EFFECTS OF PARTICULATE MATTER WITH DIFFERENT SIZES ON LUNG EPIDERMAL CELL IN FOG-HAZE WEATHER IN BEIJING

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Introduction

With the implementation of Air Pollution Prevention and Control Action Plan of China, the mean annual concentration of ambient air-borne particulate matter (PM) in 74 key cities, Beijing-Tianjin-Hebei and other regions decreased year by year from 2013 to 2017. Except for the Pearl River Delta region where the mean annual concentration of $PM_{2.5}$ is lower than that stipulated by the national Ambient Air Quality Standard (GB 3095-2012), other regions fail to meet the standards [1]. The composition of particulate matter is complex and contains a variety of persistent organic pollutants, heavy metals and so on. Many studies showed that levels of PM are positively correlated with human mortality, and millions of premature deaths each year can be attributed to inhaling large amounts of PM [2,3]. The toxicological effects and potential health risks caused by particulate matter deserve much attention [4].

According to the size of the aerodynamic diameter of PM, it can be classified into size fractions and PM_{10} (<10 μm) is a typical example. PM_{10} can be further categorized into 3 groups, including ultrafine PM ($PM_{0.1}$, <0.1 μm), fine PM ($PM_{2.5}$, < 2.5 μm) and coarse PM (2.5-10 μm) [5]. Ultrafine and fine PM is derived mainly from secondary aerosol formation and the emissions of oil refineries, power plants, domestic combustion, wildfires, and other sources of combustion, whereas coarse PM mainly come from sand dust, resuspended dust, volcanic ash, pollen and so on. Therefore, PM with different size may have different compositions. In general, ultrafine PM often consists of sulfate, elemental carbon and low volatile organic compounds, fine PM is made up of organic carbon, sulfate, nitrate, elemental carbon and water soluble organic acid, whereas fugitive dust, flying ash, crustal elemental oxides, calcium carbonate and biological fragments are the main components of coarse PM.

Currently, there are three main ways to study the effects of PM on human health, including epidemiological investigation, live animal experiment and in vitro cell experiment [6-8]. Epidemiological investigation can provide the statistical correlation between PM and human diseases by selecting representative samples and long-term data, and obtain relatively reliable conclusions on the macro level. Epidemiological studies generally cost so much and have long experimental cycles, and the results cannot directly answer the toxicity mechanism of PM, which needs to be further confirmed by animal and cell experiments. Live animal experiment is beneficial for observing the effects of PM on the individual or tissue, but this experiment has a long experiment cycle and a small sample size. In addition, it is difficult to describe some toxicity mechanisms at the molecular and cellular level because of the complexity of living system. In vitro cell experiment has the advantages of strong repeatability, strong operability and low cost. Besides, the toxicity mechanism of PM can be obtained at the cellular level. But the disadvantage is the lack of living physiological environment. Therefore, cell tests are generally used as a basic means for testing toxic effects of PM [9]. By comprehensive comparison, in vitro cell test is the most effective method to study the toxicity of PM at present.

The purpose of this study were to collect various size fractions of ambient atmospheric PM From Beijing, and to investigate cell activity, inflammatory effect, oxidative stress of the PM, focusing on the differences among the size fractions by using A549 cell lines.

Materials and methods

Cell models and Reagents. A549 human lung cancer cells were a gift from China-Japan Friendship Hospital, China and were cultured in our laboratory. RPMI Medium 1640 (RPMI) was used as culture medium for A549. The media contained 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. RPMI, FBS, Methyl Thiazolyl Tetrazolium (MTT) were purchased from Sigma, USA. Trypsin-EDTA (0.25%), Trypan Blue Stain (0.4%) were from Gibco, USA. Penicillin, streptomycin, Dulbecco's phosphate buffered saline (D-PBS) were purchased from Solarbio, China.

PM Samples. PM of nine aerodynamic diameter ranges (9.0-10, 5.8-9.0, 4.7-5.8, 3.3-4.7, 2.1-3.3, 1.1-2.1, 0.70-1.1, 0.40-0.70 and <0.40 μm) were collected from the roof of Sino-Japan Friendship Center for Environmental Protection using a nine-stage cascade impactor (TischTE-20-800, Andersen, USA) at a flow rate of 28.3 L/min for 15 days in fog-haze weather of Beijing, China. Aluminum foils (heated at 450 ℃ for 4 h) were used for collecting nine grade PMs. The nine fractions were pooled into four with nominal diameter ranges of 5.8-10 (F1), 3.3-5.8 (F2), 1.1-3.3 (F3), <0.4-1.1 μm (F4), respectively. The particles were removed from the foil by adopted mechanical method which can reduce the loss of chemical substances attached to PM.

PM Exposure and Cell Cultivation. The four PM fractions, a control were cultivated. The PM samples were diluted to proper concentrations using RPMI 1640. To each well of 96-well and 6-well tissue culture plates, approximately 30 000 and 900 000 cells were inserted and pre-cultured, respectively. The PM suspensions

(including the control and blank) were added to every wells (200 μL each). The outer peripheral wells of the plates were not used for cell test and filled with sterile PBS to reduce the evaporation of the exposure cells. Twelve wells were used as duplicates for each treatment to reduce variation among individual wells. The plates were incubated at 37 °C in a CO_2 incubator (5% CO_2, HF90, China) for 24 h [10].

MTT assay. After the PM exposure, accurate 20 μL MTT (5 mg/mL in sterilized PBS solution) was added to each well. The plates were incubated again at 37 ℃ and 5% CO_2 for 4 h, and centrifuged for 5 min (5000 r). After the supernatant was removed from the well, 200 μL DMSO was added to each well, formazan crystal desolved in DMSO was measured at 570 nm using multimode reader (SpectraMax M5, USA) [11].

Inflammatory factor. Human interleukine-8 (IL-8) in the centrifuged (5000 r for 5 min) suspension liquids were collected, and detected using double antibody sandwich method using the multimode reader at 450 nm according to the manufacturer's guidelines (Shanghai hengyuan biological technology, China) [12].

Superoxide dismutase. Superoxide dismutase (SOD) in cell of 6-well plates was detected by using enzyme linked immunosorbent assay using the multimode reader at 450 nm according to the manufacturer's guidelines (Nanjing jiancheng bioengineering institue, China) .

Data Analysis. The figures were finished by using Origin 2018C, statistical analysis was conducted by using SPSS 24.0.

Results and discussion

A549 Cell Viability. MTT relative viabilities for A549 cells after 24 exposure to the four PM fractions and a control were shown in Figure 1. At the low PM concentration (50 μg/mL), there was no significant difference in cell survival rate between each exposure group and control group (p>0.05). With the increase of the PM concentration, the toxicity of PM to A549 gradually increased. As a whole, the F3 group had the strongest cell toxicity, while the F1 group was just the opposite compared to the control group. However, Wang et al reported that PM whose diameter less than 1.1 μm could lead to the maximum survival toxicity to A549 cell [3]. The difference between two studies may be due to the different pre-treatment method and inconsistent composition of PM. According to the results of MTT assay, 100 μg/mL of PM was selected as the target concentration for subsequent experiments.

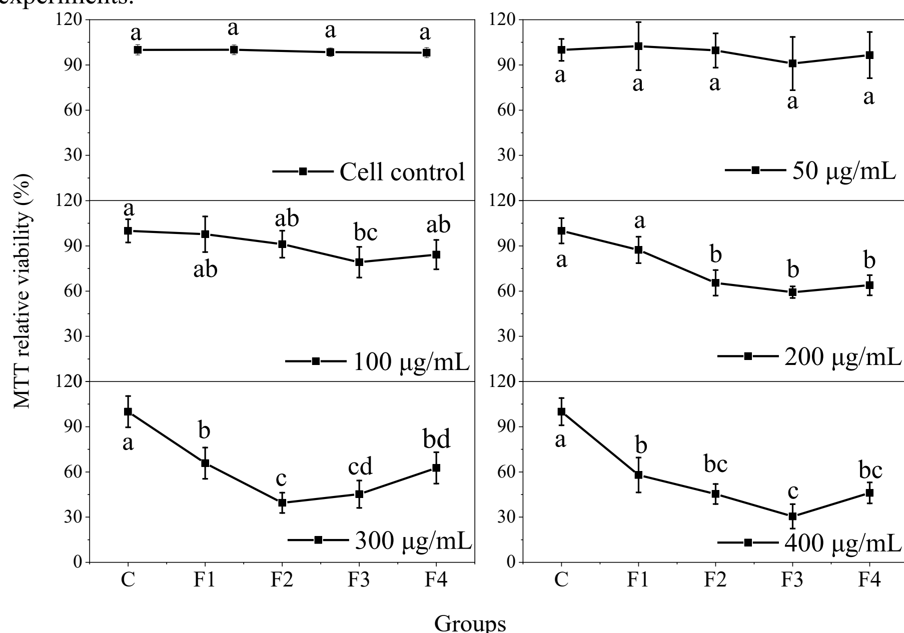

Figure 1. MTT relative viabilities of A549 cell lines after 24 h exposure with the four PM fractions and a control (C, without PM)

Cytokines. IL-8 is an activator of neutrophils and the most important chemokine in the human body. It is secreted by epithelial cells of the respiratory tract and plays an important role in the formation of inflammation. Elevated levels of IL-8 have been linked to acute lung injury, chronic bronchitis, childhood asthma and the deterioration of lung disease [12]. In this study, the levels of IL-8 as an experiment indicator of A549 cell was detected (Figure 2). By normalization, the difference between each group was more obvious (Figure 2B). There was no significant difference between F1 and the control (p>0.05), indicating that large PM could not cause significant effect on the release of inflammatory factor. For F2, F3, and F4 groups, significant difference were found compared to the control group (p<0.05), suggesting that strong inflammatory effects occurred. Besides, the measured IL-8 concentration of F3 was higher than that of other groups (p<0.05). Hetland et al found that

coarse PM could increase the release of inflammatory factor of A549 cell lines [13]. However, Wang et al reported that the secretion of cytokine of A549 induced by $PM_{0.4}$ was higher than that induced by $PM_{>5.8}$ [3]. It appeared that particle size had no correlation with the release of inflammatory cytokines. As mentioned above, this may be due to the composition of PMs, especially the contaminants absorbed in PM surfaces, rather than their size.

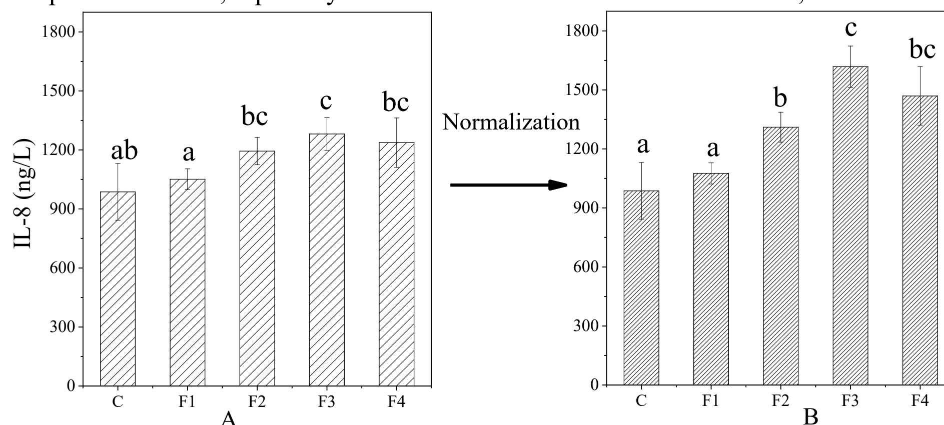

Figure 2. IL-8 expressions of A549 cell lines after 24 h exposure with five groups

SOD activity. SOD is one of the main enzymes in the cellular antioxidant enzyme system. It can catalyze the disproportionation reaction of superoxide anion free radical (•O2-) to generate hydrogen peroxide and elemental oxygen, which plays a vital role in keeping the balance of oxidation and antioxidant system of the body and avoiding free radical damage. The level of SOD in the body or cells is negatively correlated with the content of free radicals. The level of SOD can indirectly reflect the degree of oxidative damage in the body [14]. Figure 3 showed the levels of SOD for A549 cells after 24 exposure to the four PM fractions and a control. Compared with the control group, the SOD level decreased after exposure to atmospheric particles with different sizes ($p<0.05$), indicating that PM could cause oxidative damage to cells at the concentration of 100 μg/Ml. Meanwhile, no significant difference was found between F1, F2, F3, and F4 for SOD concentrations, suggesting that there was no correlation between particle size and oxidative damage degree.

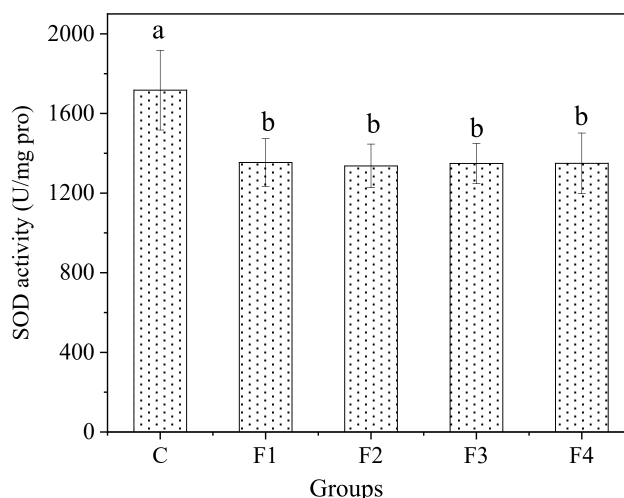

Figure 3. SOD activity of A549 cell lines after 24 h exposure with five groups

There are many hypotheses about the cytotoxicity caused by particulate matter, including the reactive oxygen free radical damage hypothesis, the inflammatory cell and inflammatory factor hypothesis, and the damage hypothesis based on physical properties. In this study, the concentration of PM, inflammatory factor and reactive oxygen free radical can all have adverse effects on cells, and It is difficult to only use a hypothesis to explain how PM is toxic to cells. Next, we will analyze the chemical composition of PM, such as heavy metal content, organic pollutant content and other indicators, and study the correlation between pollutant content and cytotoxicity.

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