Summer-winter differences of $PM_{2.5}$ cytotoxicity to human epithelial cells (A549) and the roles of transition metals

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Abstract

Atmospheric fine particulate matters ($PM_{2.5}$) induce adverse human health effects through inhalation, and the harmful effects of $PM_{2.5}$ are determined not only by its air concentrations, but also by the particle components varied temporally. To investigate seasonal differences of the aerosol toxicity effects including cell viability and membrane damage, cell oxidative stress and responses of inflammatory cytokines, the human lung epithelial cells (A549) were exposed to $PM_{2.5}$ samples collected in both summer and winter by the *in vitro* toxicity bioassays. Toxicological results showed that, the $PM_{2.5}$ led to the cell viability decrease, cell membrane injury, oxidative stress level increase and inflammatory responses in a dose-dependent manner. Temporally, the cytotoxicity of winter $PM_{2.5}$ was higher than summer of this studied industrial area

of Nanjing, China. According to the different contents of heavy metals accumulated in $PM_{2.5}$, the transition metals such as Cu might be an important contributor to the aerosol cell toxicity.

Key words: Air pollution; Fine particulate matters; Cell toxicity; Human health; Temporal variations; Heavy metals

1. Introduction

Atmospheric particulates contribute substantially to urban air pollution and have critical impacts on both environmental ecosystems and human health (Totlandsdal et al., 2014; Mukherjee et al., 2016; Jin et al., 2017; Fulgar et al., 2018). Epidemiological studies have indicated that elevated concentrations of inhalable particles were associated with increased respiratory problems, mortality, and morbidity (Feng et al., 2016; Costa et al., 2017). *In vitro* studies have shown that fine particulate matters ($PM_{2.5}$) poses greater toxicity than coarse particles due to their potential to cell membrane injury, oxidative damages, impairing the antioxidant system which results in the inflammation and immunity disorder (Kouassi et al., 2010; Davel et al., 2012; Corsini et al., 2013; Deng et al., 2013; Longhin et al., 2013; Vuong et al., 2017; Bai et al., 2018).

Aerosols are usually generated from a wide range of sources and may be composed of numerous hazardous components such as toxic heavy metals, and thereby induce varied health risks (Kan et al., 2008; Li et al., 2015; Manzanoleón et al., 2016). For instance, the overall carcinogenic and non-carcinogenic risk of $PM_{2.5}$ in winter of Tianjin in north China was higher than those in summer, because the heavy metals enriched in particles such as Fe, Cu, Cr, Co, Zn, and Mn varied among seasons (Luo et al., 2014; Zhang et al., 2015). Therefore, the effective control and management of ambient air pollution requires detailed knowledge of the distribution and health effects of $PM_{2.5}$ and the corresponding component roles. However, studies focusing on the temporal differences in $PM_{2.5}$ toxicity related to component differences are still limited. In this study, $PM_{2.5}$ samples of two distinct seasons were collected near an industrial area of Nanjing, China and conducted in vitro toxicity tests by human epithelial cells (A549). The primary objectives were: (1) to compare the seasonal

toxicity differences of ambient $PM_{2.5}$; and (2) to explore the roles of airborne metal components in $PM_{2.5}$ cytotoxicity.

2. Materials and Methods

2.1. $PM_{2.5}$ sampling

The $PM_{2.5}$ samples were collected at a university campus site in Pukou district of Nanjing, China, where chemical and metallurgical industries were concentrated nearby (Luo et al., 2017). Typical samples of July and November 2015 were selected to represent summer and winter $PM_{2.5}$, respectively. A high-volume sampler (1000 L/min) was used for daily continuous 24 h sampling each time and $PM_{2.5}$ was collected on quartz microfiber filters (QMA, 203 mm × 254 mm, Whatman, UK) that were prebaked at 400 °C for 4 h to remove organic substances before sampling. The filters were equilibrated under a constant temperature and humidity condition before and after sampling and weighted by a high-precision electronic balance. After weighing, the $PM_{2.5}$ filters were cut into subsamples by ceramic scissors and stored in refrigerator for following chemical analyses and toxicity tests.

2.2. Preparation of $PM_{2.5}$ suspension for cell exposure

For toxicity tests, each $PM_{2.5}$ sample filter was cut into small pieces, moistened with the 75% alcohol, and sonicated in 100 mL ultrapure water for 1.5 h (Zou et al., 2016). After removing the pieces of QMA materials by filtering, the extracted $PM_{2.5}$ suspension was collected into sterile centrifuge tubes which were weighed before, and then the mass of obtained particles were determined after freeze-drying. The particles were diluted in the cell culture medium to the series of concentrations (0, 1, 10, 100, 200 and 400 mg/L) for cell toxicity tests.

2.3. Cell culture

The A549 cells for cytotoxicity assays were cultured in RMPI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) and 1% antibiotics penicillin-streptomycin (100 U/mL) at 37°C with 5% CO_2. When 80%-90% cells were fused, 0.25% trypsinization was done. Cells used for the cytotoxicity assays were collected in the exponential phase of growth.

2.4. In vitro toxicity assays

The cell viability was evaluated by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide] (Mosmann, 1983). A549 cells in the exponential phase of growth were adjusted to a density of 1.0×10^5 /mL after trypsinization. The cell suspension was seeded in 96-well plate (Costar, USA) with 100 μL/well. After 24 h incubation, the $PM_{2.5}$ suspensions of different seasons were added to the 96-well plate at different concentrations (1, 10, 100, 200, 400 mg/L) and the blank control and parallel wells (n=3) were set simultaneously. After 48 h incubation, wells were washed with PBS for 3 times, and 100 μL fresh medium, 20 μL MTT (5 g/L) were added into each well. After 4 h, the supernatant was discharged, 100 μL Formazan solution was added to each well. The optical density (OD) value was measured at 492 nm by a microplate reader (Thermo MULTISKAN FC, USA). In addition, the viability of cells exposed to $PM_{2.5}$ was calculated as a percentage relative to that of control group, whose viability was seemed to be 100%.

Lactate dehydrogenase (LDH) was a ubiquitous enzyme present in the cytoplasm linked to cell viability. When the cell membrane was damaged, it can be leaked out of the cells, so the LDH activity in cell culture medium can reflect the extent of cell

membrane damage (Renz et al., 2003). For LDH assay (Kumarathasan et al., 2015), the cells were exposed to $PM_{2.5}$ in the same way, and after 24 h incubation, the supernatants were transferred to centrifuge tubes for 3 min centrifugation at 1000 r/min. The LDH level in the culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) assay. After 24 h exposure to $PM_{2.5}$, the levels of reactive oxygen species (ROS), glutathione (GSH) and superoxide dismutase (SOD) and pro-inflammatory cytokines (TNF-α and IL-6) in the supernatants were all measured by ELISA assay. The OD value of each well was measured at 450 nm by the microplate reader.

2.5. Analysis of metal contents accumulated in $PM_{2.5}$ samples and calculation of metal concentrations in air

For chemical composition analyses, metal accumulations (mg/kg) in $PM_{2.5}$ samples were analyzed (Xie et al., 2018). Filter subsamples with known $PM_{2.5}$ masses were digested by being immersed in concentrated HNO3-HClO4-HF acids with a progressive heating program and finally dissolved in 5% (v/v) high-purity HNO_3. Procedural blanks, sample replicates, and standard reference materials (NIST SRM 1648a, urban PM) were randomly inserted for quality control. The metal contents in $PM_{2.5}$ samples were determined by Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Optima 8000, PerkinElmer) and ICP-Mass Spectrometer (ICP-MS, NexION300X, PerkinElmer) for low level concentrations when needed. Then for the concentration of each airborne metal in air (ng/m^3), it can be calculated based on the particulate metal accumulation and the volume of sampled air.

2.6. Statistical analysis

Data analyses were conducted by Excel 2016, origin 2016 and SPSS software. The dose data were expressed in terms of "means ± standard deviation". T test was used to analyze the differences among different indicators, that $P<0.05$ implies statistically significant and $P<0.01$ is extremely significant.

3. Results and Discussion

3.1 Cell viability induced by various doses of $PM_{2.5}$ from different seasons

The cell viability measured by MTT assay of A549 cells exposed to summer and winter $PM_{2.5}$ at different concentrations (1, 10, 100, 200, 400 mg/L) were shown in Fig. 1. Compared with the control group, the viability of $PM_{2.5}$-treated A549 cells decreased significantly in a dose-dependent manner both for winter and summer samples. Winter $PM_{2.5}$ obviously inhibited the cell viability at low concentrations (1, 10 mg/L) compared with summer $PM_{2.5}$, and the general cell viability of summer group was slightly higher than those of winter group.

3.2. Cytotoxic effects of various $PM_{2.5}$ doses from different seasons

The levels of LDH in the supernatant of cell culture medium were showed in Fig. 2. It indicated that the LDH levels of $PM_{2.5}$-treated groups were higher than those of control group ($p<0.05$). The variation trends of LDH levels for summer group at low concentrations (1, 10 mg/L) and for the overall winter group were relatively mild, but the LDH level in cells exposed to winter $PM_{2.5}$was significantly higher than those of summer samples($p<0.05$).

3.3. Oxidative stress and damages induced by different $PM_{2.5}$

The ROS generation level and levels of antioxidant enzymes (GSH, SOD) in

supernatants of cell culture medium induced by summer and winter $PM_{2.5}$ were provided in Table 1. With the increase of $PM_{2.5}$ concentrations, the ROS generation increased for winter and summer samples, and the levels of GSH and SOD were negatively correlated with ROS generation. Compared with summer $PM_{2.5}$, the ROS generation level induced by winter $PM_{2.5}$ was higher, and the GSH level decreased more significantly. The differences of SOD level between winter and summer groups were significant compared with control group. The reduction of SOD level by winter $PM_{2.5}$ was higher than those of summer at low concentrations (1, 10 mg/L) which was opposite at high concentrations (100, 200 mg/L).

3.4. Inflammation induced by different $PM_{2.5}$

Responses of TNFα and IL-6 in supernatants of cell culture medium were showed by Figure 3. The IL-6 responses increased with the $PM_{2.5}$ concentrations in a dose-dependent manner. The IL-6 responses to winter $PM_{2.5}$ were higher than those of summer group, and the difference was significant ($p<0.05$) at the higher concentration (200 mg/L). At high $PM_{2.5}$ concentrations (100, 200, 400 mg/L), the TNF-α responses of winter group were significantly higher than those of summer group ($p<0.01$).

3.5. Distributions of heavy metals in $PM_{2.5}$ from different seasons

The average concentration of $PM_{2.5}$ in summer and winter air was 52.5 and 76.4 $\mu g/m^3$, respectively, and the levels for air concentration (ng/m^3) and particulate accumulation (mg/kg) of heavy metals were showed in Table 2. Although the particulate accumulations of most measured metals were higher in summer $PM_{2.5}$ samples than those in winter, the accumulations of the typical transition metals such as Cu, Mn and Co were higher in winter $PM_{2.5}$, the fold difference of which compared

to the summer metal was 2.23, 1.09 and 3.98, respectively. They might be related to the $PM_{2.5}$ cytotoxicity differences. For example, there were evidences that the pulmonary toxicity effects of $PM_{2.5}$ to mice was associated with Cu concentrations (Sun et al., 2017), and the transition metal Co was related with the decrease of lung density (Sullivan, 2012).

3.6. Health implications of $PM_{2.5}$ differences and roles of transition metals

Results above confirmed that the $PM_{2.5}$ samples collected near the industrial district located in Nanjing, China during summer and winter induced a series of adverse health effects in a dose-dependent manner. The MTT assay showed that the toxic effects were stronger with the increasing of $PM_{2.5}$ concentration in cell culture medium, and the cell viability induced by winter $PM_{2.5}$ was observed lower than summer samples. Since the air concentrations of $PM_{2.5}$ in winter was significantly higher than summer, together with the stronger particulate cytotoxicity in winter, finally the human health risks of air $PM_{2.5}$ pollution in winter would be doubly higher than summer in this area.

Moreover, related indexes of cell oxidative damages showed that both the summer and winter $PM_{2.5}$ could induce the ROS generations and decrease the levels of metabolism and antioxidant enzymes such as SOD and GSH. Compared with winter $PM_{2.5}$, the ROS generation in the summer group was lower, and the SOD reduction in summer group was higher than those in winter. SOD could catalyze disproportionation of anionic radicals which played an important role in eliminating free radical damage (Gheddouchi et al., 2015), thereby explaining why the lower ROS generation level in summer than winter. The GSH level induced by $PM_{2.5}$ in winter was lower than those in summer. The GSH is an important metabolic regulator within

the cell that could reduce the damage of free radicals by combing with peroxides and free radicals in the body, therefore the GSH level decrease is the signal of early apoptosis (Zhang et al., 2016). Meanwhile, the generation of free radicals could induce expressions of inflammatory cytokines (IL-6 and TNF-α) resulting in cell viability decline or even apoptosis. Compared with summer, the oxidative damages and inflammation induced by winter $PM_{2.5}$ were severer, that may be related to higher accumulations of some transition metals (Cu, Mn, Co) in winter $PM_{2.5}$ samples. It could be supported by evidences that Cu acted as an important part in PM-related inflammation which can stimulate inflammatory cytokines expression (Aung et al., 2011), and Cu has also been found to be a vital factor in medicating the generation of ROS which could lead to oxidative stress and damages finally (Vidrio et al., 2008). Therefore, the airborne component of transition metals accumulated in particles would contribute significantly to the $PM_{2.5}$ cytotoxicity.

On a separate note, although the accumulations and air concentration levels of transition metals were different in winter and summer may explain partially the seasonal differences of cytotoxic effects induced by $PM_{2.5}$ in Nanjing of this study, the $PM_{2.5}$ organic extracts have also been reported to exert its toxicity resulting inflammation (Huang et al., 2017; Chi et al., 2018), and the toxicological properties of both inorganic and organic components are affected by emissions and atmospheric processes (Rönkkö et al., 2018). The detailed independent and combined effects of various air particle components in cytotoxicity, and how do they affect cell signaling pathways that caused different cell outcomes were not clear, that need further study.

4. Conclusions

In conclusion, the $PM_{2.5}$ from summer and winter both induced varied degrees of

toxic effects on A549 cells in a dose-dependent manner, but their cytotoxicities were different. Our study found that the differences in the accumulation of some transition metals in air particle may be one of the key parameters for the seasonal differences of $PM_{2.5}$ cytotoxicity. We suggested that, Cu, Mn and Co would play important roles in $PM_{2.5}$ cytotoxicity. Of course, the compositions of $PM_{2.5}$ were quite complex, and there were complicated interactions among various components. Therefore, evaluating human health risks of $PM_{2.5}$ should consider both the temporal sources and compositions, and the toxicological effects of major transition metals and organic components

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