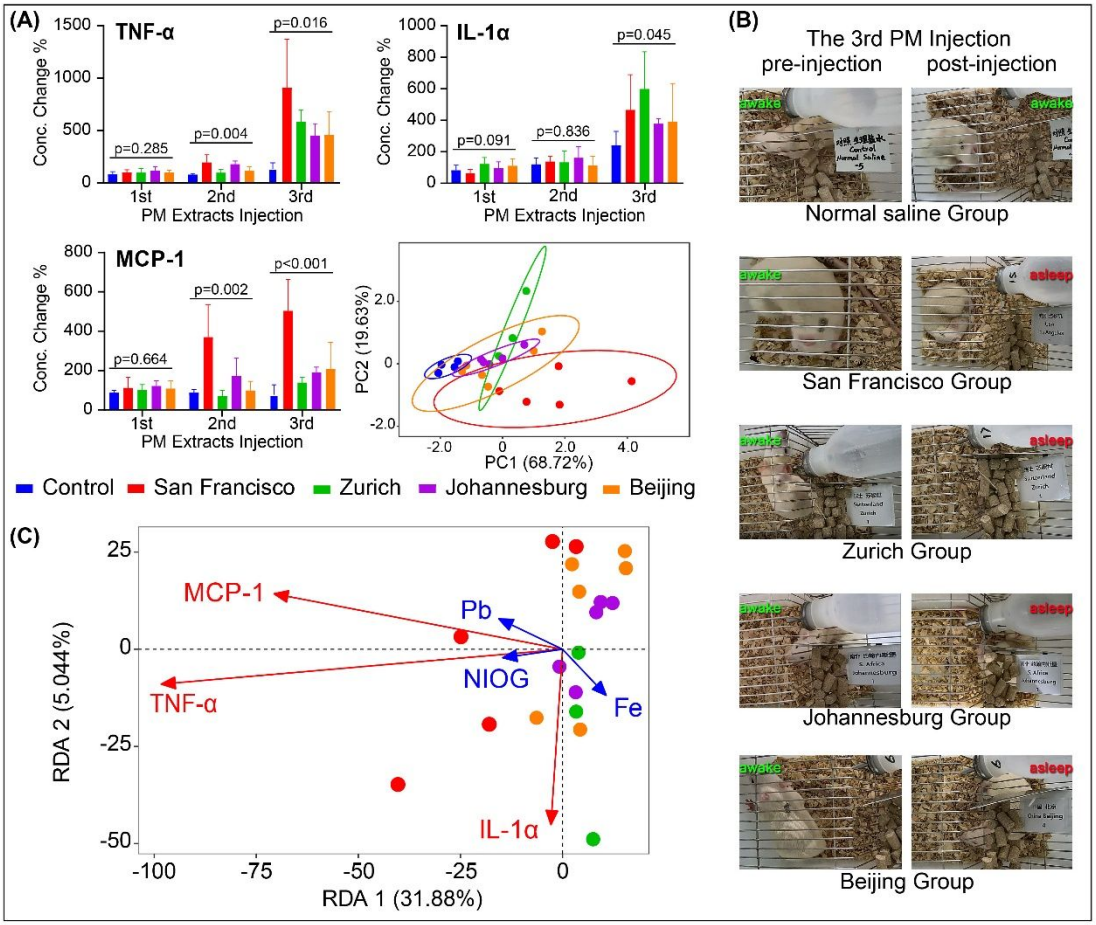

Figure 1. (A) Concentration percentage changes and PCA analysis of serum TNF-α, MCP-1 and IL-1α of rats from different groups (five groups: Normal saline, San Francisco, Zurich, Johannesburg, and Beijing, each group originally (day 0) consisted six rats) on three times' injections (day 0, 3 and 7). Data points represent the average results from at least 3 rats after eliminating rats with outliers by Grubbs test and sampling failures (Table S2 in Supporting Information), and error bars stand for the

standard deviations from at least three independent measurements. The first injection with 1 mL of 1mg/mL extracts, second and third injections with 1 mL of 2mg/mL extracts each time. The PCA ordinations of biomarker expression profiles were based on the biomarker results on the 3rd time injection (each group had at least 3 rats, a total of 22 rats as listed in Table S2) under exposures to PMs from four different cities. PC1 (68.72%) and PC2 (19.63%) are the first and second principal components. (B) Video snapshot of rats of different groups in 1 h after the 3rd injection. In general, rats injected with normal saline appeared to be more active than those injected with PM extracts. Videos are provided in Supporting Information Video S1. (C) Redundancy (RDA) analysis results of the three biomarker expression after the 3rd injection and PM characteristics (Normalized index of oxidant generation (NIOG) and metal). The PM characteristic variables were selected by backward method according to the variance inflation factor. NIOG determined by the DTT assay of samples from San Francisco, Zurich, Johannesburg and Beijing are 0.0220, 0.0152, 0.0187 and 0.0114, respectively.[4] Fe and Pb levels were determined by ICP-MS a previous study.[8] Each dot in the figure represents an independent rat injection test.

For all biomarkers, the concentration percentage changes generally increased and differences between five groups were shown to be increasingly substantial with the sequential PM extract injection as shown in Figure 1(A). With regard to the biomarker concentration percentage changes after the third injection, biomarker levels between five groups were statistically different (TNF-α, and MCP-1 were more sensitive) (*p-values*<0.05). Zurich group had the highest increase rates in IL-1α; while San Francisco group had the highest for TNF-α and MCP-1. On the other hand, Johannesburg and Beijing groups tended to have comparable percentage increases in their corresponding serum biomarkers. PCA results revealed a clear contrast in biomarker expression profiles of rats between different groups. The biomarker expression profile of the San Francisco group was very different from that of the control group (normal saline) and the biomarker expression profile of the Beijing group was the closest to

that of the control group. In our previous work using the same PM samples, breath-borne IL-6 was shown to be higher in exhaled breath collected from rats injected with PM samples collected from Zurich and San Francisco than those from Beijing and Johannesburg.[8] Innate immune cells are believed to recognize PM extracts in the blood to activate immune and acute phase response with the expression of inflammatory mediators such as TNF-α to defend against foreign agents and repair tissue injury.[19] Different biomarkers can be also synergistic and mutually promoted. For example, TNF-α can regulate the production of IL-6 and MCP-1,[20] while MCP-1 as a chemokine in return indirectly promoted the secretion of IL-1 and IL-6 by recruiting leucocytes and leading them to inflammatory sites in the body.[21] Besides, the increased IL-1α observed in this study might be due to acute tissue injury as the IL-1α was known as an injury indicator rather than a pro-inflammatory mediator in the immune and defense system.[22] Therefore, these findings indicate rats from Zurich and San Francisco groups injected even with the same mass PM as other groups yet suffered a much more serious inflammatory responses, which was most likely due to more toxic PM samples collected from the corresponding cities. Exposure to PMs from different cities caused different expression of biomarkers in rats' blood. As shown in Figure 1 (C), RDA analysis showed that TNF-α and MCP-1 were more sensitive to PM toxicity (NIOG) than IL-1α; while among the metals Pb and Fe were shown to be involved in the expressions of the biomarkers. In an *in vitro* study, it was shown that $PM_{2.5}$ from Beijing with higher burden of metals and PAHs exhibited different toxic potencies than Guangzhou at equal mass concentrations.[23] In another work, it was found that the oxidative potential by acellular assays of PM per unit of mass from Beijing was even lower compared to that of Zurich.[24] In a toxicology study based on rat model, $PM_{2.5}$ collected from California, USA was shown to have greater lung toxicity than $PM_{2.5}$ from Shanxi, China at equal mass concentration which appears to be driven by more oxidized organic carbon and copper content.[25] The experimental data showed that rats responded differently to PMs from different cities with different biomarker expressions, which on the other hand revealed the differences in PM

303 toxicity and health mechanisms.

304

305 In general, for exposure groups, there were significant increases in the average
306 concentration percentage changes of biomarkers for the three separate injections as
307 shown in Figure 2 (A), (*p-value*<0.001, Kruskal-Wallis One Way Analysis of Variance on
308 Ranks). In contrast, concentration percentage changes of biomarkers for the control
309 group did not change significantly (*p-value*=0.260). In other studies, it was also shown
310 that repeated PM exposure caused 10 times higher levels of biomarkers than single
311 exposure such as TNF-α, suggesting stronger inflammatory response in rats due to
312 repeated exposures.[26] These results indicate that prior PM exposure is also an
313 important factor that influences the responses to subsequent exposures of the PMs
314 from the same sources.

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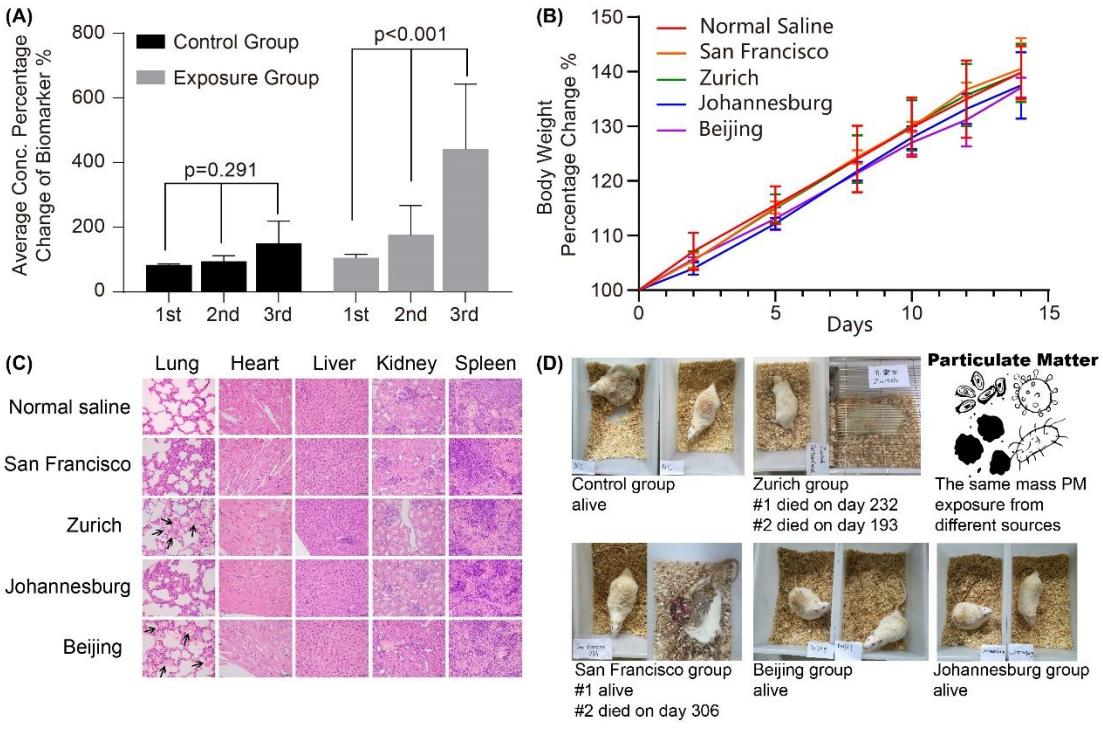

316

317 **Figure 2.** (A) Average biomarker concentration percentage changes of control and
318 exposure groups for three biomarkers (TNF-α, MCP-1 and IL-1α) at three injections.
319 The sample size (N>=3) for each group after excluding outliers using Grubbs test were
320 shown in Table S2 (Supporting Information). (B) Average rat body weight percentage

changes between different groups (five groups, each group consisted six rats) in the first fourteen days. (C) The histology images of organs of rats from different groups by HE stains (40X). Hemorrhage in lung alveoli was marked with black arrows in the figure. (D) Photo of rats left for long-term observation until day 473 (five groups, each group had two rats left for observation after the exposure experiment). Two rats from the Zurich group and one rat from the San Francisco group died during the observation period. One rat from the control group and one rat from the San Francisco group developed a tumor. "*" indicates a significant difference at 95% confidence level by t-test. Data points represent the average results from at least 3 rats for Figure 2 (A), and 6 rats for Figure 2 (B) , and error bars stand for the standard deviations.

In addition to the observation of the acute health effects in the 14 days experiments, 2 rats from each group (due to lab space limitation) were used as an additional evidence to observe how the PM injection affects the rats' health over a longer time period. Body weight of rats were recorded consequently throughout the study. As shown in Figure 2 (B), in the exposure experiment stage (three injections), the rat body weight percentage between different groups are similar as of day 14 (*p-value*= 0.669, one-way ANOVA analysis). In the following 459 days, as shown in Figure S2, two rats of San Francisco and Zurich groups continued to gain the most weight, followed by the control group, the Johannesburg group, and the Beijing group. In a previous rat study, it was also found that breathing polluted air resulted in metabolic dysfunction and weight gain.[27] In another study, hemorrhage was observed in the lung alveoli of rats injected with $PM_{2.5}$ extracts from samples collected on haze days.[15] In this study, after the rats were sacrificed by anesthesia, hemorrhage was also observed in lung alveoli of rats from Zurich and Beijing groups (as shown in black arrows in Figure 2 (C)). While for other organs such as heart, liver, kidney and spleen, there were no obvious injuries. It's known that the lung is the organ directly suffering from PM by inhalation. The results of our study suggested that the PM extracts entering blood would further spread and directly or indirectly damage the lung. The reason might be

that alveoli as well as the apillary vessels have a single layer of epithelial cells, which makes them more vulnerable to be attacked. Decreased blood flow velocity might have also contributed to more interaction of PM extracts and alveoli tissue.

In addition, as shown in Figure 2 (D), two rats of Zurich group died naturally on day 193 and 232, respectively. There are three rats from the control, San Francisco and Beijing group have developed tumors (not known if they were benign and malignant). Nonetheless, studies have shown that there is a significant correlation between the occurrence of cancer and the exposure of PM.[28] In the follow-up experiments, any behavior observations after 14 days since the initial exposure did not have a statistical power, but only served as additional information as mentioned for the rats with PM exposure from different sources. Overall, the results from Figures 1, 2 indicate that source-specific PMs indeed caused different health effects.

PM Toxicity is Correlated with Expression of Specific MicroRNAs

In order to investigate the mechanism of rats' responses to PM injection exposure, microRNA levels in blood samples were analyzed. Fold percentage changes of concentrations of blood microRNAs of rats from different groups before and 1h after the third injection are shown in Figure 3 (A).

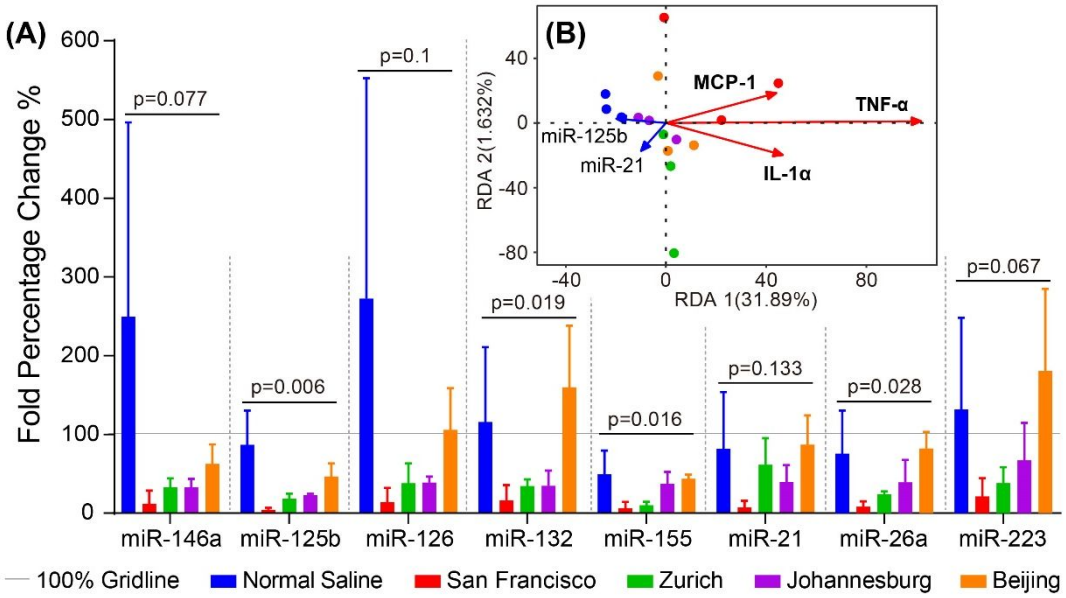

Figure 3. (A) Concentration fold percentage changes of blood microRNAs of rats from five groups before and 1h after the third injection of 1 mL 2mg/mL PM extract. Each group consisted six rats at the beginning of the third injection experiments. Data points represent averages and standard deviations of measurements from at least 3rats after eliminating rats with data outliers or rats with catheter blockage without samples. (B) Redundancy (RDA) analysis results of the biomarker expression profiles (Figure 1 (A)) and microRNA levels. The microRNA variables were selected by backward method according to the variance inflation factor. The miR-155 and miR-21 were shown to be negatively correlated with three biomarkers (TNF-α, MCP-1 and IL-1α).

Down-regulation of the microRNAs among all the exposed groups were observed 1h after the third injection. In general, the down-regulation of microRNA expression indicated the development of immune and inflammatory reactions during the third injection exposure. Similar findings were reported, e.g., several microRNAs such as miR-21, miR-26, miR-132 and miR-126, etc., could mute immune or inflammatory responses via inhibition of targeted mediators such as protein PDCD4, P300 and VEGF.[29-31] In particular, miR-146a and miR-125b are thought to inhibit the production of IL-6 and TNF-α.[32-34] In this study, the decline of these two microRNAs are in support

of the elevated serum MCP-1 and TNF-α levels. Previously, it was also reported that miR-125b was down-regulated while miR-155 was up-regulated in both mice and Raw 264.7 macrophages under Lipopolysaccharide(LPS)-induced inflammation.[34] In our study, as shown in Figure 3 (B), the RDA (Redundancy analysis) results revealed that the miR-125b and miR-21 levels were negatively correlated with the three biomarkers suggesting that miR-125b and miR-21 might have played important roles in the regulation of biomarker production. And miR-125b was observed to be more closely related to TNF-α as observed in the figure. Regarding the microRNA levels, San Francisco and Zurich groups generally had the largest downregulation rates, suggesting that PM samples from San Francisco and Zurich were more toxic than those from Beijing and Johannesburg. These results are generally consistent with the biomarker results above. The oxidative potential of the same PM samples from these cities but without the filtration were measured using dithiothreitol (DTT) assay in our previous study.[4] Normalized index of oxidant generation (NIOG) determined by the DTT assay of samples from San Francisco, Johannesburg, Zurich and Beijing are 0.0220, 0.0187, 0.0152 and 0.0114,respectively.[4] The PM samples with stronger oxidative potential (NIOG), e.g., those from Zurich, are more likely to generate more ROS, thus inducing stronger oxidative damage. The dose-response type relationship between NIOG and different microRNAs levels are shown in Figure 4. Generally, all microRNA expression levels were shown to decrease with increasing PM oxidative potential presented in NIOG (PM toxicity) (*p-values*<0.05), except for the miR-155 (*p-value*=0.1239). Compared to the RDA analysis results of the microRNA regulation and biomarker expression, the miR-155 expression did not well correlate with the results obtained using the DTT assay (*p-value*=0.1239). This partially could be due to the limitation of the DTT assay in measuring overall PM toxicity. Among all studied miRNAs, miR-125b and miR-21 had the best correlations (r^2=0.6332, and 0.5705, respectively) with the oxidative potentials of PMs measured using the DTT assay, and the correlations were also statistically significant (p-value=0.0007 and 0.0018, respectively). By searching available gene targets at http://www.microrna.org, we

found miR-125b has 1,254 gene targets for Rattus norvegicus, including Wee1 and H3f3b genes; while miR-155 has 5,445 gene targets for Homo sapiens, including ASF1A and ARID2 (Top 20 genes are attached in Supporting Information Table S3). And specific targets for each miRNA could not be simply derived using the data from this work. These results indicated that different oxidative potentials of PMs could lead to different down-regulations of specific miRNA expressions, thus causing different toxicological effects.

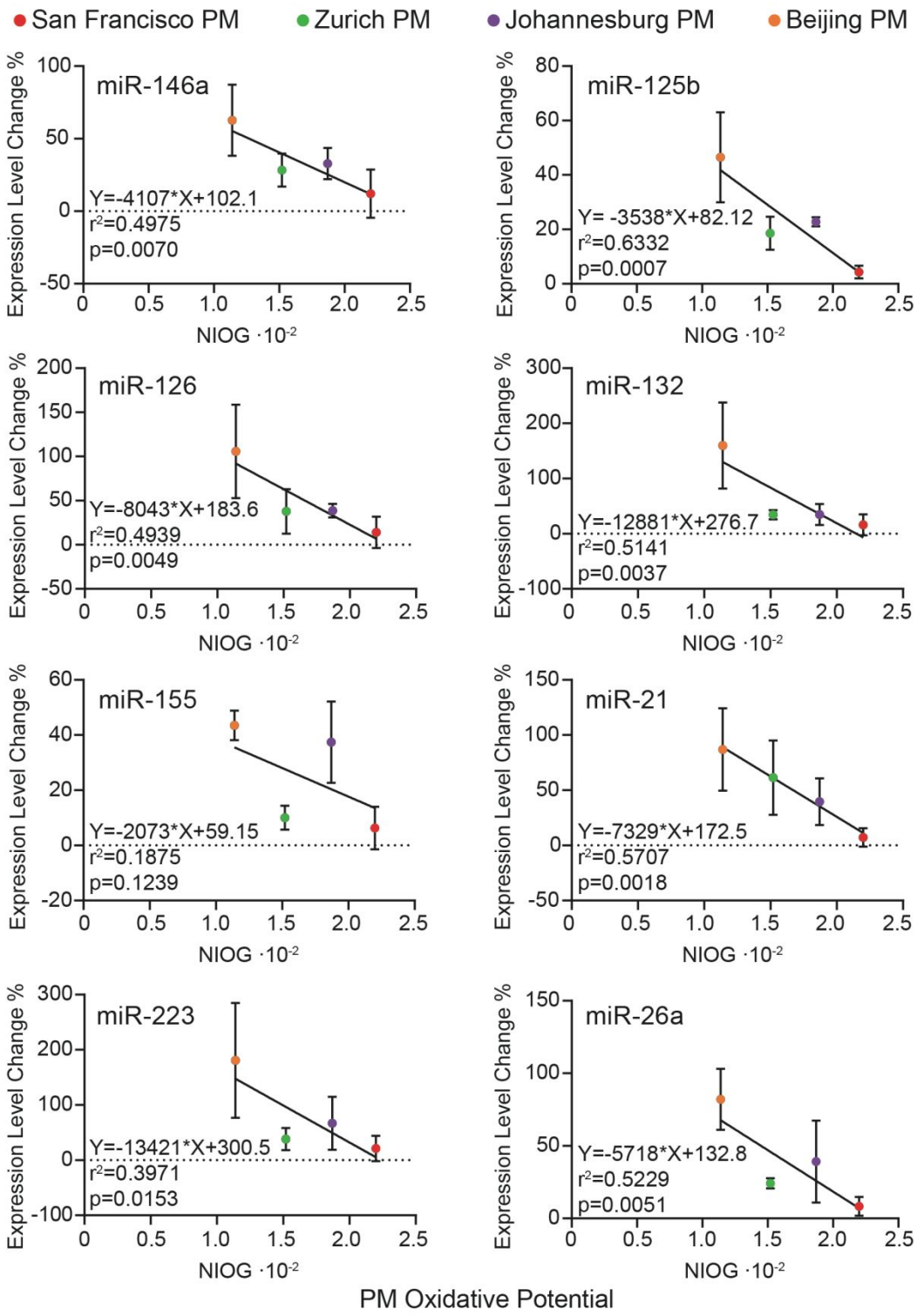

Figure 4. Linear regressions between oxidative potentials of PM samples and different microRNA expression level changes before and 1 hour after the third injection. The oxidative potentials of the same PM samples were determined by DTT assay in our previous study[4] and presented in normalized index of oxidant generation (NIOG). Data

points represent averages and standard deviations from at least three rats.

On the other hand, results from Figure 4 hint that some miRNAs can be used as a target for novel method against the pollutant exposure, such as PMs here. In the past, protein biomarkers have been already investigated in fighting against the diseases. For example, the IL-6 inhibitor, tocilizumab, has been used for the treatment of rheumatoid arthritis, juvenile idiopathic arthritis, and Castleman disease.[19] Therapeutic blockade of TNF-α is highly beneficial in case of chronic inflammatory conditions including rheumatoid arthritis.[35] An earlier study also suggested that IL-1 inhibitor can be used to treat severity of sepsis, colitis, arthritis and diabetes.[36] Since the production of biomarkers are regulated by microRNAs, it would be more efficient to modulate the expression of microRNAs using corresponding inhibitors or promotors. Such efforts have been already ongoing. For instance, enforced expression of miR-125b was shown to have resulted in remarkable reversal of LPS-induced increases in lung permeability as assessed by reductions in total protein, albumin and IgM in bronchoalveolar lavage fluid, indicating potential application for miR-125b-based therapy to treat clinical acute respiratory distress syndrome (ARDS).[37] Another relevant study used miR-155 for suppressing expression of programmed death ligand-1 (PD-L1), showing remarkable efficiency and improvement in treating solid cancers.[38-39] Here, we found that PM exposure led to down-regulations of both miR-125b and miR-21, which were apparently influenced or gated by the PM toxicity. Accordingly, in the future controlling the expression of these two microRNAs can offer a solution to protect people against ambient PMs.

Health mechanisms of different sourced PMs with different toxicity

PM is a complex and heterogeneous mixture whose composition varies greatly from one place to another.[4] In a recent study, PMs from different countries were shown to have varying toxicity as determined by DTT method.[4] Among PM contents, metals are generally studied due to its resistance to biodegradation, potential

462 damages to the nerve system and genotoxicity to DNA damage.[40-42] The redox
463 potential of transitive metals, such as Fe, Cu, Zn, Mn etc., were thought to play an
464 important role in the oxidative and inflammatory injuries as characterized with
465 increasing biomarkers including C-reactive protein (CRP), IL-6, TNF-α, IL-8, 8-OHdG,
466 etc.[43-45] In our previous study, the concentrations of metals in normal saline and PM
467 extracts of the same PM samples from different cities were analyzed.[8] Concentrations
468 of metals such as Cu, Zn, Fe, Cr, Ni, Mo and Mn from PM extracts were significantly
469 higher than those from normal saline. Among all the metals studied for the PMs, Fe
470 was the most dominant species, followed by Zn and Cu. Previous studies have shown
471 that metals in $PM_{2.5}$ can induce oxidative stress and cause inflammatory injury, as
472 characterized with evaluated biomarker levels such as IL-6 and TNF-α in airways.[46-50]
473 The PMs from Zurich (City A) and Johannesburg (City D) had relatively higher level of
474 Fe, Zn, Mo, Co; while PM from Beijing (City B) had relatively high level of Mg, Cu, V, Ni,
475 and San Francisco had the highest Pb level.[8] As discussed above, Figure 1 (A) shows Pb
476 and Fe could have been more involved in down-regulations of miRNAs such as miR-
477 125b and miR-21. Different metal levels might contribute to different toxicity of PMs.
478 However, the specific contribution of single metal and possible synergy mechanisms
479 of different metals are not clear and need to be further investigated.
480
481 In addition to metals, biological components (endotoxin, viruses, bacteria etc.)
482 and organics such as polycyclic aromatic hydrocarbons (PAHs) of PMs are also able to
483 induce adverse health effects to human.[51] In our previous study, the bacterial species
484 of the PM samples from these cities were measured using high-throughput gene
485 sequencing analysis.[8] Among the top 10 bacteria phyla, the proportion of Gram-
486 negative bacteria in samples from San Francisco, Zurich, Johannesburg and Beijing are
487 68.19%, 57.15%, 62.59% and 54.07%, respectively.[8] Endotoxins released by dead and
488 damaged Gram-negative bacteria can induce the release of acute response
489 biomarkers such as CRP and promoting the inflammatory reaction.[52-53] Our previous
490 study found in the PM samples used here but without the filtration that total bacteria,

491 culturable fungi and certain metals such as Cr, Mo, and Na strongly influenced the
492 oxidative potential of PM.[4] The filtration in this work would have removed those
493 insoluble and larger particles such as bacteria and fungi, which could influence the
494 miRNA expressions otherwise. In addition, the size distribution of PM components is
495 another important factor for their toxicity. For example, a recent work showed that
496 Zurich PM samples had a particle peak at the size of ~40 nm, while Beijing's PM
497 samples did not have such a peak.[24] Particles in small sizes such as $PM_{2.5}$ and
498 nanoparticles (NPs) are thought to be more harmful to humans due to its ability to
499 enter the deep respiratory system, and even permeate into blood circulation through
500 gas-blood barrier and possibly translocate to the brain.[54-58] Further studies about the
501 contribution of different components of various sizes including those soluble organics
502 to the PM toxicity are warranted.
503
504 Adverse health effects of PMs have been widely investigated during the past
505 decades.[59] It is well known that inflammation and oxidative stress play pivotal roles in
506 PM-induced health effect.[60-62] Here, we employed a PM-toxicity protocol based on rat
507 intravenous injection to investigate different toxicities of PMs from different cities,
508 and also its dose-response relationship with microRNA regulations. Our study revealed
509 that PM water-soluble extracts could induce both acute and chronic adverse health
510 effects when injected into blood circulation. Repeated exposure can make immune
511 response more sensitive as characterized by higher increase rate of inflammation
512 biomarkers. Recognized in playing important roles in the immune and inflammatory
513 process[9,12], down-regulation of microRNAs triggered a "brake release" effect, causing
514 increases in levels of biomarkers such as MCP-1 and TNF-α as observed in our study.
515 However, it should be noted that the signal pathways by which expressions of specific
516 miRNAs influence the biomarker levels such as MCP-1 and TNF-α needs to be further
517 explored. Using the same protocol, previously we have shown that different mass
518 levels of the same source PMs resulted in different levels of health effects.[15] Here, we
519 further demonstrated that the same mass PMs yet from different sources were shown

to have exhibited clearly different toxicities. These results in general agree with those results obtained using a DTT assay for these cities. Influenced by chemicals, biologicals and their size distributions, differences in PM toxicities among the studied cities suggest that current environmental air quality standards of PMs should be revised according to its local PM toxicity, and current PM mass level policy only tells one side of the whole story. Our data for the first time showed that there exists a dose-response type relationship between PM toxicity and microRNA regulation, and different sourced PMs selectively influence the expressions of specific miRNAs. The findings from our study imply that controlling the expressions of certain microRNAs such as miR-125b and miR-21 through novel inhibitors can possibly offer a solution to protect people from the adverse health effects of ambient PMs. Such a potential application from this work warrants future investigations. While a lot of epidemiological studies investigated the health effects of PM exposure, here we developed an animal based protocol, i.e., injecting the PM extract directly into the blood circulation, for studying the toxicity of PMs, and used it to analyze the differences in toxicities of PMs collected from four different cities with different air pollution conditions. However, the results from this work do not represent the true exposure of PM and can be only interpreted as the comparison of toxicity differences of PMs from different cities through the same exposure procedure. Nonetheless, the results to some extent can serve as a reference for discussing the true exposure in addition to providing a comparison. The results from this work would provide a valuable reference for considering the PM toxicity difference when dealing with PM associated air quality for many different cities.

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549 **Ethics Approval and Consent to Participate**

550 All animal experiments in this study were approved by the Laboratory Animal
551 Ethics Committee of Peking University (Granted Number: LA2017204), and were
552 performed in accordance with the Guideline for Animal Experiments of Peking
553 University.

554

555 **Competing Interests**

556 The authors declare that they have no competing interests.

557

558 **Supporting Information**

559 The microRNA extraction and qRT-PCR detection procedure, including the cycling
560 conditions and primers;

561 Scheme of the exposure experiment procedure;

562 Body weight percentage change of single rat after the exposure experiment;

563 Surveillance video of the rats before and after the injection of PM extracts from
564 different cities;

565 The sample size of biomarker analysis in each group on each time injections;

566 The top 20 potential mRNAs targeted by miR-125b and miR-155 and their genes'
567 information.

568

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