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Seasonal Variations in $PM_{2.5}$-induced Oxidative Stress and Up-regulation of Pro-inflammatory Mediators

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ABSTRACT

Inhaling particulate matter with an aerodynamic diameter of ≤ 2.5 μm ($PM_{2.5}$) has been demonstrated to induce season-dependent adverse health effects. As inflammation and oxidative stress play a critical role in $PM_{2.5}$-induced health effects, this study used a human monocytic cell line, THP-1, to investigate whether the $PM_{2.5}$-induced oxidative stress and pro-inflammatory response varied by season. $PM_{2.5}$ was collected during April (spring), July (summer), September (fall) and December (winter) of 2014. The cytotoxicity was assessed with a lactate dehydrogenase (LDH) release assay. The levels of pro-inflammatory mediators, including tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β), were measured with ELISA, and the reactive oxygen species (ROS) were identified with flow cytometry. Sulforaphane (SFN), an antioxidant, was used to determine whether ROS regulated the $PM_{2.5}$-induced expression of pro-inflammatory mediators. The $PM_{2.5}$ from winter exhibited the highest potency in inducing cytotoxicity as well as the production of TNF-α and IL-1β from THP-1 cells; the same was true for ROS production. Further experiments demonstrated that pretreating THP-1 cells with SFN markedly mitigated the winter-$PM_{2.5}$-induced release of TNF-α and IL-1β. Composition analysis revealed that the $PM_{2.5}$ contained higher levels of anions (NO_3^- and SO_4^{2-}) and water-soluble metals (Al, Ca, Mg, Zn and Cr) during summer and winter than spring and fall. In summary, $PM_{2.5}$-induced oxidative stress and the subsequent production of pro-inflammatory mediators vary by season.

Keywords: $PM_{2.5}$; THP-1 cells; Cytotoxicity; Oxidative stress; Inflammation.

INTRODUCTION

Air pollution has become a major global public health concern. $PM_{2.5}$ is the primary component of urban air pollutants in China (van Donkelaar *et al.*, 2015). Epidemiological studies revealed that exposure to $PM_{2.5}$ increases cardiopulmonary mortality and morbidity (Schwarze *et al.*, 2006; Lippmann, 2014). A survey carried out in 2015 indicated that ambient $PM_{2.5}$ was the fifth-ranking mortality risk factor. The report also pointed out that deaths which is attributable to long-term exposure to $PM_{2.5}$ varied with country (Cohen *et al.*, 2017). Additionally, previous studies have shown that adverse health effects exposed to $PM_{2.5}$ vary with season (Becker *et al.*, 2005; Peng *et al.*, 2005).

$PM_{2.5}$, a heterogeneous mixture of constituents, includes diverse organics, metals and biological components. The constituents of $PM_{2.5}$ vary with origin and season (Becker *et al.*, 2005; Pardo *et al.*, 2018). Previous report has demonstrated that the $PM_{2.5}$ from different seasons presented different toxicity profiles (Hetland *et al.*, 2005). There is evidence that PM-induced increase in mortality is higher in the warm seasons compared with the cold seasons (Smith *et al.*, 2000; Danielsen *et al.*, 2011). These observations were further supported by an *in vivo* study showing that particles from warm/sunny days had greater inflammatory and cytotoxic activities in mouse lung than the particles from cold/wet seasons (Happo *et al.*, 2008). However, inconsistent results were also observed. For example, previous epidemiological and toxicological studies demonstrated that winter PM have more potency in cytotoxicity, especially in Asian countries (Chen *et al.*, 2013; Kurai *et al.*, 2016).

Oxidative stress as well as inflammation is regarded as critical events in PM-induced adverse health risk (Mazzoli-Rocha *et al.*, 2010). It is an effective way to evaluate $PM_{2.5}$-induced oxidative stress and inflammatory response by establishing a cell system. Assays of oxidative stress and inflammatory responses in PM-exposed cell systems, for example, could be used to provide an integrative assessment

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of biological activity of the PM mixture at modest cost. Assays reflecting different biological pathways of effect could be used to examine the mechanisms underlying epidemiological associations of PM with both acute and chronic disease. Associations in plausible pathways would also help reduce uncertainty in the causal interpretation of epidemiological findings (Manzano-Leon *et al.*, 2016).

Our previous studies have shown that $PM_{2.5}$ exposure can induce oxidative stress and inflammation in rats or BEAS-2B cells (Yan *et al.*, 2016; Li *et al.*, 2019). Alveolar macrophages (AMs) are regarded as the initial cells which can phagocytose inhaled particulates. PM deposited in lung could activate AMs via binding of ligands to receptors. Subsequently activate signaling pathways and potentially mediate inflammatory or cytotoxic outcomes (Wu *et al.*, 2014). AMs are a critical type of airway cells derived from blood monocytes (Hocking and Golde, 1979). The present study *in vitro* was to explore whether $PM_{2.5}$-induced oxidative stress and inflammatory effects exhibited seasonal variations using a human acute monocytic leukemia cell line (THP-1), which is an economical, high-throughput system associated to airway monocytes and AMs (Tsuchiya *et al.*, 1980).

MATERIALS AND METHODS

Reagents

IL-1β and TNF-α ELISA kits were purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). Lactate dehydrogenase (LDH) and superoxide dismutase (SOD) assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The 5(6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) was procured from Beyotime Biotechnology (Shanghai, China). Sulforaphane (SFN) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

$PM_{2.5}$ Collection

The $PM_{2.5}$ samples were provided by Dr. Ruiqin Zhang from Zhengzhou University, China. They were collected onto quartz microfiber filters (20.3 × 25.4 cm; PALL, USA) for a continuous 24 h using a $PM_{2.5}$ high-volume air sampler (KC-1000; Laoshan Mountain Electronic Instrument Factory Company, Qingdao, China) from April 2015 and December 2015 on non-rainy days, with proximity to a variety of small- and medium-size factories including machinery, chemical manufacturing, boiler industry, and power plant, indicating a characteristic mixed pollutant area. Before and after PM extraction, the quartz filters were equilibrated in a conditioning room at 22°C and at 33% relative humidity for 48 h (XS205; Mettler Toledo, Switzerland). Each filter was weighed twice, and the difference of the weights was less than or equal to 0.03 mg. The $PM_{2.5}$ was extracted from the filters by a 15 min sonication for three times with 2 min intervals. The extraction rate of $PM_{2.5}$-loaded quartz filters was between 87–92%. The $PM_{2.5}$ was recovered through a vacuum freeze-drying procedure and used for the following *in vitro* exposure studies.

$PM_{2.5}$ Constituent Analysis

To specify the physicochemical properties of $PM_{2.5}$, anions in $PM_{2.5}$ were determined with ion chromatography (ICS-90; Dionex, USA) using 8 mM Na_2CO_3/1 mM $NaHCO_3$ as eluent at 0.5 mL min^{-1}. Particles were digested by nitric acid and hydrogen peroxide. The metal elements analysis was carried out using an inductively coupled plasma mass spectrometry (ICP-MS; Thermo Fisher, USA).

Cell Culture and $PM_{2.5}$ Exposure

THP-1 cells were purchased from American Type Tissue Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C, 5% CO_2 in a humidified incubator. For determination of cytotoxicity and inflammatory response, the cells were seeded in 96-well plates (2×10^4 cells per well). After 24 h incubation, the cells were exposed to different doses of $PM_{2.5}$ from different seasons (0, 12.5, 25, 50, 100 μg mL^{-1}) for 24 h. Diesel exhaust particles (DEP) were used as the positive control. The rationale for using DEP as the positive control is that the associations of air pollution with cardiopulmonary diseases are strongest for $PM_{2.5}$, of which the combustion-derived ultrafine particles from diesel exhaust are an important component (Laden *et al.*, 2000). The final concentration for oxidative stress assay was 100 μg mL^{-1}.

Evaluation of Cytotoxicity

LDH is a stable cytoplasmic enzyme that is present in all kinds of cells. Once plasma membrane of cell is damaged, the LDH will instantly be released to the outside of the cells. The cytotoxicity was evaluated with LDH assay kit following the manufacturer's instructions.

Determination of Inflammatory Cytokines with ELISA

TNF-α and IL-1β are bio-markers of pro-inflammatory response. After the exposure of THP-1 cells to $PM_{2.5}$ for 24 h, the cell culture media were collected and the supernatants obtained through centrifugation at 300 g for 10 min. Levels of TNF-α and IL-1β were determined with ELISA according to the manufacturer's instructions.

Determination of Oxidative Stress

Levels of intracellular ROS were measured with flow cytometry. Briefly, the cells were incubated with 10 μM carboxy-H2DCFDA in a 37°C, 5% CO_2 humidified incubator for 30 min before exposed to $PM_{2.5}$ for 4 h. Then the cells were washed three times with PBS. Mean fluorescence (MFI) was measured by using BD Flow Cytometer (Becton Dickinson, NY, USA).

Effect of SFN on $PM_{2.5}$-induced Production of ROS and Over-expression of Pro-inflammatory Mediators

THP-1 cells were incubated with 5 μM SFN for 8 h before further exposure to 100 μg mL^{-1} $PM_{2.5}$ for 4 h and 24 h, respectively. The supernatants of cell media were collected for measurement of TNF-α, IL-1β and ROS as described above.

Statistical Analysis

Analysis of variance (ANOVA) was used to make multiple comparisons followed by LSD analysis. Student's

t-test was used to compare two comparisons. Data were presented as mean ± standard deviation (SD) and *P*-value less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 21.0.

RESULTS

Water-soluble Chemical Composition of $PM_{2.5}$

April, July, September and December in 2015 were chosen to represent spring, summer, fall and winter, respectively. The elemental and water-soluble ions of $PM_{2.5}$ from these four months are shown in Table 1. The water-soluble metal analysis uncovered twelve metals (Fe, Al, Ca, Mg, Cr, Mn, Ni, Cu, Zn, As, Pb, Cd) in the $PM_{2.5}$ samples. Mg, Ca, and Zn were the most abundant metals presented in fall and winter $PM_{2.5}$. The $PM_{2.5}$ from summer contained more Cr. As shown in Table 2, $PM_{2.5}$ from summer contained more NO_3^- and SO_4^{2-}, while those from spring and winter contained more Cl^-.

Seasonal Variation of $PM_{2.5}$ Cytotoxicity

Fig. 1(d) indicated that the $PM_{2.5}$ from winter induced a dose-dependent increase in cytotoxicity in THP-1 cells. In comparison with the $PM_{2.5}$ from winter, the $PM_{2.5}$ from other three seasons showed less cytotoxicity at a concentration of 50 μg mL^{-1} (Figs. 1(a), 1(b) and 1(c)).

Seasonal Variation of $PM_{2.5}$-induced Over-expression of Pro-inflammatory Mediators

As shown in Figs. 2(a), 2(b), 2(c) and 2(d), $PM_{2.5}$ (100 μg mL^{-1}) from different seasons all induced significant increases in the expression of TNF-α. Of them, winter $PM_{2.5}$ was the most potent in inducing the expression of TNF-α. As shown in Fig. 3, all the $PM_{2.5}$ samples increased the release of IL-1β compared with the control.

Seasonal Variation of ROS Production Induced by $PM_{2.5}$

ROS production was measured by MFI using flow cytometry in THP-1 cells. Intracellular levels of ROS were obviously increased when exposure of THP-1 cells to $PM_{2.5}$ (100 μg mL^{-1}) compared with control, especially from winter (Fig. 4(a)).

Inhibition of SFN on $PM_{2.5}$-induced ROS Production and Expression of Pro-inflammatory Mediators

ROS is regarded as a key factor in inducing the release of inflammatory mediators (Mazzoli-Rocha *et al.*, 2010). As expected, the release of ROS was significantly inhibited by pretreatment of THP-1 cells with SFN (Fig. 4(b)). Importantly, the TNF-α and IL-1β release were decreased by the pretreatment with SFN in THP-1 cells exposed to 100 μg mL^{-1} $PM_{2.5}$ from winter (Fig. 5(a) and 5(b)), indicating that ROS were required for winter-$PM_{2.5}$-induced pro-inflammatory response.

DISCUSSION

$PM_{2.5}$ has been considered to be a key contributor to the formation of haze in most parts of China. Although the association between $PM_{2.5}$ exposure and adverse human health effects is well known (Lawal, 2017; Mukherjee and Agrawal, 2018), the mechanisms of adverse effects caused

Table 1. Elemental constituents of $PM_{2.5}$ from different seasons (ng m^{-3}).

	Apr.	Jul.	Sep.	Dec.
Mg	856.38	854.76	1594.87	3036.29
Al	1171.68	1399.02	1433.78	512.20
Ca	6545.41	6007.75	6917.61	7704.00
Cr	295.16	298.29	98.62	141.71
Zn	1123.79	640.94	1441.32	1435.21
Cu	40.28	53.56	56.51	33.68
Pb	58.98	86.64	142.30	43.95
As	9.70	10.35	9.74	4.04
Cd	2.48	3.39	3.89	2.55
Ni	20.11	44.79	17.92	18.04
Mn	50.34	43.61	76.33	18.95

Table 2. Concentrations of water-soluble ions in $PM_{2.5}$ (μg m^{-3}).

	Apr.	Jul.	Sep.	Dec.
Cl^-	44.8348	0.9522	1.1493	10.2458
SO_4^{2-}	4.5722	15.7290	11.4700	11.1790
NO_3^-	2.8521	14.9048	5.9180	7.1120
F^-	0.2480	0.1929	0.2650	0.9733
Ac^-	1.3191	0.9918	1.1874	1.1465
NO_2^-	-	-	0.0802	-
Br^-	-	-	-	0.0543
PO_4^{3-}	4.0051	-	-	-

-: no data.

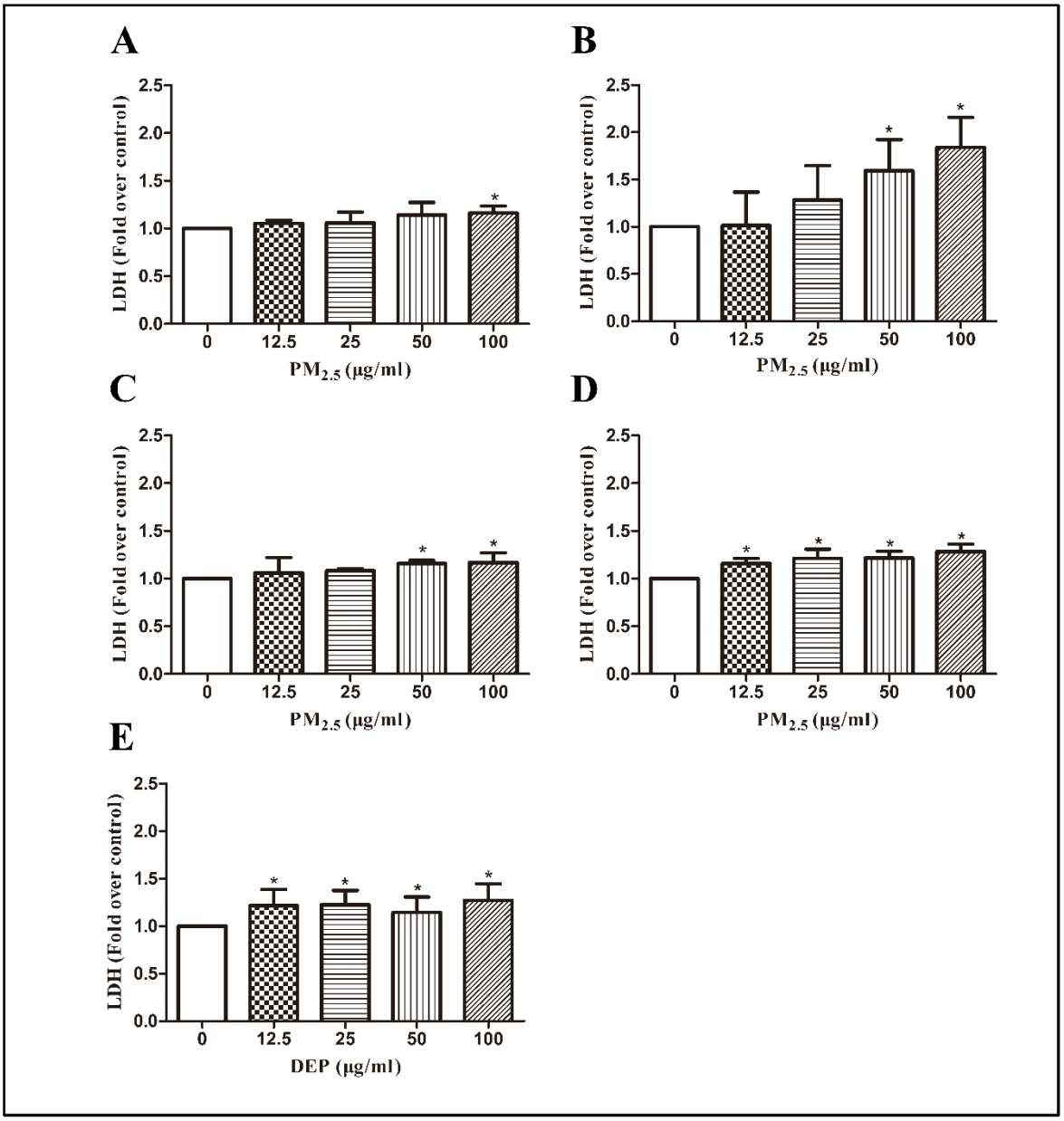

Fig. 1. Cytotoxicity of THP-1 cells exposed to $PM_{2.5}$. THP-1 cells were stimulated with $PM_{2.5}$ from (a) spring, (b) summer, (c) fall and (d) winter or (e) DEP at an indicative concentration for 24 h, separately. Cell viability was evaluated by using an LDH assay kit. LDH levels were represented by fold over control. All values represent the mean ± SD in three independent experiments. $^{*}p < 0.05$, compared with control.

by $PM_{2.5}$ is still unclear. Thus far, oxidative stress and inflammation have been recognized to play vital roles in $PM_{2.5}$-induced adverse effects. Season is an important modifying factor when investigating the acute health effects of air pollution. Documented studies have shown season-associated short-term health effects of PM (Roosli *et al.*, 2000). However, the evidence of seasonal variation in these associations is inconsistent (Hetland *et al.*, 2004; Molinelli *et al.*, 2006). A previous epidemiological study in China indicated that the acute effect of PM air pollution varied by seasons with the largest effect in winter and summer (Roosli *et al.*, 2000). In contrast, a recent *in vitro* study employed human lung epithelial carcinoma cells (A549), hepatocellular liver carcinoma cells ($HepG_2$), and neuroblastoma cells (Sh-Sy5y) to evaluate the toxicological properties of the collected $PM_{2.5}$ in Nanjing, China, showing that the viability inhibition in A549, Sh-Sy5y, and $HepG_2$ cells was more prominent in summer, and the induction of ROS in A549 and Sh-Sy5y cells was also more evident in summer (Zhang *et al.*, 2019).

In the present study with human blood monocytic cells, we showed that $PM_{2.5}$ from different seasons presented variable potencies in oxidative stress and inflammation, with the strongest potency for $PM_{2.5}$ from winter. Uniquely, this study found that oxidative stress was involved in $PM_{2.5}$-induced pro-inflammatory response since SFN, a naturally occurring potent inducer of antioxidant Phase II enzymes,

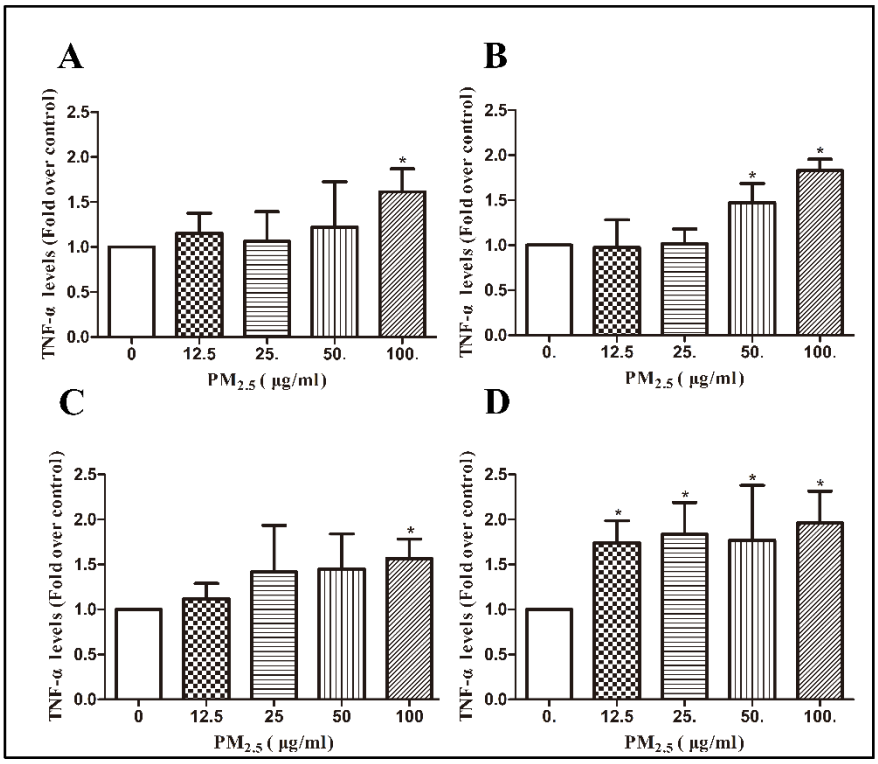

Fig. 2. Effect of $PM_{2.5}$ on TNF-α expression in THP-1 cells supernatant. THP-1 cells were exposed to $PM_{2.5}$ from (a) spring, (b) summer, (c) fall and (d) winter at an indicative concentration for 24 h, separately. The production of TNF-α was determined by using ELISA. TNF-α levels were represented by fold over control. All values represent the mean ± SD in three independent experiments. $^{*}p < 0.05$, compared with control group.

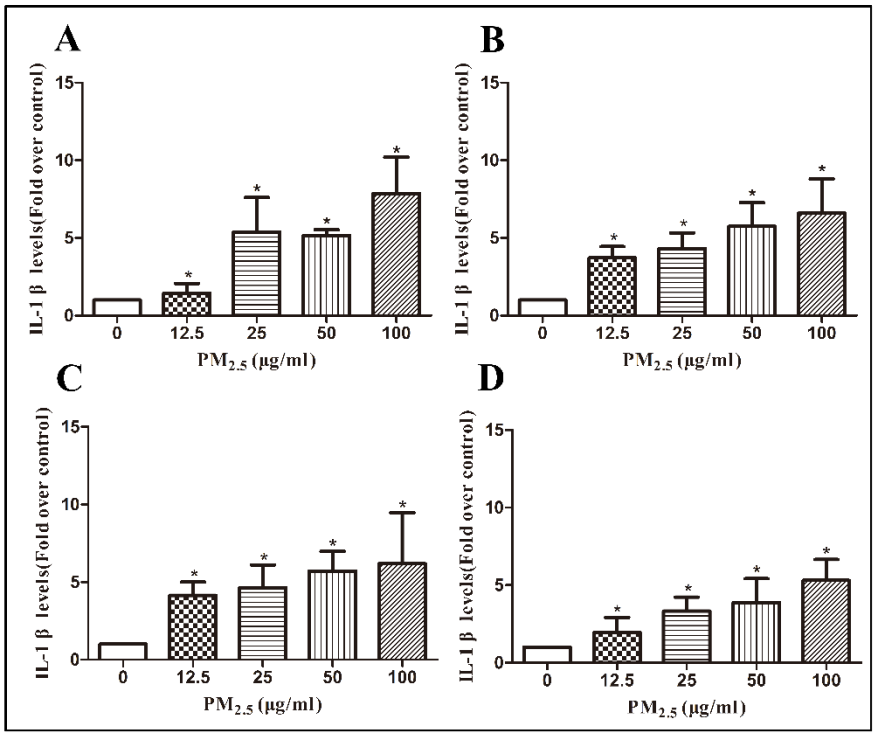

Fig. 3. Effect of $PM_{2.5}$ on IL-1β expression in culture medium of THP-1 cells. THP-1 cells were exposed to $PM_{2.5}$ from (a) spring, (b) summer, (c) fall and (d) winter at an indicative concentration for 24 h, separately. The production of IL-1β was determined by using ELISA. IL-1β levels were represented by fold over control. All values represent the mean ± SD in three independent experiments. $^{*}p < 0.05$, compared with control group.

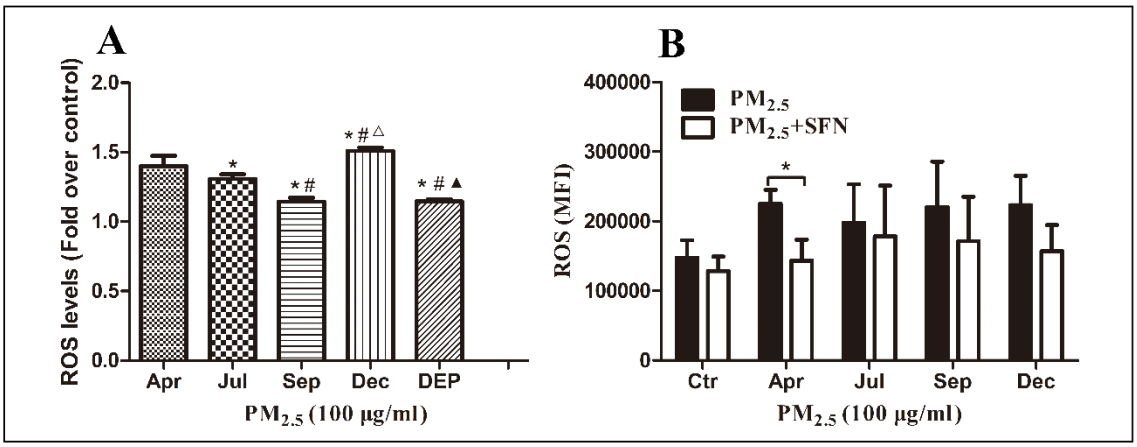

Fig. 4. Production of ROS triggered by $PM_{2.5}$ exposure in THP-1 cells. (a) THP-1 cells were exposed to 100 μg mL^{-1} $PM_{2.5}$ from different seasons for 4 h after incubated with carboxy-H2DCFDA for 30 min. ROS generation was determined by flow cytometry. ROS levels were represented by fold over control. (b) Before exposure to 100 μg mL^{-1} $PM_{2.5}$, 5 μM SFN was supplied to inhibit the production of ROS. All values represent the mean ± SD in three independent experiments. $^{*}p < 0.05$, compared with Apr group; $^{\#}p < 0.05$, compared with Jul group; $^{\triangle}p < 0.05$, compared with Sep group; $^{\blacktriangle}p < 0.05$, compared with Dec group.

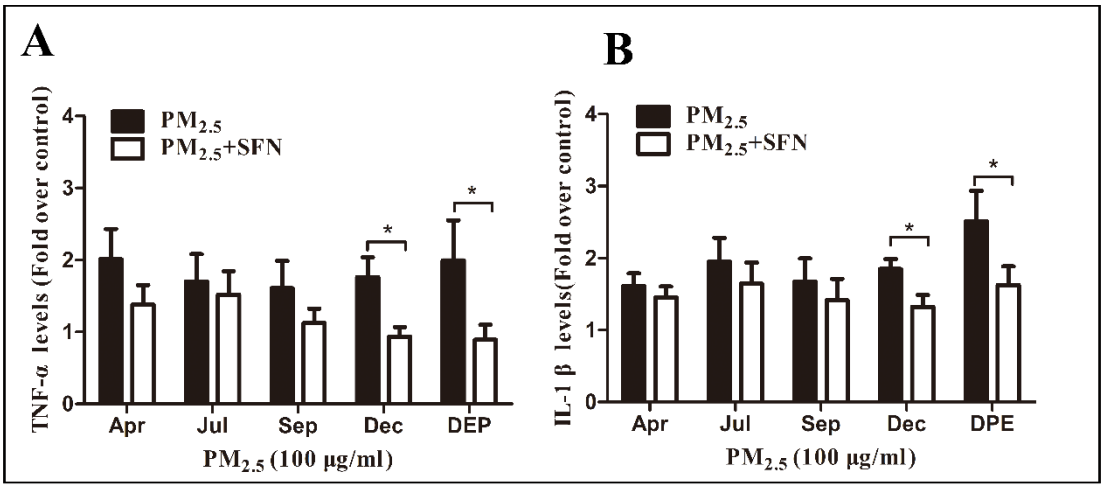

Fig. 5. Effect of SFN treatment on TNF-α and IL-1β expression induced by $PM_{2.5}$ exposure in THP-1 cells. TNF-α and IL-1β levels were represented by fold over control. All values represent the mean ± SD in three independent experiments. $^{*}p < 0.05$, compared with $PM_{2.5}$ group.

from broccoli sprouts, significantly block $PM_{2.5}$-induced ROS production and subsequent expression of pro-inflammatory mediators (Riedl *et al.*, 2009).

TNF-α and IL-1α/β are regarded as key mediators in lung, which will up-regulate the induction of secondary mediators including IL-6 and TGF-β1 and initiate the following inflammation cascade (Becker *et al.*, 1996; Grivennikov *et al.*, 2006; Barksby *et al.*, 2007). Previous studies have shown that $PM_{2.5}$ is capable of inducing TNF-α and IL-1β release (Ovrevik *et al.*, 2009; Dieme *et al.*, 2012). Chen *et al.* (2018) found cytotoxicity of $PM_{2.5}$ from winter was more potent than $PM_{2.5}$ from summer in Nanjing, China. The observation from Chen and his colleagues is supported by the present study, showing that $PM_{2.5}$, especially from winter, induces the release of inflammatory cytokines. Remarkably, several studies have indicated that PM from warmer seasons has a greater cell toxicity and pro-inflammatory response (Becker *et al.*, 2005; Happo *et al.*, 2008). The inconsistent findings suggest that mixture of components may participate in PM-induced heterogeneous health outcomes. The pathways regulating PM-induced inflammatory effect were not elucidated. Previous studies have shown inflammatory mediators induced by PM exposure through activating transcription factors including NF-kB and AP-1 (Donaldson and Stone, 2003; Ohyama *et al.*, 2007). In addition, the release of IL-6 or TNF-α induced by PM exposure was associated with Cu and Zn content (Becker *et al.*, 2005). Our previous study found 20% of IL-8 production was reduced after chelating of zinc of $PM_{2.5}$ (Yan *et al.*, 2016). In our study, Zn (varied in different seasons) is the highest metal in all the samples, especially winter ones. $PM_{2.5}$ from winter can obviously induce ROS release and up-regulation of TNF-α and IL-1β.

Although mechanisms of $PM_{2.5}$-induced health effects are not fully understood, ROS production has been well recognized to involve in inflammatory response upon exposure to PM (Nel, 2005). ROS is regarded as an important messenger to activate several cell signal transduction pathways which can initiate inflammatory gene expression (Dagher *et al.*, 2007). We found that $PM_{2.5}$ can lead to the generation of ROS which is in line with other studies (Becker *et al.*, 2005). Moreover, $PM_{2.5}$-induced TNF-α and IL-1β expression was significantly inhibited by the antioxidant SFN, implying that oxidative stress is required for release of pro-inflammatory cytokines in THP-1 cells. However, how PM induces oxidative stress is still to be investigated. A plethora of studies pointed out that PM components may account for oxidative stress. Metals, especially transition metals, which are present on PM are related to $PM_{2.5}$-induced airway injury, inflammation and oxidative stress (Hetland *et al.*, 2000; Steenhof *et al.*, 2011). Our present study found that Zn and Cr are enriched in $PM_{2.5}$ from Zhengzhou. Zinc is an important metal element detected in traffic-derived $PM_{2.5}$, and it could mediate ROS generation (Chen and Lippmann, 2009; Donadelli *et al.*, 2009). In conclusion, these studies indicate that differential cytotoxicity, inflammation and oxidative stress may be related to water-soluble metals.

It should be noted that although quartz filter is recommended for air sampling and chemical analyses, its highest efficiency of absorption and promotion of on-filter degradation of air pollutant components poses potential limitations on evaluation of PM toxicity (Grosjean, 1983; Parshintsev *et al.*, 2011).

CONCLUSIONS

In summary, the present study uses THP-1 cells to demonstrate that the ability of $PM_{2.5}$ to induce oxidative stress and inflammation differs by season. Our findings, which suggest that PM during winter in Zhengzhou (China) produces stronger toxic effects, support the conclusions of previous epidemiological studies. The variation in the $PM_{2.5}$ composition across different seasons must be considered in future toxicological and epidemiological research.

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