Oxidative Damage to Lung Tissue and Peripheral Blood in Endotracheal $PM_{2.5}$-treated Rats[1]

ZHI-QING LIN[*], ZHU-GE XI[*,#,2], DAN-FENG YANG[*], FU-HUAN CHAO[*], HUA-SHAN ZHANG[*], WEI ZHANG[*], HUANG-LIANG LIU[*], ZAI-MING YANG[*], AND RU-BAO SUN[*]

[*]*Institute of Health and Environmental Medicine, Tianjin 300050, China;* [#]*State Environmental Protection Key Laboratory of Urban Ambient Air Particulate Matter Pollution Prevention and Control, Tianjin 300071 China*

Objective To investigate the oxidative damage to lung tissue and peripherial blood in $PM_{2.5}$-treated rats. **Methods** $PM_{2.5}$ samples were collected using an auto-sampling instrument in summer and winter. Treated samples were endotracheally instilled into rats. Activity of reduced glutathione peroxidase (GSH-Px) and concentration of malondialdehyde (MDA) were used as oxidative damage biomarkers of lung tissue and peripheral blood detected with the biochemical method. DNA migration length (μm) and rate of tail were used as DNA damage biomarkers of lung tissue and peripheral blood detected with the biochemical method. **Results** The activity of GSH-Px and the concentration of MDA in lung tissue significantly decreased after exposure to $PM_{2.5}$ for 7-14 days. In peripheral blood, the concentration of MDA decreased, but the activity of GSH-Px increased 7 and 14 days after experiments. The two indicators had a dose-effect relation and similar changing tendency in lung tissue and peripheral blood. The DNA migration length (μm) and rate of tail in lung tissue and peripheral blood significantly increased 7 and 14 days after exposure to $PM_{2.5}$. The two indicators had a dose-effect relation and similar changing tendency in lung tissue and peripheral blood. **Conclusion** $PM_{2.5}$ has a definite oxidative effect on lung tissue and peripheral blood. The activity of GSH-Px and the concentration of MDA are valuable biomarkers of oxidative lung tissue damage induced by $PM_{2.5}$. The DNA migration length (μm) and rate of tail are simple and valuable biomarkers of $PM_{2.5}$-induced DNA damage in lung tissues and peripheral blood. The degree of DNA damage in peripheral blood can predict the degree of DNA damage in lung tissue.

Key words: $PM_{2.5}$; Endotracheal instillation; Oxidative damage; Oxidative DNA damage; Biomarker; DNA repair

INTRODUCTION

Attention has been paid to the relation between environmental pollution by atmospheric particle matters and human health in recent years. Fine particle matters ($PM_{2.5}$) are thought to be the most hazardous air pollutants not only because of their high concentration in atmosphere but also because of them carrying many kinds of metal and organic elements[1-2]. They can penetrate bronchiole, pulmonary alveoli, and blood circulation due their small size and produce serious biological toxicity. Some serious diseases such as irritable asthma, leukemia, lung cancer, and cardiovascular diseases, are related with the pollution of $PM_{2.5}$[3-9]. So far, the multi-pathogenic mechanisms of $PM_{2.5}$ are not well understood. In an attempt to study the oxidative damage induced by $PM_{2.5}$ in animals, we used the activity of GSH-Px and the concentration of MDA as biomarkers and the levels of these biomarkers in lung tissue and peripheral blood of rats exposed to $PM_{2.5}$ by endotracheal instillation were determined. The resulting oxidative DNA damage may be implicated in cancer risk and serve as a marker of oxidative stress relevant to other ailments caused by particulate air pollution. There are various preparations of ambient air $PM_{2.5}$ inducing oxidative DNA damage *in vitro* systems, whereas *in vivo* studies are scarce. Oxidative stress-induced DNA damage appears to an important mechanism of action of urban particulate air pollution. In an attempt to study the DNA damage induced by $PM_{2.5}$ in animals, we used the DNA migration length and the rate of tail as biomarkers, and measured their levels in lung tissue and

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[2]Correspondence should be addressed to Zhu-Ge XI, E-mail: zhugexi2003@sina.com

Biographical note of the first author: Zhi-Qing LIN, born in 1976, Ph. D. candidate, majoring in environmental toxicology. E-mail: wolinzq@sina.com

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peripheral blood of rats exposed to $PM_{2.5}$ by endotracheal instillation. In addition, the molecular mechanism of $PM_{2.5}$ on health effects was explored.

METERIALS AND METHODS

Reagents

Normal and low melting point agarose, RPMI-1640medium were purchased from Promega. Acridine orange and Triton X-100 were bought from Sigma. GSH-Px and MDA detecting kit were purchased from Nanjing Jiancheng Bio-Corporation.

Sampling Procedure

$PM_{2.5}$ samples were collected in winter and summer. The sampler with a medium rate of flow placed on a 30 m high platform was used to collect $PM_{2.5}$, 24 h a day for 30 days. The sampling rate was set at 77.7 L/min. The sampling membranes were glass fiber membranes.

$PM_{2.5}$ Suspension Preparation

Thirty glass fiber filtration membranes were suspended in distilled water and treated with ultrasound for 15 min, then centrifuged at 10 000 r/m at 4 ℃ for 20 min. The supernatant was treated repeatedly. The condensing supernatant and all particle matters in bottom were collected totally to freeze dry under vacuum conditions. The samples were maintained in a low temperature refrigerator for future use.

Entrotracheal Instillation Treatment

Fifty male Wistar rats were randomly divided into control group (CT), low-dose group in winter (HL), high-dose group in winter (HH), low-dose group in summer (NL), and high-dose group in summer (NH). After anesthesia with ether, the rats were exposed to $PM_{2.5}$ suspension by entrotracheal instillation at the dose of 1.5 mg/kg (body weight) and 7.5 mg/kg (body weight) for 7 and 14 days. The control group was treated with 0.3 mL normal saline (NS).

Detection of Oxidative Damage in Lung Tissue and Peripheral Blood

Activity of GSH-Px was detected with the dithio-dinitro-benzoic acid colorimetric method, while the MDA content was detected using the TBA method. Concrete operation steps were performed according to instructions for the kits from Nanjing Jiancheng Bio-Corporation.

Detection of DNA Damage in Lung Tissue and Peripheral Blood

Comet assay was performed under alkaline conditions as previously described[3-5]. Three slides were prepared for each experimental point and 30 cells per slide were randomly scored. ①*Slide preparation*: Briefly, 85 μL of 0.8% normal agarose (NA) was added onto a microscope slide (Sigma, USA) pre-coated with 1.5% NA. Cell suspensions (1×10^6 cells/mL) were mixed with 75 μL of 0.5% low melting-point agarose (LMPA) kept at 37 ℃ and added onto the microscope slide. A top layer of 75 μL of 0.5% LMPA was finally added. After the top layer of agarose was solidified, the slides were immersed for at least 1 h at 4 ℃ in the dark into a lysing solution consisting of 2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris (pH 10), to which 1% Triton X-100 and 10% DMSO were freshly added (pH adjusted to 10 with NaOH). ②*Electrophoresis and staining*: The slides were placed on a horizontal gel electrophoresis unit which was filled with freshly prepared alkaline buffer (1 mmol/L EDTA and 300 mmol/L NaOH, pH>13) to approximately 0.25 cm above the slides. Cells were exposed to the alkaline buffer for 20 min to allow DNA unwinding and expression of single-strand breaks and alkali-labile sites. Next, electrophoresis was conducted for 20 min by applying an electric current of 0.7 V/cm (25 V/300 mA). After electrophoresis, the slides were neutralized with 0.4 mol/L Tris buffer (pH 7.5) and then dipped in absolute ethanol for 5 min in order to preserve them. Subsequently, the slides were air-dried and stored at room temperature until they were scored for DNA migration; ③*Image analysis*: The slides were examined at 250 × magnification under a fluorescence microscope (Leica Microscopy and Scientific Instruments Group, Heerbrugg, Switzerland) equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm, connected via a gated CCD camera to a Comet image-analysis system (version 5.0 software, Meta Imaging Ltd., Olympus, Japan). Images of 30 selected cells from each investigated slide were randomly analyzed. For each experimental point, 300 cells were scored, i.e. 30 cells per slide, 2 slides per concentration and 5 animals per experimental point. ④*Tail parameters*: The olive tail moment (OTM) was used to evaluate DNA damage. The OTM, expressed in arbitrary units, was calculated by multiplying the percent of DNA (fluorescence) in the tail by its length in micrometer. The tail length was measured between the edge of comet head and the end of comet tail. The rate of tail was measured among 30 cells. A major advantage of using the OTM (instead of the conventional tail

moment) as an index of DNA damage is that both the amount of damaged DNA and the distance of migration of the genetic material in the tail are represented by a single number. ⑤*Statistical analysis*: Since the OTM frequencies and other tail parameters did not follow a Gaussian distribution, we verified that there was a significant dose-effect correlation using the non-parametric Kruskall-Wallis test. The statistical significance of differences in the median values between each group *versus* the control was determined with ANOVA and the non-parametric *Q*-test.

RESULTS

Oxidative Lung and Peripheral Blood Damage Induced by $PM_{2.5}$

After endotracheal instillation of MDA for 7 days, the content of MDA in lung homogenate had no difference between the experiment and control groups, but the content of MDA induced by the $PM_{2.5}$ in peripheral blood collected in summer and winter were higher than those in the control group. The contents of MDA induced by the summer $PM_{2.5}$ were higher than those induced by the winter $PM_{2.5}$. However, after endotracheal instillation for 14 days, the contents of MDA in lung homogenate and peripheral blood of the experiment groups were lower than those of the control group. There was no difference between the $PM_{2.5}$ collected in different periods at the same dosage (Figs. 1a and 1c). The activity of GSH-Px in experiment group was lower than that in control group. However, there was no difference between the same dosages of $PM_{2.5}$ in different periods. The activities of GSH-Px in peripheral blood of experiment groups were lower than those in the control group. The GSH-Px activities induced by the summer $PM_{2.5}$ were lower than those induced by the winter $PM_{2.5}$, but the activities of GSH-Px were lower both in summer group and in winter group than in the control group. The GSH-Px activity induced by the summer $PM_{2.5}$ was higher than that induced by the winter $PM_{2.5}$ (Figs. 1b and 1d).

Oxidative DNA Damage Induced by $PM_{2.5}$

After endotracheal instillation of DNA for 7 days, the DNA migration length of lung cells at high dosage in the summer group was greater than that in the control group. The rate of tail in the experiment group was higher than that in the control group. The rate of tail for the high dosage was higher than that for the low dosage in the same period. The rate of tail

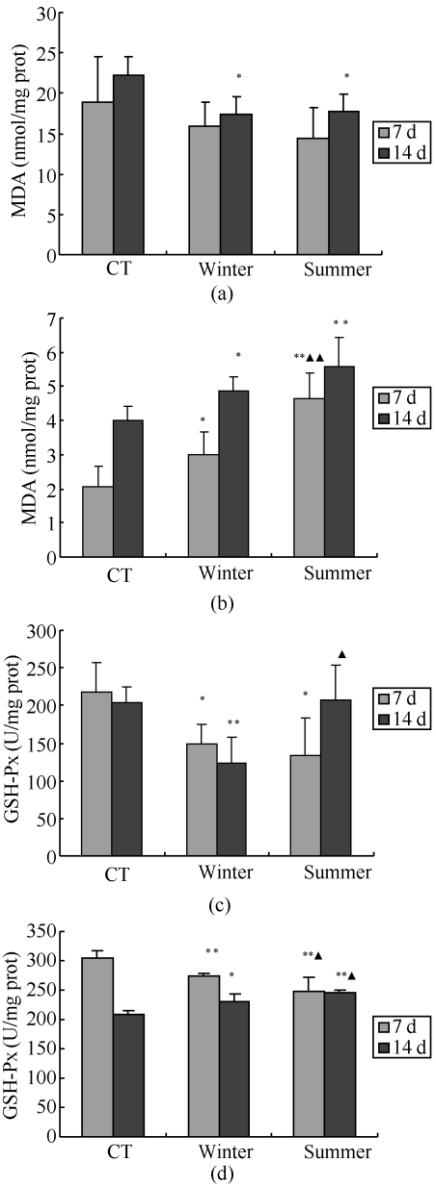

FIG. 1. Oxidative lung and peripheral blood damage induced by $PM_{2.5}$ after 7 and 14 days. The data are expressed as $\bar{x} \pm s$. $^{*}P<0.05$, $^{**}P<0.01$ *vs* CT; $^{▲}P<0.05$, $^{▲▲}P<0.01$ *vs* the same dose in winter.

in the summer was higher than that in the winter. However, after intratracheal instillation of DNA for 14 days, the DNA migration length of lung cells at a high dosage in the summer and winter groups was greater than that in the control group. The rate of tail for the experiment group was higher than that for the control group. The rate of tail for high dosage was higher than that for low dosage in the same period, and that the rate of tail for summer period was higher than that for winter period at the same dosage (Figs. 2a and 2c).

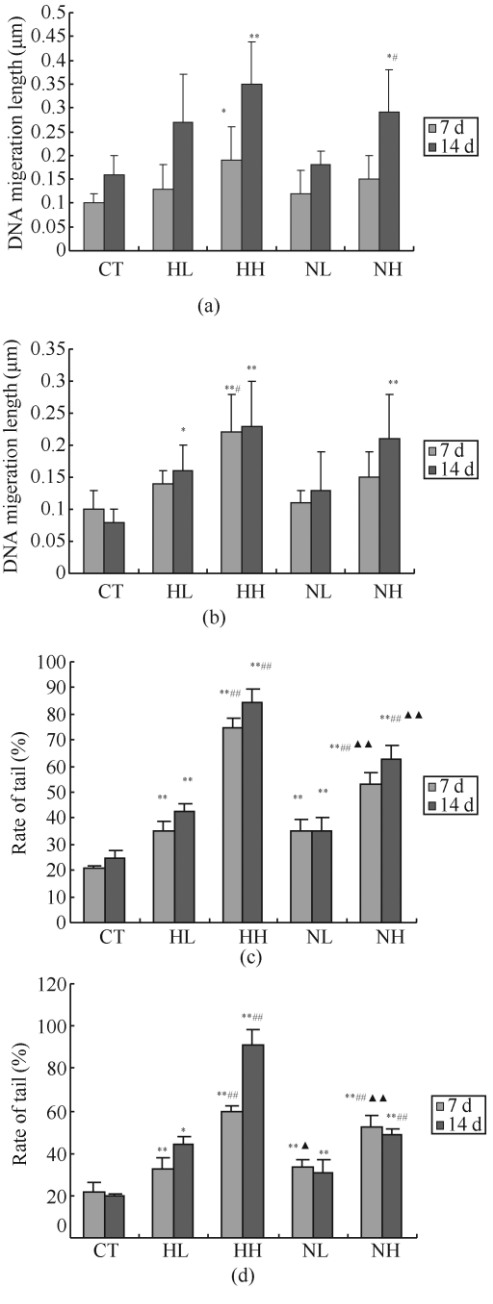

FIG. 2. DNA damage in lung cells and peripheral blood 7 and 14 days after endotracheal instillation with $PM_{2.5}$. CT: control test; HL: low dose in summer; HH: high dose in summer; NL: low dose in winter; NH: high dose in winter. The data are expressed as $\bar{x} \pm s$. $^{*}P<0.05$, $^{**}P<0.01$ *vs* CT; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* low-dosage in the same period; $^{\blacktriangle}P<0.05$, $^{\blacktriangle\blacktriangle}P<0.01$ *vs* the same dose in summer.

After intratracheal instillation of DNA for 7 days, the DNA migration length of lymph cells in the rest experiments, except for low dosage in winter, was greater than that in the control group. The rate of tail for the experiment group was higher than that for the control group. The rate of tail for high dosage was higher than that for low dosage in the same period. However, after intratracheal instillation of DNA for 14 days, the DNA migration length of lymph cells at high dosage in summer group was greater than that in control group. The rate of tail for the experiment group was higher than that for the control group. The rate of tail for high dosage was higher than that for low dosage in the same period and the rate of tail in summer period was higher than that in winter period at the same dosage (Figs. 2b and 2d).

DISCUSSION

Although it is generally believed that particulate matters, especially $PM_{2.5}$, threaten health[13-14], their toxic components and biological mechanism(s) involved remain unclear. The current study investigated the biological effects of $PM_{2.5}$ in winter and summer and the oxidative damage was compared betweent the samples collected monthly.

Our oxidative damage test showed that changes occurred in the activity of GSH-Px and the content of MDA in lung cells and peripheral blood cells, especially in the high dosage group in summer. However, the results were somewhat perplexed, mainly due to more dusty days in summer, when mainly SiO_2 resided in $PM_{2.5}$, which is difficult to degrade in organisms. SiO_2 provokes neutrophilic granulocytes and macrophages to release free oxygen radicals (ROS) from mitochondria where the ROS are generated by functioning as a substrate for the electron transport chain. There is additional evidence that supports the hypothesis that PM increases ROS within mitochondria at both complexes I and III. Antimycin, a mitochondrial respiratory inhibitor, has been used to demonstrate the role of cytochrome C reductase (complex III) in generating excessive ROS during PM oxidation. Since antimycin inhibits electron transfer from cytochrome b_H to ubiquinone and its semiubiquinone anion during the "Q" cycle of complex III, it has two effects within cells. It inhibits ATP synthesis by preventing electron flow through the Q cycle of complex III and increases the concentration of ubiquinone semiubiquinone anion in the Q cycle, which would facilitate the transfer of unpaired electrons present in the semiquinone to molecular oxygen to form superoxide. Thus, in the presence of antimycin, mitochondrial ROS production would be elevated due to the continuous oxidation of endogenous substrates and further stimulated by the added substrate, $PM_{2.5}$:

$$SQ\bullet + O_2 \rightarrow Q + O_2^{-}\bullet$$

In this study, chronic exposure to particle matters

decreased the viability of lung cells, demonstrating that $PM_{2.5}$ increases ROS production in lung cells and chronic $PM_{2.5}$ exposure might create an oxidative stress within cells, particularly in mitochondria. Furthermore, chronic exposure to $PM_{2.5}$ decreases the production of mitochondrial antioxidants, which may also lead to increased susceptibility to oxidative mitochondrial injury. Proteins are readily oxidized by ROS and may represent a critical target of oxidative damage within lung cells because oxidative inactivation of proteins could have very rapid and detrimental effects on cell viability due to their catalytic function. Administration of a single, large dose of $PM_{2.5}$ could increase carbonyl content in lung cytosolic and mitochondrial protein. Chronic exposure to $PM_{2.5}$ exposure can greatly increase the content of carbonyl groups in mitochondrial proteins. The reasons for this increased susceptibility of mitochondrial proteins to $PM_{2.5}$-related oxidative damage include the possibility that the oxidative insult of PM-elicited ROS might be greatest at the level of mitochondrion where the ROS are generated. The results of metal analysis on samples collected in winter and summer indicate that there were generous metals such as Fe and Cu, *etc*. For example, if there is Fe ion, particulate matters can form typical Fenton reaction system, causing circulating reaction:

$$ADP-Fe(\Pi)+2H_2O_2 \rightarrow ADP-Fe(\Pi\Pi) + O_2^{-}\bullet + 2\,H_2O$$

$$Fe^{2+}+O_2 \leftrightarrow Fe^{3+}-O_2^{-}\bullet \leftrightarrow Fe^{3+}+O_2^{-}\bullet$$

$$O_2^{-}\bullet+H^{+}\rightarrow HO_2\bullet$$

$$HO_2\bullet + HO_2\bullet \rightarrow H_2O_2+O_2$$

$$O_2^{-}\bullet + HO_2\bullet \rightarrow O_2+HO_2^{-}$$

$$HO_2^{-}+H^{+}\rightarrow H_2O_2$$

In addition, PAH and phenol absorbed on $PM_{2.5}$ can produce FOR during their metabolism to cause cell oxidative damage. At the same time, some physical action of itself can concentrate neutrophilic granulocytes to lung and trachea to produce mass FOR which can cause physiological and pathological changes, even nucleic acid damage.

Excessive generation of FOR overwhelming the antioxidant defense system can oxidize cellular biomolecules. Free radicals generate a large number of oxidative modifications in DNA, including SB and base oxidations. Among the oxidative DNA damage products, 8-dihydro-2-deoxyguanosine (8-oxodG) is probably the extensivelyt studied oxidation product due to its relative ease of measurement and premutagenic potential. In DNA, 8-oxodG may be formed by oxidation of guanine or incorporation during replication or repair as oxidized nucleotides (8-oxodGTP). Oxidative DNA damage is repaired by a number of different enzymes, indicating that the level of oxidative DNA damage in intact cells or in animal experimental models exposed to particles must be interpreted as a steady level. DNA base lesions are mainly repaired by base excision repair enzymes. The main guardians against damage due to cellular metabolism are ROS, methylation, deamination, and hydroxylation. OGG1, a base excision repair enzyme involved in removal of 8-oxodG, is expressed in all tissues, although the resulting enzyme activity is lowier in some cells, such as testicular cells. OGG1 is the major repair enzyme for 8-oxodG base. The *MTH1* gene encodes an 8-oxodGTPase that hydrolyzes 8-oxodGTP. Other repair pathways for 8-oxodG include nucleotide excision repair processes, mismatch repair and NEIL proteins.

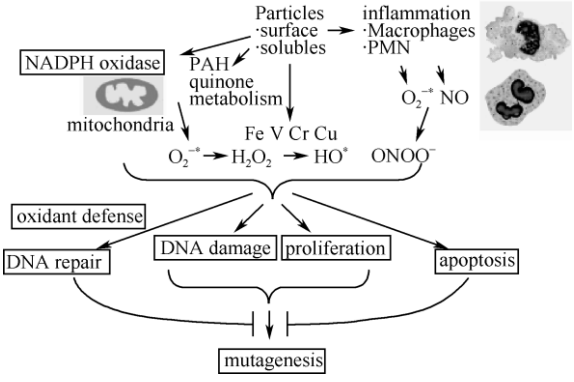

FIG. 3. Possible mechanisms of $PM_{2.5}$ by which oxidative stress and DNA damage are induced.

DNA chain breakage is a general index of DNA damage. Some exogenous materials can directly or indirectly cause breakage of DNA chain and the repair system of organisms can ablate the damage parts posed by DNA-DNA, DNA-protein and DNA bulky adducts, which cause DNA chain breakage, suggesting that DNA breakage can be used as an end-point marker of early genotoxicity of exogenous materials[15-17]. In our study, DNA damage was detected with SCGE to observe DNA breakage in lung cells and peripheral blood cells. The results showed that the degree of DNA damage in the two kinds of cells increased in a dose-dependent manner. Our previous study *in vitro* confirmed that some significant metal ions play an important role in DNA damage caused by particulate matters. The results of metal analysis of samples collected in summer and winter indicate that there were metals such as Fe, and Cu, *etc*. In addition, PAH and phenol absorbed on $PM_{2.5}$ can produce FOR during their metabolism to cause cell oxidative damage. At the same time, some physical actions can concentrate neutrophilic granulocytes to lung and trachea to produce massive FOR which can cause physiological and pathological

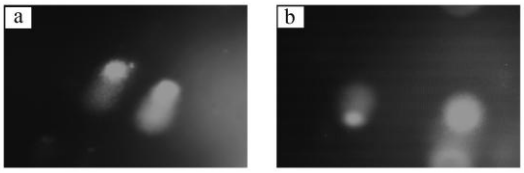

FIG. 4. DNA damage caused by intratracheal instillation of $PM_{2.5}$. a, SCGE image of lung cells; b, SCGE image of peripheral blood cells.

changes, even nucleic acid damage.

In conclusion, our study provides data needed to guide additional toxicity studies and to support the design of environmental hygiene procedures. $PM_{2.5}$ causes serious health hazards in animals and exposures to PM_{25} should be avoided.

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