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Heavy Metals Bound to Fine Particulate Matter from Northern China Induce Season-Dependent Health Risks: A Study Based on Myocardial Toxicity

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Abstract:

Substantial epidemiological evidence has consistently reported that fine particulate matter ($PM_{2.5}$) is associated with an increased risk of cardiovascular outcomes. $PM_{2.5}$ is a complex mixture of extremely small particles and liquid droplets composed of multiple components, and there has been high interest in identifying the specific health-relevant physical and/or chemical toxic constituents of $PM_{2.5}$. In the present study, we analyzed 8 heavy metals (Cr, Ni, Cu, Cd, Pb, Zn, Mn and Co) in the $PM_{2.5}$ collected during four different seasons in Taiyuan, a typical coal-burning city in northern China. Our results indicated that total concentrations of the 8 heavy metals differed among the seasons. Zn and Pb, which are primarily derived from the anthropogenic source, coal burning, were the dominant elements, and high concentrations of these elements were observed during the spring and winter. To clarify whether these heavy metals in the locally collected $PM_{2.5}$ were associated with health effects, we conducted health risk assessments using validated methods. Interestingly, Pb was responsible for greater potential health risks to children. Because cardiovascular disease (CVD) is a main contributor to the mortality associated with $PM_{2.5}$ exposure, we performed experimental assays to evaluate the myocardial toxicity. Our *in vitro* experiments showed that the heavy metal-containing $PM_{2.5}$ induced season-dependent apoptosis in rat H9C2 cells through a reactive oxygen species (ROS)-mediated inflammatory response. Our findings suggested that heavy metals bound to $PM_{2.5}$ produced by coal burning play an important role in myocardial toxicity and contribute to season-dependent health risks.

Keywords: fine particulate matter ($PM_{2.5}$); heavy metals; health risk assessment; myocardial toxicity

Heavy metals bound to the $PM_{2.5}$ from coal combustion caused H9C2 apoptosis via ROS-mediated inflammatory response and contributed to season-dependent health risks for children.

1. Introduction

With the rapid economic development, industrial expansion and urbanization in China during the last few decades, increasingly frequent incidents of haze or smog episodes characterized by high fine particulate matter ($PM_{2.5}$) levels and reduced visibility have been reported at the national scale in this country (Liu et al., 2013; Zhang et al., 2012). As both the total amounts and the proportions of $PM_{2.5}$ have increased in Chinese cities, the adverse health effects of this major urban air pollutant have attracted increasing concern due to its influence on not only cardiorespiratory system but also cerebrovascular events (Stafoggia et al., 2014). These two conditions constitute two major causes of high rates of hospitalization and mortality (Ito et al., 2011; Zheng et al., 2015). The World Health Organization (WHO) has reported that ambient air pollution was responsible for 3,700,000 deaths in 2012, including 16% of the lung cancer deaths, 11% of the chronic obstructive pulmonary disease-related deaths, 29% of the heart disease and stroke deaths, and approximately 13% of the deaths that were due to respiratory infections (Lee et al., 2014). Lelieveld et al. (2013) calculated a global mortality of approximately 773,000/year due to respiratory disease, 186,000/year due to lung cancer and 2,000,000/year due to cardiovascular disease (CVD) resulting from exposure to anthropogenic $PM_{2.5}$ (Lelieveld et al., 2013). Obviously, CVD is a key contributor to mortality. A substantial body of epidemiological studies have provided consistent evidence that indicates that exposure to $PM_{2.5}$ is associated with an increase in cardiovascular mortality (Crouse et al., 2012; Madrigano et al., 2013). A previous study by the Women's Health Initiative (WHI) reported a 24% increase in the risk of a cardiovascular event [hazard ratio (HR) = 1.24; 95% confidence interval (CI), 1.09-1.41] and a 76% increase in the risk of death from CVD

(HR=1.76; 95% CI, 1.25-2.47) for each 10 μg/m^3 change in the $PM_{2.5}$ level (Miller et al., 2007). Chen et al. (2008) found that long-term exposure to $PM_{2.5}$ increases the risk of cardiovascular mortality by approximately 12-14% per 10 μg/m^3 increase in the $PM_{2.5}$ level (Chen et al., 2008). Moreover, Hoek et al. (2013) concluded that per 10 μg/m^3 increase in $PM_{2.5}$ exposure is associated with 11% (95% CI 5, 16%) for cardiovascular mortality (Hoek et al., 2013). Specifically, increasing evidences have shown that CVDs such as acute myocardial infarction (MI) and heart failure (HF) are associated with a sustained loss of cardiac cells through apoptosis (Hilfiker-Kleiner et al., 2006; Ale et al., 2013). Therefore, the potential risk of myocardial toxicity caused by $PM_{2.5}$ exposure should received more attention.

$PM_{2.5}$ is a complex mixture of extremely small particles and liquid droplets that include various components including acids (such as nitrates and sulfates), organic chemicals, heavy metals, and soil or dust particles (Yue et al., 2015). Although heavy metals account for only a small fraction of the $PM_{2.5}$, they not only are non-biodegradable when adherent to particles but also can bio-accumulate through the food chain and contribute to the toxicity of $PM_{2.5}$ (Fang et al., 2013; Abuduwaili et al., 2015). In earlier studies, Chen and Lippmann (2009) reported an association between PM-bound metals and potential health effects (Chen and Lippmann, 2009). Moreover, Niu et al. (2013) reported that specific metals might be important components that are responsible for the $PM_{2.5}$-induced cardiovascular effects (Niu et al., 2013). The Comparative Toxicogenomics Database (CTD) revealed that air pollutants that are composed of particulate metal ions are associated with cardiac arrhythmia, myocardial ischemia, myocardial infarction, stroke, and thrombosis (Meng et al., 2013). These findings suggested that heavy metal-containing $PM_{2.5}$ plays an important role in the myocardial

toxicity-associated health risks.

$PM_{2.5}$ is highly heterogeneous, and various characteristics of the materials from different pollution sources induce differences in corresponding biological effects. We postulated that the myocardial toxicity-associated health risk resulting from heavy metal-containing $PM_{2.5}$ might depend on the characteristics of these pollutants, particularly in northern China, where the coal-based heating systems that are used in spring and winter have become the dominant sources of $PM_{2.5}$ (Song et al., 2015). Huang et al. (2011) reported that $PM_{2.5}$ from coal-burning heat sources caused the increase of mortality; among these, CVDs accounted for approximately 46% (Huang et al., 2011). However, few studies have examined the relationship between the heavy metal-containing $PM_{2.5}$ produced by coal burning and CVDs. Thus, the aims of the present study were to analyze the concentrations and sources of heavy metals in $PM_{2.5}$ in Taiyuan, a typical coal-burning city in northern China, assess the associated health risks, and determine the potential biological responses based on myocardial toxicity.

2. Materials and methods

2.1. Collection of $PM_{2.5}$ samples

Sampling was performed between 2012 and 2013 in Taiyuan, a city in northern China. The $PM_{2.5}$ samples were collected on quartz filters (Φ90 mm, Munktell, Falun, Dalarna, Sweden) with PM middle-volume air samplers (TH-150CIII, Wuhan, China). Subsequently, 1/8 of the filters from each season were used for the heavy metal measurement, and another 1/8 of the filters were used for the *in vitro* experiments. The details of the $PM_{2.5}$ collection and filter treatment are provided in the text of the Supporting Information (SI).

2.2. Determination of heavy metals in $PM_{2.5}$

The concentrations of 8 heavy metals (chromium (Cr), nickel (Ni), copper (Cu), cadmium (Cd), lead (Pb), zinc (Zn), manganese (Mn) and cobalt (Co)) in the $PM_{2.5}$ were determined using inductively coupled plasma-mass spectrometry (ICP-MS). The detailed procedures are presented in the text of the SI.

2.3. Calculation of the enrichment factor (EF)

The enrichment factor (EF) is an important indicator for the quantitative assessment of the levels and sources of heavy metal pollution, and this value is calculated as indicated below (Liu D. et al., 2012):

$$EF = \frac{(C_i/C_{ref})_{samples}}{(B_i/B_{ref})_{baseline}}$$

where EF is the enrichment level of a certain heavy metal, C_i is the measured concentration of heavy metal i in the $PM_{2.5}$ (mg/kg), C_{ref} is the measured concentration of the reference element (mg/kg), B_i is the background value of heavy metal i in the local region (mg/kg), and B_{ref} is the background concentration of the reference element in the same region (mg/kg). In this study, Al was used as the reference element because the steel factories present in Taiyuan could produce significant amounts of Fe and the filters used for collection are made of quartz, which has a high content of Si. The background values of Cr, Ni, Zn, Mn, Cu, Cd, Pb and Co in the local region (B_i) were 68.48, 28.7, 83.6, 600, 26.7, 0.2208, 24.9 and 13 mg/kg, respectively (Li X. et al., 2014; Li L. et al., 2014).

2.4. Principal component analysis (PCA)

Principal component analysis (PCA), a method that describes variables with a minimum loss of information, is commonly used to evaluate the specific sources of pollutants (Zhao et al., 2006). The concentration data obtained for each congener in the sample analysis was

expressed as a fractional part of the total and normalized to a sum equal to 100. This normalization minimizes the influence of the total concentration and permits the comparison of compositional similarities among samples. The eigenvectors were normal-varimax rotated to facilitate the interpretation of the results (Sakurai et al., 1998). The statistical analyses were performed using SPSS 17.0 for Windows (SPSS Inc.).

2.5. Non-carcinogenic risk assessment of heavy metal-containing $PM_{2.5}$

$PM_{2.5}$ primarily induces health risks to humans by three routes: ingestion, inhalation, and dermal contact. In the present study, we adapted the health risk assessment models of the U.S. EPA to evaluate the health risks of the heavy metals. The average daily doses through ingestion (ADD_{ing}) (mg/kg/day), inhalation (ADD_{inh}) (m^3/kg/day), and dermal contact (ADD_{dermal}) (mg/kg/day) were calculated as follows (Cao et al., 2015):

$$ADD_{ing} = C \times \frac{IngR \times EF \times ED}{BW \times AT} \quad (1)$$

$$ADD_{inh} = C \times \frac{InhR \times EF \times ED}{PEF \times BW \times AT} \quad (2)$$

$$ADD_{dermal} = C \times \frac{SA \times AF \times ABS \times EF \times ED}{BW \times AT} \quad (3)$$

where C represents the concentration of the heavy metal (mg/kg), IngR is the ingestion rate (mg/day), InhR is the inhalation rate (m^3/day), PEF is the inhalation factor for inhalable particles (m^3/kg), SA is the surface area of the skin exposed to pollutants (cm^2), AF is the skin adherence factor (mg/cm^2/day), ABS is the dermal absorption factor, EF is the exposure frequency (days/year), ED is the exposure duration (year), BW is the body weight (kg), and AT is the average time (days). The values of these parameters were obtained from the literature, as shown in Table S1.

The hazard quotient (HQ) reveals the non-carcinogenic risk of a single contaminant and

was calculated by dividing the ADD from each exposure route based on a specific reference dose (RfD). The HQ is defined as follows:

$$HQ = \frac{ADD}{RfD} \quad (4)$$

where RfD is the estimated maximum permissible risk to humans through daily exposure (Li L. et al., 2014). If HQ ≤ 1, adverse health effects would be unlikely to be experienced, whereas potential non-carcinogenic effects would occur when HQ > 1 (Cao et al., 2015).

To evaluate the potential non-carcinogenic effects induced by many metals (e.g., i), the HQ values of all metals were calculated and expressed as a hazard index (HI):

$$HI = \sum_{1}^{i} HQ \quad (5)$$

The total exposure hazard index (HIt) was used to reflect the non-carcinogenic risks through different pathways and is expressed as follows:

$$HIt = \sum_{1}^{i} HI(Exposure \quad Pathway) \quad (6)$$

When HIt ≤ 1, chronic risks are unlikely to occur, whereas non-carcinogenic risks are likely to occur when HIt > 1; thus, an analysis segregating the contaminants and separating the HIt would be relevant (Cao et al., 2015).

2.6. Cell culture and exposure

The rat H9C2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) that was supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% carbon dioxide (CO_2). The cells were seeded at the appropriate density for each experimental design. The cells were randomly separated into a control group and various treatment groups. The control group was incubated only in DMEM with 10% FBS. One treatment groups were treated with $PM_{2.5}$ (10 μg/mL) from the four seasons, respectively, and

the other treatment groups were treated with $PM_{2.5}$ from the spring or winter at various concentrations (0, 1, 3, and 10 μg/mL). For the interference experiments, the groups were treated with the winter $PM_{2.5}$ (10 μg/mL) for 24 h in the absence or presence of a reactive oxygen species (ROS) inhibitor, N-acetyl-L-cysteine (NAC, Sigma, USA), which was added to the medium 1 h prior to the $PM_{2.5}$ treatment. We performed three independent experiments, and each independent experiment was conducted in triplicate.

2.7. ROS assay

After seeding 5×10^5 cells per mL onto 35-mm Petri dishes in DMEM with 10% FBS, the cells were treated with winter $PM_{2.5}$ at various concentrations (0, 0.1, 0.3, 1, 3 and 10 μg/mL) for 24 h. Subsequently, the harvested cells were incubated with 2′, 7′-dichlorofluorescin diacetate (DCFH-DA, 10 μM) at 37°C for 30 min in the dark. Then, the formation of the fluorescent-oxidized derivative of 2′, 7′-dichlorofluorescein (DCF) was determined using a flow cytometer (C6, BD, USA) with an emission wavelength of 530 nm and an excitation wavelength of 488 nm. The ROS generation was quantified as the median fluorescence intensity of the fluorescent compound DCF formed by 1×10^4 cells (Yue et al., 2015).

2.8. Real-time quantitative reverse transcription-PCR

Approximately 5×10^5 H9C2 cells per mL were plated onto 35-mm Petri dishes for 24 h in DMEM supplemented with 10% FBS. The spring or winter $PM_{2.5}$ at various concentrations (0, 1, 3, and 10 μg/mL) was added to the culture media. After incubation for 24 h, the cells were washed twice with Dulbecco's Hank’s Balanced Salt Solution (D-Hanks) (1 mL in each well) and subsequently harvested. We extracted the total RNA using the Trizol Reagent (Invitrogen, USA) and synthesized first-strand complementary DNA (cDNA) using a reverse transcription

kit (Takara, China) (Lan et al., 2014). The relative quantification of the gene expression (*bcl-2*, *bax*, and *p53*) was determined using a qTOWER 2.2 Real-Time PCR instrument (Analytik Jena AG, Jena, Germany) with glyceraldehyde-phosphate dehydrogenase (*GAPDH*) as an internal control (Guo et al., 2014). The detailed procedures and primer sequences are presented in the text of the SI and Table S2, SI.

2.9. Western blotting

The H9C2 cells were seeded at 5×10^5 per mL onto 60-mm Petri dishes for 24 h before exposing them to the $PM_{2.5}$. After treatment for 24 h, the proteins were extracted from the cultured cells using ice-cold lysis buffer containing 4 mM ethylene glycol tetraacetic acid (EGTA), 0.5 mM ethylene diamine tetraacetic acid (EDTA), 2.5 μg/mL aprotinin, 1 μg/mL pepstatin, 10 μg/mL leupeptin, 25 μM phenyl methane sulfonyl fluoride (PMSF), 25 μg/mL trypsin inhibitor, and 1 μM 1, 4-dithiothreitol (DTT). The protein concentrations were determined to ensure equal loading for assessment using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, 35 μg of total protein was separated using SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 3% nonfat milk and then incubated with rabbit polyclonal antibodies specific for GAPDH, bax, bcl-2, p53, inducible nitric oxide synthase (iNOS) or intercellular adhesion molecule-1 (ICAM-1) at a dilution of 1:1000 (for GAPDH, p53, iNOS and ICAM-1) or 1:200 (for bax and bcl-2) at 4°C overnight. After exposure to a fluorescently labeled secondary antibody (1:2000) (IRDye 800CW goat anti-rabbit IgG (H + L), LI-COR), the membranes were subjected to scanning and detection using a LI-COR Odyssey Infrared Fluorescent System (Yun et al., 2015).

2.10. TUNEL assay

The KeyGen Biotech TUNEL Apoptosis Detection Kit (KeyGen, China) was used to assess the levels of apoptosis of the H9C2 cells *in vitro*. The cells (5×10^4) were plated onto poly-D-lysine-coated coverslips. After incubation for 24 h, the cells were fixed with 4% paraformaldehyde and permeabilized with Triton X-100. After being rinsed, the cells were incubated with the TUNEL reagents and stained with 4', 6-diamidino-2-phenylindole (DAPI). The images were subsequently acquired using a fluorescence microscope (Olympus, Japan) with Image-pro Express 6.0 software (Media Cybernetics, USA). The percentages of TUNEL-positive cells were calculated under 200× magnification in randomly selected fields from three separate coverslips for each experiment.

2.11. Statistical analyses

Our data are presented as the means ± standard error (SE) of three independent experiments and evaluated using one-way ANOVA. Differences were considered to be significant if $p < 0.05$.

3. Results and discussion

3.1. Analysis of the concentrations and sources of heavy metal-containing $PM_{2.5}$

We first determined the overall concentrations of the $PM_{2.5}$ from the various seasons to investigate the regional pollutant levels. Figure 1 shows the season-dependent trend of the $PM_{2.5}$ concentrations for the whole year: the highest value was observed in the winter. Similar seasonal trends have also been determined in some Asian cities including Beijing (Duan et al., 2012), Chengdu (Li et al., 2015), and Changchun (Fang et al., 2014), China, and Chennai (Srimuruganandam and Nagendra, 2011), India. Importantly, the average daily concentration of $PM_{2.5}$ was 121 μg/m^3, which was much higher than the Air Quality Standard of China ($PM_{2.5}$

≤ 75 μg/m^3). To further characterize the toxic components in the collected $PM_{2.5}$ samples, we then determined the concentrations of 8 heavy metals bound to the $PM_{2.5}$ collected during the various seasons. The results are summarized in Table 1. The total contents of the 8 heavy metals in $PM_{2.5}$ samples were 5456.30, 3505.39, 4556.60 and 5231.15 mg/kg in the spring, summer, autumn, and winter, respectively. Mn, Zn and Pb accounted for the highest percentages. Zn and Pb were the predominant elements for the highest concentrations observed during the spring and winter. Yu et al. (2008) also reported that Zn and Pb were the major components of the particulate matter in Liaoning, North China (Yu et al., 2008). The average annual concentration of these 8 heavy metals in $PM_{2.5}$ in the present study was 4687.36 mg/kg (0.80 μg/m^3: the formula for this calculation is shown in Text 1, SI), which was higher than that of cities in southern China, including Hong Kong (0.30 μg/m^3) (Ho et al., 2006) and Shenzhen (0.38 μg/m^3) (Hagler et al., 2007), but similar to two cities in Liaoning province (Shenyang, 0.75 μg/m^3 and Anshan, 0.80 μg/m^3), another heavily industrialized site in northern China (Han et al., 2010).

However, the type and contents of the heavy metals varied between the southern and northern cities, reflecting different pollution sources and characteristics (Chen et al., 2008; Tan et al., 2014; Xiao et al., 2015; Xu L. et al., 2012). Accordingly, we analyzed the sources of the heavy metal constituents of the $PM_{2.5}$ by calculating the EF value, an important indicator that quantitatively assesses the levels and sources of the heavy metal pollution. According to a previous study, an EF>10 indicates that heavy metals are significantly enriched and that anthropogenic pollutants were most likely the primary source of these compounds. In contrast, an EF<10 indicates compounds that are primarily derived from the natural environment (Liu D.

et al., 2012). In the present study, according to the data shown in Table 2, the sources of Cd, Pb and Zn might be anthropogenic, whereas the other metals, including Cr, Mn, Ni, Co and Cu, in our study may have been derived from the natural environment. Li et al. (2014) also reported that Cd, Zn and Pb were from anthropogenic sources (coal dusts) during the heating seasons in local area (Li et al., 2014). Furthermore, Sun et al. (2015) indicated that the source of the abundant Zn and Pb observed in samples collected from Datong, Shanxi was anthropogenic coal burning (Sun et al., 2015). In addition, Hong et al. (2011) observed that heavy metals Zn and Pb in the particulate matter from Shengyang were derived from anthropogenic sources (Hong et al., 2011).

To determine the precise source of these heavy metals, we analyzed the inter-element relationships using Pearson correlation analysis. A strong correlation between contaminants indicates that the two substances are derived from the same source (Table S3). Strong positive correlations were observed between the Ni and Co (0.985, $p<0.01$), Co and Cu (0.982, $p<0.05$), Cu and Cr (0.959, $p<0.05$), Cr and Mn (0.972, $p<0.05$), and Zn and Pb (0.956, $p<0.05$). In addition, the heavy metal Cd was highly correlated with Zn and Pb, which indicated that Zn, Pb and most Cd were derived from a common source. To make the results more easily interpretable, a PCA analysis of the heavy metals in the $PM_{2.5}$ was conducted, and the results are shown in Figure 2. As Zhao et al. (2006) reported, two principal components with eigenvalues >1 were extracted from the variables and retained for further analysis (Table S4) (Zhao et al., 2006). Table S5 shows the factor loadings for the heavy metals generated by this PCA, and the principal components that accounted for more than 95.08% of all of the variance were extracted. The first factor accounted for 58.86% of the total variance and included Cu, Ni,

Cr, Co, and Mn. Co and Cu were the predominant components of factor 1, whereas Pb and Zn were the predominant components of factor 2. These data, coupled with the EF values, Pearson's correlative analysis, and the reported evidence suggested that Cu, Ni, Cr, Co, and Mn were primarily derived from natural sources, such as ore, soil parent material and parent rocks (Mico et al., 2006; Xiao et al., 2015). Pb, Zn and most Cd were primarily derived from the anthropogenic source, coal burning (Xu H. et al., 2012). These data indicate that factor 2 may represent coal ash. These conclusions were consistent with the results found for the Xi'an, Shanxi, province (Han et al., 2006), Changchun, Jilin province (Yang et al., 2015) in China.

3.2. Non-carcinogenic risk assessment for heavy metal-containing $PM_{2.5}$

Epidemiological findings coupled with animal inhalation studies have revealed that heavy metals are associated with non-carcinogenic health risks (Niu et al., 2013; Meng et al., 2013). To determine whether the heavy metals in the $PM_{2.5}$ from the various sources in the study area have health risks for local residents and to clarify the major contributors, we estimated the non-carcinogenic health risks of the metals by calculating the HQ. This model is a valuable method used by the U.S. EPA to assess the non-carcinogenic risk of heavy metals to human health based on the intake rate, exposure frequency, and duration (Cao et al., 2015). The HQs for Cr, Ni, Cu, Cd, Pb, Zn, Mn and Co in the $PM_{2.5}$ samples from the various seasons collected for the present study are shown in Table 3.

According to the risk assessment, for adults, the HIt values (sum of three exposure pathways) for these 8 heavy metals from each single season were approximately 1 (0.8 - 1.2), indicating that potential health risks for adults are unlikely. However, the HIt values for children were approximately 5 (4.9 - 5.3) times higher than those for adults, suggesting that children

face greater potential health risks from the heavy metal-containing $PM_{2.5}$. In addition, Pb presented the highest HI value and was the predominant source of the increased risks to children. There is strong epidemiological evidence that Pb exposure appears to be associated with various health effects, including hypertension and clinical cardiovascular end points (Navas-Acien et al., 2007; Schober et al. 2006).

3.3. Effects of heavy metal-containing $PM_{2.5}$ on H9C2 cell apoptosis

Numerous studies have indicated that exposure to heavy metal-containing $PM_{2.5}$ is associated with various CVDs (Niu et al., 2013; Meng et al., 2013), and high mortality due to CVDs was attributed to early lesions. Increasing evidences have shown that the sustained loss of cardiac cells through apoptosis, even when it occurs in a low-grade manner, can cause cardiac dysfunction and ultimately lead to heart disease (Hilfiker-Kleiner et al., 2006; Ale et al., 2013). Here, we first determined whether the heavy metal-containing $PM_{2.5}$ could cause H9C2 cell apoptosis. As shown in Figure 3A, TUNEL staining revealed that the apoptosis induced by heavy metal-containing $PM_{2.5}$ from the spring and winter was more obvious than the other seasons. These results suggest a positive association between the levels of heavy metal-containing $PM_{2.5}$ and the cell apoptosis and that the samples from spring and winter caused the most distinct effects.

Apoptosis of cells is controlled by complex interactions among numerous pro-survival and pro-death signals, including the bcl-2 family of proteins, some of which are anti-apoptotic (bcl-2 and bcl-xL) and others of which are pro-apoptotic (bax, bak and bid), as well as dysfunction of the tumor suppressor gene p53 (Amaral et al., 2010). In the present study, the expression of apoptosis-associated proteins was examined to further evaluate the apoptotic process. Both

the elevated levels of p53 and the decreased ratios of bcl-2/bax showed season-dependent patterns, and the most obvious effects were observed for samples collected during the spring and winter (Figure 3B). Subsequently, we treated the H9C2 cells with various concentrations (0, 1, 3, or 10 μg/mL) of the winter and spring $PM_{2.5}$ and evaluated the expression of the apoptosis-associated genes, including *p53*, *bax*, and *bcl-2*. Figure 4 shows the concentration-dependent cell apoptosis after 24 h of treatment with either the spring or winter samples, in which significant differences occurred after exposure to 3 or 10 μg/mL samples of the heavy metal-containing $PM_{2.5}$. Previous studies have demonstrated that decreased expression of the anti-apoptotic protein bcl-2 and elevated expression of the pro-apoptotic proteins bax and p53 could collaboratively trigger an evolutionarily conserved process, cell apoptosis (Li et al., 2011; Yun et al., 2010b). Therefore, our results imply that the heavy metals associated with the $PM_{2.5}$ derived from coal burning caused H9C2 cell apoptosis even at a low dose.

3.4. Mechanism of heavy metal-containing $PM_{2.5}$ H9C2 cell toxicity

Cell apoptosis can be initiated by diverse signals (such as oxidative stress and inflammation) and executed through various biological processes (Dagher et al., 2006). Previous studies have shown that a decrease in the bcl-2/bax ratio is partially regulated by a sustained generation of nitric oxide (NO) by iNOS, which is critical for apoptosis (Semmler et al., 2005). ICAM-1 is an endothelial cell- and leukocyte-associated transmembrane protein that has long been known to be important in stabilizing cell-cell interactions and is also a factor closely associated with cell apoptosis (Li et al., 2011; Pan et al., 2012). To explore the possible mechanism of the H9C2 cell apoptosis induced by the heavy metal-containing $PM_{2.5}$, we

evaluated the expression of the inflammatory mediators, including *iNOS* and *ICAM-1*. The results in Figure 5 indicated that $PM_{2.5}$ caused a dose-dependent change in the levels of these inflammatory markers. Specifically, the *iNOS* expression increased 2.17- and 4.64-fold relative to the control levels, and the *ICAM-1* expression increased 1.78- and 2.24-fold relative to the control levels after 24 h of treatment with the highest concentrations of the spring and winter samples. Over-expression of *ICAM-1* contributes to the process of leukocyte recruitment to the sites of inflammation (Li et al., 2011). During the development of inflammation, endothelial cells actively participate in this process by regulating the leukocyte recruitment by enhancing the expression of pro-inflammatory enzymes, such as *iNOS* (Yun et al., 2010a). An elevated activity of *iNOS* promotes NO production and ultimately leads to cell apoptosis (Brown and Bal-Price, 2003; Chen et al., 2012). Additionally, inflammatory factors including TNF-α, iNOS and ICAM-1 also act as pro-apoptotic proteins (Alexander et al., 2005). Therefore, our results clearly demonstrated that the inflammatory molecules *iNOS* and *ICAM-1* play indispensable roles in the H9C2 cell apoptosis induced by heavy metal-containing $PM_{2.5}$.

It has been well documented that an increase in intracellular ROS leads to inflammation and cell apoptosis, which play major roles in the progression of cardiovascular dysfunction (Busik et al., 2008; Oyinloye et al., 2015). Therefore, we hypothesized that ROS generation might be an important modulator for the myocardial toxicity promoted by the heavy metal-containing $PM_{2.5}$. To test this hypothesis, we first analyzed the intracellular ROS levels after exposure to the winter $PM_{2.5}$ at 0, 0.1, 0.3, 1, 3 or 10 μg/mL for 24 h. Figure 6A shows that a positive concentration-dependent increase in ROS production was observed, and a significant difference from the control group was observed at 3 μg/mL.

Low levels of these free radicals can be cleared by antioxidants. However, at high concentrations of ROS, the oxidant/antioxidant balance can be disturbed (Valko et al., 2007). The oxidative stress induced by excessive levels of ROS could cause further induction of inflammatory mediators, DNA damage and apoptosis. These effects may cause downstream molecular events that trigger vasculature permeabilization and, eventually, cardiovascular disorders (Yan et al., 2015; Tseng et al., 2016). There are some fragmentary studies that have also indicated that the association between PM-associated metals and biological processes is related to oxidative stress and other biological processes. Kodavanti et al. (2008) implied that PM-associated zinc is responsible for myocardial effects, including oxidative stress and altered cell signaling (Kodavanti et al., 2008). Liu C.M. et al. (2012) reported that the inflammation induced by the heavy metal Pb is partly due to oxidative stress (Liu C.M. et al., 2012). In the context of these reports, our results from the H9C2 cell toxicity experiments suggest that the ROS-mediated inflammatory and apoptotic responses were critical modulators of the increased health risk of exposure to heavy metal-containing $PM_{2.5}$. To provide stronger evidence for the involvement of ROS in the cell apoptosis induced by the heavy metal-containing $PM_{2.5}$, we evaluated the effects of pretreatment of the H9C2 cells with or without a ROS inhibitor (NAC) (Tseng et al., 2015) and then evaluated the H9C2 cell inflammation and apoptosis after a 24-h exposure to winter $PM_{2.5}$ (10 μg/mL). As expected, both the inflammation and apoptosis were significantly attenuated in the H9C2 cells that had been pretreated with NAC (Figure 6B).

Our results indicated that total concentrations of 8 heavy metals in $PM_{2.5}$ demonstrated a season-dependent pattern, and Zn and Pb, which are primarily derived from the anthropogenic

source, coal burning, were the dominant elements, present at the highest concentrations, in the samples collected in the spring and winter. Interestingly, Pb was responsible for greater potential health risks to children. Furthermore, based on myocardial toxicity, we showed that the myocardial cell apoptosis caused by the ROS-mediated inflammatory responses could contribute to the health outcomes. These findings suggested that heavy metal-containing $PM_{2.5}$ produced by coal burning plays an important role in myocardial toxicity and contributes to season-dependent health risks.

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Supporting information

Additional information on some supporting tables and texts can be found in the Supporting Information document.

Reference

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Figure legends

Fig. 1 $PM_{2.5}$ concentrations in different seasons ($\mu g/m^3$).

Fig. 2 Two-dimensional principal component loading plot obtained from the data for 8 heavy metals.

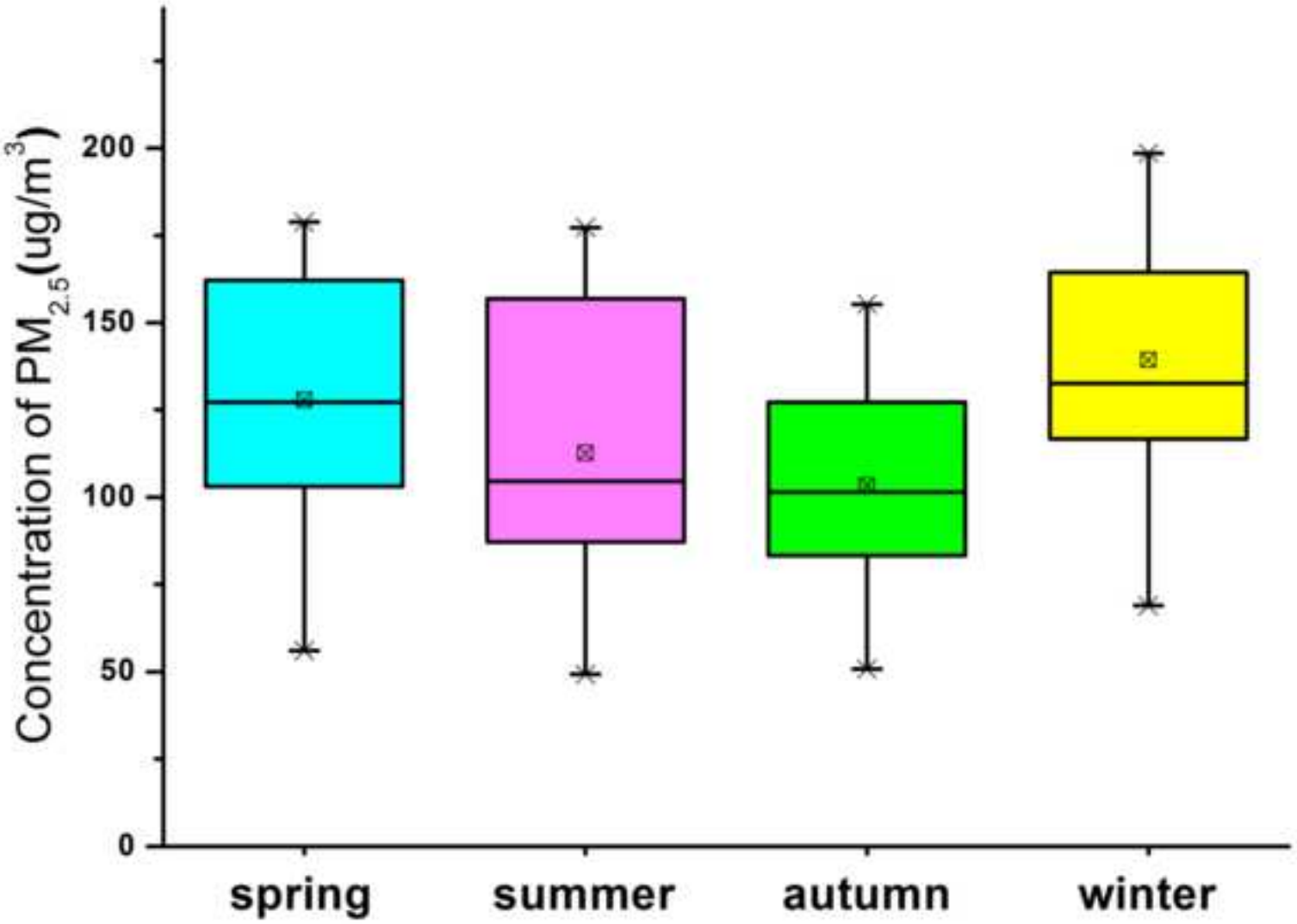
Fig. 3 Effects of the heavy metal-containing $PM_{2.5}$ from various seasons on H9C2 cell apoptosis. A. Representative TUNEL images of rat H9C2 cells after exposure to $PM_{2.5}$ from the various seasons. The H9C2 cells were treated with $PM_{2.5}$ samples (10 μg/mL) collected during spring, summer, autumn or winter, and the control group was treated with same amount of a DMEM vehicle (bar = 10 μm). B. Effects of $PM_{2.5}$ from different seasons on p53 and bcl-2/bax protein expression in rat H9C2 cells. H9C2 cells were treated with $PM_{2.5}$ samples (10 μg/mL) collected during spring, summer, autumn and winter, and the control group was treated with same amount of a DMEM vehicle. The resulting values for each treated group are expressed as the fold-increase compared to the mean values of the control groups, which was assigned an arbitrary value of 1. The data are expressed as the means ± SE (n = 3); $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus the vehicle control.

Fig. 4 Effects of $PM_{2.5}$ with different concentrations from spring (A, B) and winter (C, D) on mRNA expression of *p53* and *bcl-2/bax* in rat H9C2 cells. H9C2 cells were treated with $PM_{2.5}$ (0, 1, 3, and 10 μg/mL) samples from spring and winter, and the control group was treated with same amount of the DMEM vehicle. The resulting values for each treated group are expressed as the fold-increase compared to the mean value of the control group, which was assigned an arbitrary value of 1. The data are expressed as the means ± SE (n = 3); $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus the vehicle control.

Fig. 5 Effects of a range of concentrations $PM_{2.5}$ collected during the spring (A, B) or winter (C, D) on the expression of *ICAM-1* and *iNOS* mRNA in rat H9C2 cells. The H9C2 cells were treated with the $PM_{2.5}$ samples (0, 1, 3, and 10 μg/mL) from the spring or winter, and the control group was treated with the same amount of the DMEM vehicle. The resulting values for each treated group are expressed as the fold-increase compared to the mean value of the control group, which was assigned an arbitrary value of 1. The data are expressed as the means ± SE (n = 3); *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus the vehicle control.

Fig. 6 ROS-mediated H9C2 cell toxicity of the heavy metal-containing $PM_{2.5}$. A. Effects of a range of concentrations of winter $PM_{2.5}$ on the intracellular ROS production. The H9C2 cells were treated with $PM_{2.5}$ samples (0, 0.1, 0.3, 1, 3, and 10 μg/mL) collected during the winter, and the control group was treated with the same amount of the DMEM vehicle. The resulting values for each treated group are expressed as the fold-increase compared to the mean value of the control group, which was assigned an arbitrary value of 1. The data are expressed as the means ± SE (n = 3); *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus the vehicle control. B. Expression of bcl-2, bax, ICAM-1 and iNOS proteins, and the TUNEL assay after exposure to the winter $PM_{2.5}$ in the presence or absence of NAC. The H9C2 cells were treated with the $PM_{2.5}$ sample (10 μg/mL) collected during the winter, and the control group was treated with the same amount of the DMEM vehicle. Where indicated, NAC was added to the medium 1 h prior to the $PM_{2.5}$ treatment. The resulting values for each treated group are expressed as the fold-increase compared to the mean value of the control groups, which was assigned an arbitrary value of 1. The data are expressed as the means ± SE (n = 3); *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus the vehicle control (bar = 10 μm).

Figure 1


Concentration of $PM_{2.5}$ (ug/m^3)
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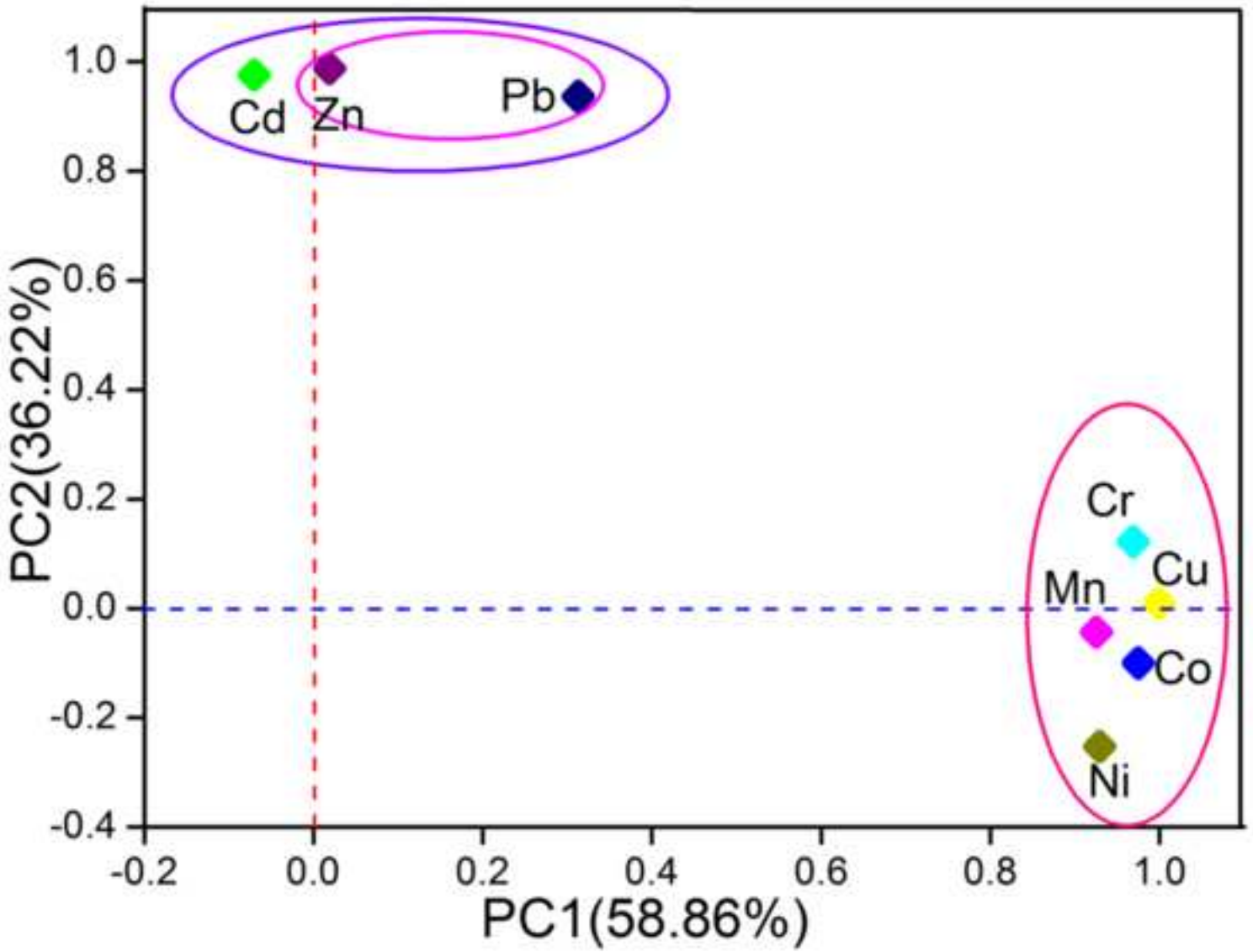
150
100
50
0
spring
summer
autumn
winter

Figure 2


Cd
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Cu
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0.8
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-0.4
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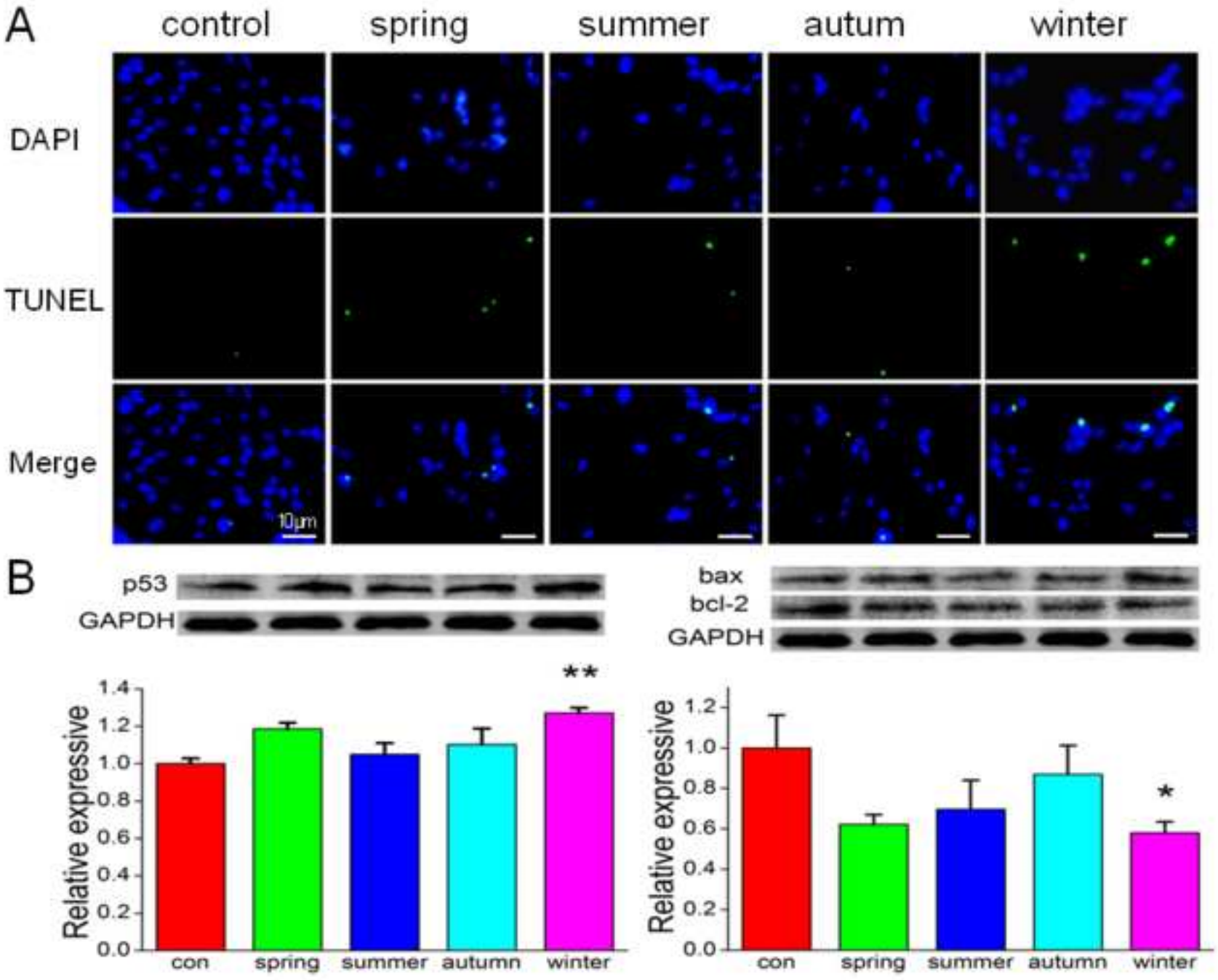
PC2(36.22%)
PC1(58.86%)

Figure 3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Relative expressive
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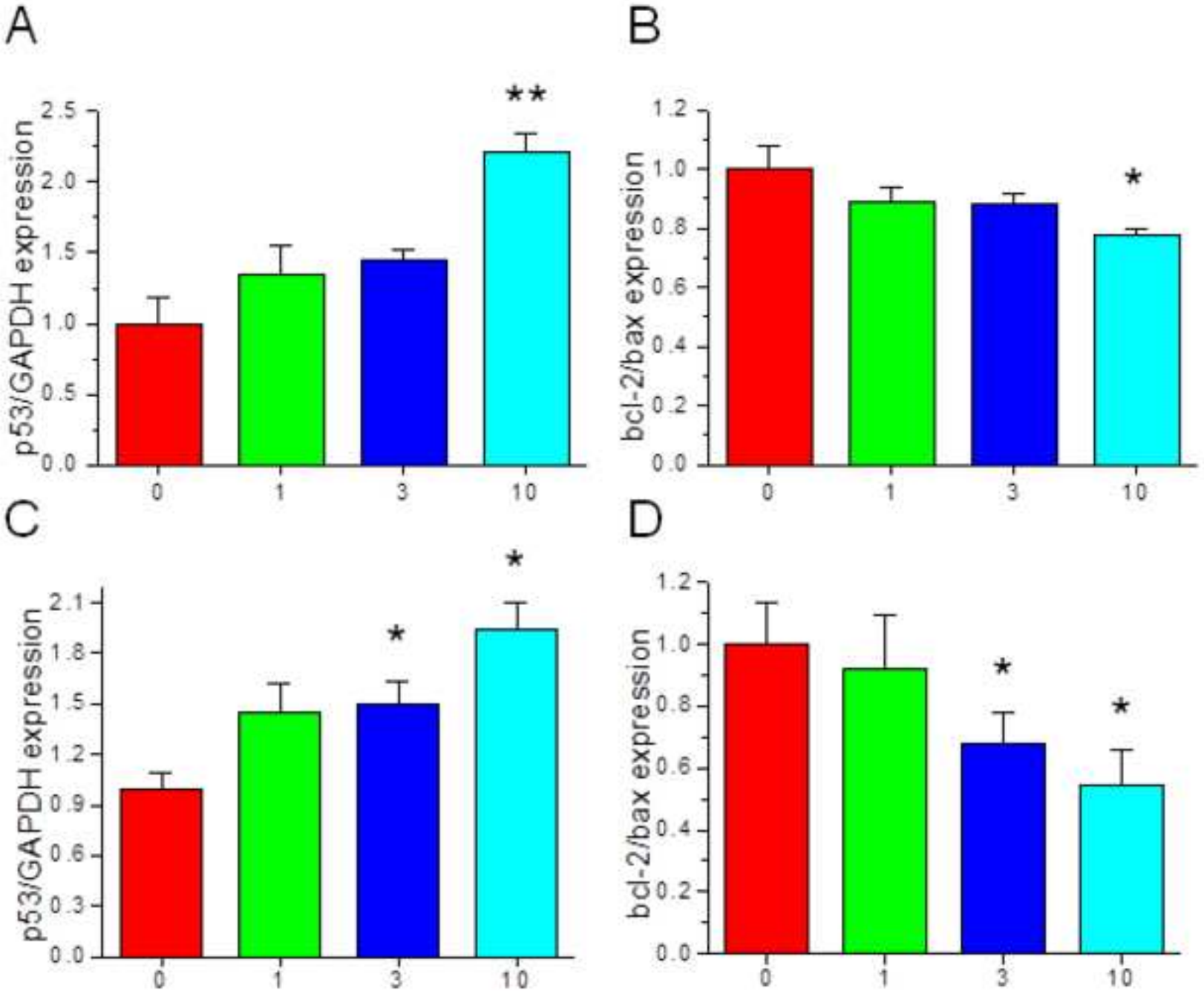
Figure 4


A
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2.5
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bcl-2/bax expression
1.2
1.0
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p53/GAPDH expression
2.1
1.8
1.5
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bcl-2/bax expression
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Figure 5

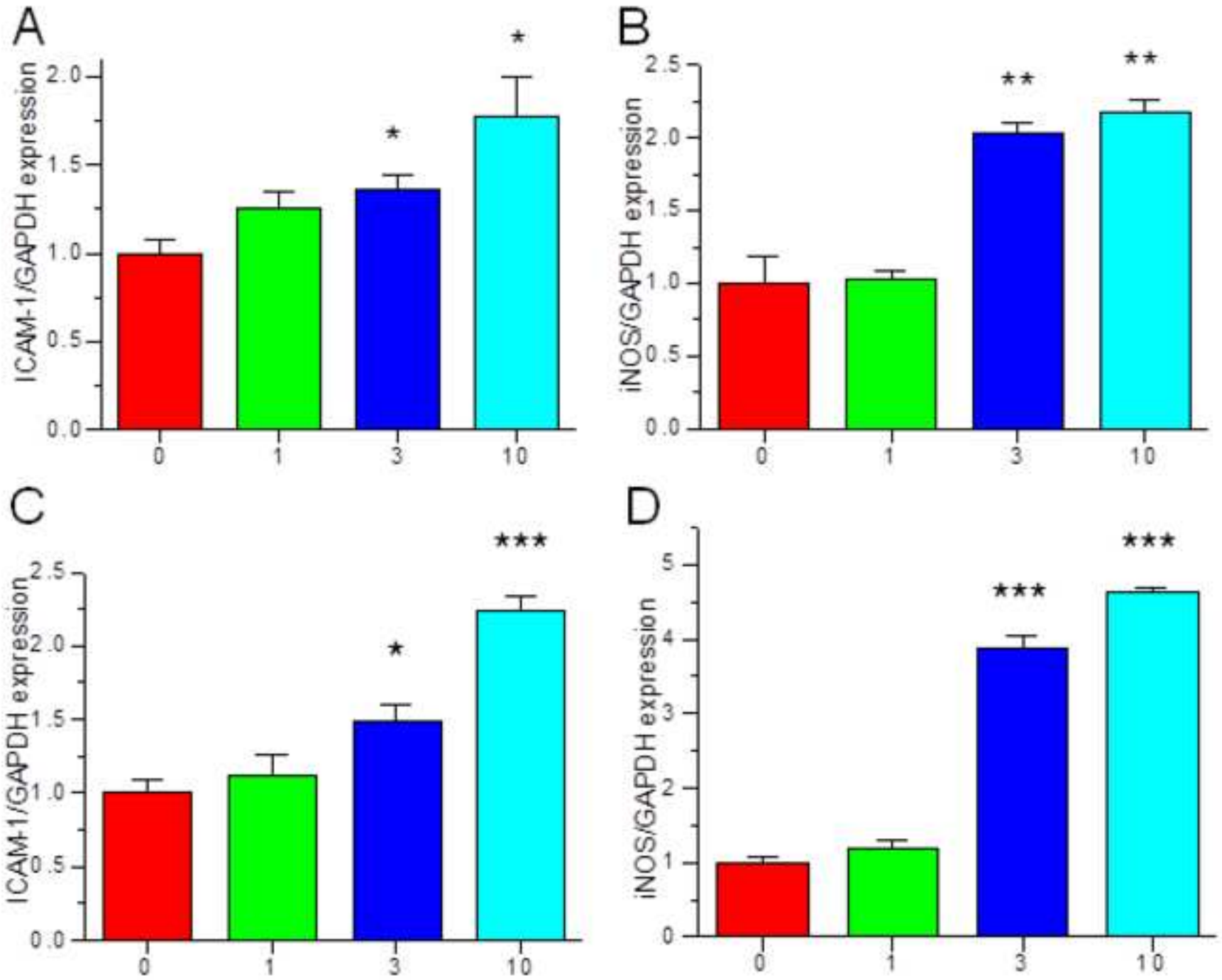
A
ICAM-1/GAPDH expression
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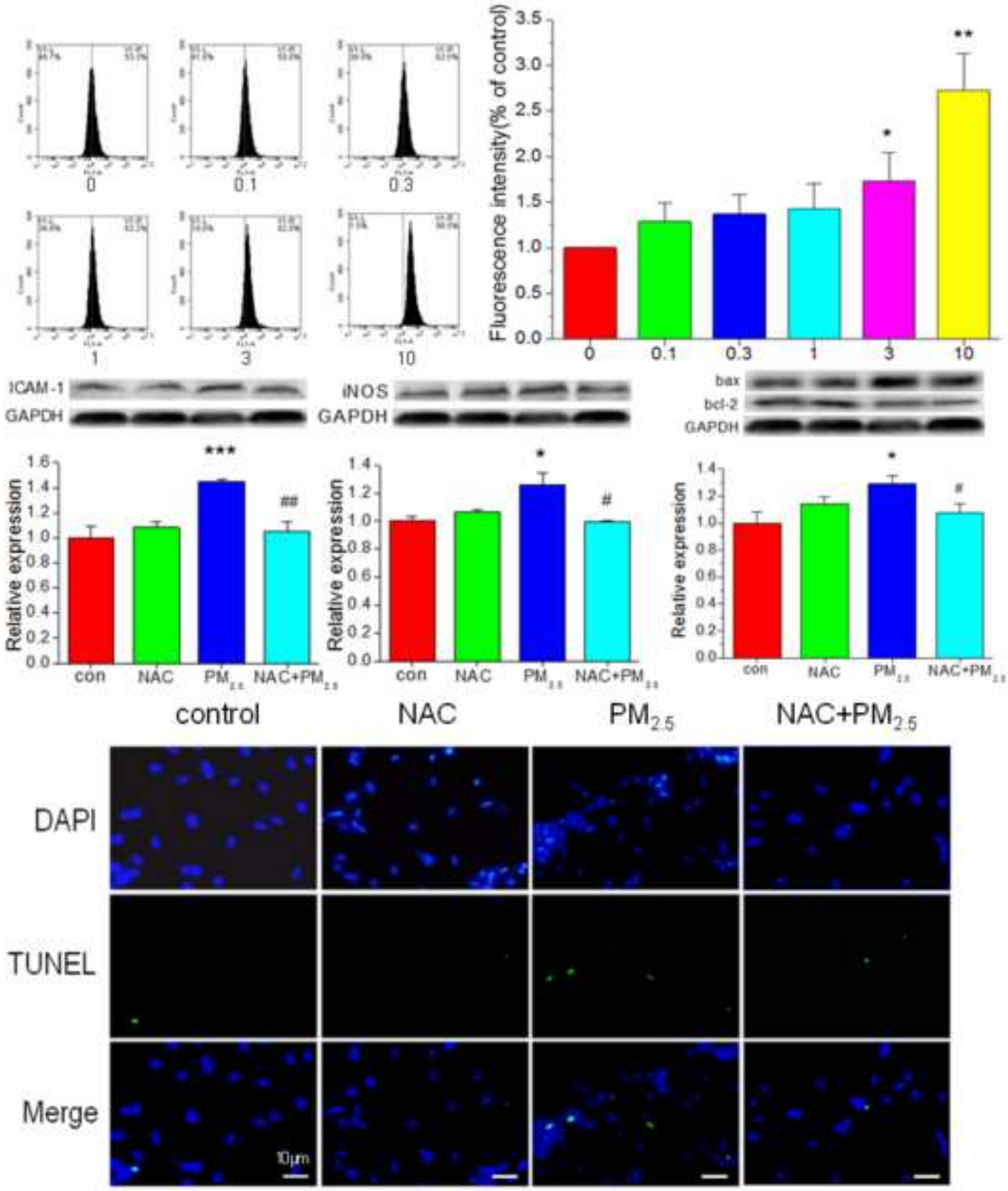
Figure 6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1
3
10
Fluorescence intensity(% of control)
ICAM-1
GAPDH
iNOS
GAPDH
bax
bcl-2
GAPDH
Relative expression
con
NAC
PM2.5
NAC+PM2.5
control
NAC
PM2.5
NAC+PM2.5
DAPI
TUNEL
Merge
10μm

Tables

Table 1. Concentrations of 8 heavy metals in $PM_{2.5}$ from different seasons (mg/kg)

Elments	spring	summer	autumn	winter
Cd	13.29	9.78	13.87	15.11
Co	25.32	14.95	11.79	10.27
Cr	449.46	325.52	367.52	311.24
Mn	1292.98	959.31	1109.51	834.28
Cu	167.76	134.58	134.53	135.98
Ni	129.77	80.52	45.77	37.77
Pb	943.22	640.69	793.30	950.33
Zn	2434.50	1340.04	2080.32	2946.18
Total	5456.30	3505.39	4556.60	5231.15

Table 2. EF value of each heavy metal

Elments	spring	summer	autumn	winter	mean
Cd	34.0	34.8	38.5	39.9	36.8
Co	1.0	0.8	0.5	0.4	0.7
Cr	3.7	3.4	3.3	2.7	3.3
Mn	1.2	1.1	1.1	0.8	1.0
Cu	3.5	3.6	3.1	2.8	3.2
Ni	2.6	2.0	1.0	0.8	1.6
Pb	21.4	18.5	19.5	22.3	20.4
Zn	16.4	11.5	15.3	20.6	16.0

Table 3. HQ, HI and HIt for heavy metals in $PM_{2.5}$ from study area

Heavy metal	RfD_{ingest}	RfD_{inh}	RfD_{dermal}	Season	HQ_{ingest}		HQ_{inh}		HQ_{dermal}	
					Children	Adult	Children	Adult	Children	Adult
Cr	5.0.E-03	2.9.E-05	2.5.E-04	spring	1.1E+00	1.5E-01	3.8E-03	4.2E-03	2.1E-01	1.5E-01
				summer	8.3E-01	1.1E-01	2.8E-03	3.0E-03	1.5E-01	1.1E-01
				autumn	9.4E-01	1.3E-01	3.1E-03	3.4E-03	1.7E-01	1.3E-01
				winter	8.0E-01	1.1E-01	2.6E-03	2.9E-03	1.4E-01	1.1E-01
Ni	2.0.E-02	2.1.E-02	1.0.E-03	spring	8.3E-02	1.1E-02	1.5E-06	1.7E-06	1.5E-02	1.1E-02
				summer	5.1E-02	6.9E-03	9.5E-07	1.0E-06	9.3E-03	6.9E-03
				autumn	2.9E-02	3.9E-03	5.4E-07	5.9E-07	5.3E-03	3.9E-03
				winter	2.4E-02	3.2E-03	4.4E-07	4.9E-07	4.3E-03	3.2E-03
Cu	3.7.E-02	4.0.E-02	1.9.E-03	spring	5.8E-02	7.8E-03	1.0E-06	1.1E-06	1.0E-02	7.6E-03
				summer	4.7E-02	6.2E-03	8.1E-07	8.9E-07	8.2E-03	6.1E-03
				autumn	4.6E-02	6.2E-03	8.1E-07	8.9E-07	8.1E-03	6.1E-03
				winter	4.4E-02	5.8E-03	7.6E-07	8.4E-07	7.6E-03	5.7E-03
Cd	1.0.E-03	1.0.E-03	5.0.E-05	spring	1.7E-01	2.3E-02	3.2E-06	3.5E-06	3.1E-02	2.3E-02
				summer	1.3E-01	1.7E-02	2.4E-06	2.6E-06	2.3E-02	1.7E-02
				autumn	1.8E-01	2.4E-02	3.4E-06	3.7E-06	3.2E-02	2.4E-02
				winter	1.9E-01	2.6E-02	3.7E-06	4.0E-06	3.5E-02	2.6E-02
Pb	3.5.E-03	3.5.E-03	5.3.E-04	spring	3.4E+00	4.6E-01	6.5E-05	7.1E-05	2.1E-01	1.5E-01
				summer	2.3E+00	3.1E-01	4.4E-05	4.9E-05	1.4E-01	1.0E-01
				autumn	2.9E+00	3.9E-01	5.5E-05	6.0E-05	1.7E-01	1.3E-01
				winter	3.5E+00	4.7E-01	6.5E-05	7.2E-05	2.1E-01	1.6E-01
Zn	3.0.E-01	3.0.E-01	6.0.E-02	spring	1.0E-01	1.4E-02	2.0E-06	2.2E-06	4.7E-03	3.5E-03
				summer	5.7E-02	7.7E-03	1.1E-06	1.2E-06	2.6E-03	1.9E-03
				autumn	8.9E-02	1.2E-02	1.7E-06	1.8E-06	4.0E-03	3.0E-03
				winter	1.3E-01	1.7E-02	2.4E-06	2.6E-06	5.7E-03	4.2E-03
Mn	4.7.E-02	1.4.E-05	2.4.E-03	spring	3.5E-01	4.7E-02	2.2E-02	2.4E-02	6.2E-02	4.6E-02
				summer	2.6E-01	3.5E-02	1.6E-02	1.8E-02	4.6E-02	3.4E-02
				autumn	3.0E-01	4.0E-02	1.9E-02	2.1E-02	5.3E-02	4.0E-02
				winter	2.3E-01	3.0E-02	1.4E-02	1.6E-02	4.0E-02	3.0E-02
Co	2.0.E-02	5.7.E-06	1.6.E-02	spring	1.6E-02	2.2E-03	1.1E-03	1.2E-03	1.8E-04	1.4E-04
				summer	9.6E-03	1.3E-03	6.3E-04	7.0E-04	1.1E-04	8.0E-05
				autumn	7.5E-03	1.0E-03	5.0E-04	5.5E-04	8.5E-05	6.3E-05
				winter	6.6E-03	8.8E-04	4.4E-04	4.8E-04	7.4E-05	5.5E-05
Parameter				**Season**	**Children**	**Adult**	**Children**	**Adult**	**Children**	**Adult**
HI (ΣHQ)[(a)]				spring	5.4E+00	7.8E-01	2.7E-02	3.0E-02	5.4E-0	4.0E-01
				summer	3.7E+00	5.4E-01	2.0E-02	2.2E-02	3.8E-01	2.8E-01
				autumn	4.5E+00	6.6E-01	2.2E-02	2.5E-02	4.6E-01	3.3E-01
				winter	4.9E+00	7.1E-01	1.6E-02	1.7E-02	4.4E-01	3.3E-01
HIt (ΣHI)[(b)]		Children		Spring 5.9		summer 4.1		autumn 5.0		winter 5.3
		Adult		Spring 1.2		summer 0.8		autumn 1.0		winter 1.0

Graphical Abstract