

## Research Paper

# PM<sub>2.5</sub> Exposure Induces More Serious Apoptosis of Cardiomyocytes Mediated by Caspase3 through JNK/P53 Pathway in Hyperlipidemic Rats

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## Abstract

Exposure to airborne particulate matter with an aerodynamic diameter less than or equivalent to 2.5 microns (PM<sub>2.5</sub>) easily induces acute myocardial infarction in populations with high-risk cardiovascular diseases such as hyperlipidemia, but its mechanism remains unclear. In this study, hyperlipidemic rats were used to examine the effects of PM<sub>2.5</sub> exposure on the cardiovascular system and the mechanism for its induction of cardiovascular events. We found that PM<sub>2.5</sub> exposure resulted in bigger changes in the myocardial enzyme profile (cTnl, LDH, CK, CK-MB) in hyperlipidemic rats than that of control rats, as well as a significant increase in the C-reactive protein (CRP) level and a decrease in the superoxide dismutase (SOD) activity. It promoted a hypercoagulable state, significantly increased blood pressure and heart rate, while decreased the variability of heart rate in hyperlipidemic rats. In addition, pathological test showed that PM<sub>2.5</sub> exposure more easily deteriorated myocardial injury in hyperlipidemic rats. It upregulated the phosphorylation levels of myocardial c-Jun NH2-terminal kinase (JNK) and P53, resulting in the elevated expression of downstream effector protein Bax and the decreased expression of Bcl-2, and then increased caspase3 level leading to cardiomyocyte apoptosis, while little change of caspase2 was observed. Taken together, PM<sub>2.5</sub> exposure induced more serious inflammation and oxidative stress in the circulation system of hyperlipidemic rats, promoted a hypercoagulable state and triggered cardiomyocyte apoptosis, in which JNK/P53 pathway played a key role.

Key words: PM<sub>2.5</sub> exposure, hyperlipidemia, cardiacmyocyte apoptosis, JNK/P53 pathway, caspase3

## Introduction

Particulate matter with an aerodynamic diameter less than or equivalent to 2.5 microns (PM<sub>2.5</sub>) is a major air pollutant that causes approximately seven million premature deaths worldwide [1]. The surface of PM<sub>2.5</sub> absorbs a large quantity of toxic and hazardous substances (including organic, inorganic and biological components), which can not only remain in the air for a long period of time, subsequently enter and accumulate in the alveoli, but eventually enter the blood circulation system through respiration, thus affecting the health of organism [2-5].

PM<sub>2.5</sub> exposure has a greater impact on the susceptible population of cardiovascular diseases than the healthy population, such as people with high cholesterol or diabetes [6-7]. PM<sub>2.5</sub> is associated with cardiovascular diseases (CVD), and the CVD morbidity is also relative with PM<sub>2.5</sub> exposure concentration. A study found that there was strong association between particulate and congestive heart failure, a 0.72% increase in risk per 10 µg/m<sup>3</sup> elevation [8]. Brook reported that congestive heart failure increased 0.18% per 10 µg/m<sup>3</sup> in the general population [9]. Annual

average exposure to higher levels of PM (per 0.26  $\mu\text{g}/\text{m}^3$  elevation), was closely correlated with a 1.1% increase in carotid intima media thickness (CIMT) in a cohort of elderly men [10]. It induces cardiovascular events by promoting inflammation and oxidative stress, leading to a hypercoagulable state, causing autonomic nervous tension and vasomotor changes, thus resulting in the lack of blood or oxygen supply to the cardiovascular system, ultimately leading to acute cardiovascular events such as myocardial injury or infarction [11-13]. Clinical diagnostic criteria of acute cardiovascular events include cardiac troponin (cTnI), creatine kinase (CK), creatine kinase-MB isoenzyme (CK-MB) and lactate dehydrogenase (LDH) levels, which are usually combined with blood pressure and electrocardiogram (ECG) for diagnosis [14-16]. Among them, cTnI is currently considered the most cardiac-specific and valuable indicator of myocardial injury [17]. In addition, several inflammatory markers also have important reference values during cardiovascular events. For example, C-reactive protein (CRP), a typical marker reflecting the body's inflammatory state, has an important reference value in evaluating acute myocardial infarction and cerebral infarction [18-20]. PM<sub>2.5</sub> exposure can induce oxidative stress, in which superoxide dismutase (SOD) is an important factor for removing oxidative damage and for cell protection. Moreover, changes in SOD activity can reflect the body's oxidative stress status [21]. In addition, PM<sub>2.5</sub> exposure can also affect the coagulation and fibrinolytic system, resulting in a hypercoagulable state, represented by elevating fibrinogen (FIB), reduced prothrombin time (PT) and activated partial thromboplastin time (APTT), as well as increased blood viscosity, which causes symptoms of ischemia or hypoxia [22-23]. Taken together, PM<sub>2.5</sub> exposure can induce the body's acute inflammatory and oxidative stress reaction, as well as change the coagulation status, leading to cardiac ischemia, which causes disordered autonomous heart rhythm and myocardial apoptosis, eventually leading to myocardial injury and necrosis. Studies have shown that the C-Jun NH2-terminal protein kinase (JNK) or tumor suppressor factor P53 (P53) is the main pathway regulating the progression of cardiovascular diseases [24-25]. P53 induces cell apoptosis and plays a central role in regulating the apoptotic signaling pathway, which is important in regulating cardiomyocyte apoptosis in myocardial ischemia-reperfusion injury, myocardial infarction and hypertension [26-28]. Its downstream genes, such as Bcl-2 and Bax, are important effector molecules regulating apoptosis. In this study, by comparing the differences between hyperlipidemic rats and normal rats after exposure to PM<sub>2.5</sub> collected in Beijing during

the winter (October to December) of 2012, the toxic effects of PM<sub>2.5</sub> on the cardiovascular system of rats with different pathological conditions, as well as its impact on inflammation and oxidative stress were analyzed. Our research would clarify the mechanism of PM<sub>2.5</sub> exposure in inducing acute cardiovascular events in population with high-risk cardiovascular diseases.

## Materials and Methods

### PM<sub>2.5</sub> collection and preparation

The ambient PM<sub>2.5</sub> was sampled at flow rate of 77.59 L/min on a glass fiber filter (Whatman® 41 filters, Whatman Inc, Maidstone, UK) by a middle flowmeter (model:TSP/PM10/PM<sub>2.5</sub>-2, Beijing Geological Instrument-Dickel Co, Ltd) at the altitude of 40 meters between 2nd and 3rd ring road of Beijing city from October to December in the year 2012 (winter). The filter was immersed in deionized water after sampling, from which the sample was eluted out by ultrasonic cleaner (TP250B, Tianpeng Electronics Co. Ltd) and lyophilized, then stored at -20°C. A certain quantity of PM<sub>2.5</sub> was weighed and suspended in 0.9% normal saline, homogenized for 15 min by ultrasonicator, then stored at 4°C. The method for sampling PM<sub>2.5</sub> is according to literature [29].

### Chemical analysis of PM<sub>2.5</sub>

The inorganic components were detected by plasma emission spectroscopy (ICP-AES, Model: ULTIMA, JOBIN-YVON Company, France), and polycyclic aromatic hydrocarbon (PAHs) were detected by HPLC (Water® 2690 Separations Module, Water® 474 Scanning Fluorescence Detector and Symmetry® C1839 mm×150 mm, 65  $\mu\text{m}$  column) using US EPA method (US EPA, 1998).

### Animal grouping and exposure to PM<sub>2.5</sub>

All procedures used in this research were approved by the Institutional Animal Care and Use Committee of Institute of Military Cognitive and Brain Sciences. 70 Wistar male 8-weeks-old rats were purchased from the Animal Experiment Center of AMMS, and the code for the animals was SCXK(Jing)2012-0001, the inclusion criteria were as follows: 1. Body weight: 180-200g. 2. No abnormal appearance by a comprehensive observation. The selected rats were kept at constant temperature (20±2°C) and humidity (50%±5%), free drinking water, artificial lighting for 12 h a day, and adaptive feeding for one week, from which 35 rats were taken randomly and fed on high fat diet<sup>[30]</sup> (20% lard, 5% sugar, 3% cholesterol 0.5% sodium cholate, 0.2% propylthiouracil, 1% methionine, 1% vitamin D, 2% egg yolk powder, 68% the basic forage) for 24 weeks, 5

of which were utilized to evaluate the modeling of hyperlipemia. The other 35 rats were fed on the basic diet, 5 of which were used as the control. The remaining 30 with hyperlipemia and 30 normal rats were divided each into 3 groups (10 rats/group), labeled as the normal saline, low dose, and high dose group, and the biochemical indexes were determined before feeding on high fat diet and instilling the PM<sub>2.5</sub>. After setting up the model, the toxicant exposure to PM<sub>2.5</sub> was performed by endotracheal instillation once per two days for continuous 3 times. The dose of endotracheal instilling PM<sub>2.5</sub> suspension in the rat was 0, 4, 40 mg/kg respectively. The 10% chloral hydrate (ShangHai Hushi Laboratorial Equipment company) was used for anesthesia by intraperitoneal injection according to 0.27ml/100g weight at 24 h after the final exposure, SMUP/E biological signal processing system to monitor the rat's blood pressure and electrocardiogram, the blood samples were collected via abdominal aorta by the 2 ml specific anticoagulant vial (Institute of Medical Instruments, Beijing) and centrifuged at 4°C, 3000 r/min for 10 min, then the plasma was stored at -20°C.

### Biochemical assay of blood

The serum hs-CRP was measured with a commercial kit (Rat CRP ELISA Kit, Invitrogen, CA, USA), serum cTn-I, LDH, CK and CK-MB were determined by mini vidas-IDAS full automatic fluorescence ELISA instrument (BioMerieux Company, France), All the assays were completed by the same physician strictly following the instruction manual. HITACHI-7060 Automatic Biochemical Analyzer (JAPAN) was used to detect the serum levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) using the reagents supplied by RANDOX LABORATORIES LTD, UK. FIB, APTT and PT were assayed by Sysmex CA-530 coagulation analyzer (JAPAN), the reagents were supplied by Dade-Behring (GERMAN) and the serum SOD was determined according to the instruction manual provided by Nanjing Jiancheng Biotechnologies Co. Ltd. All the assays were repeated twice.

### HE and TUNEL staining

The rat's left ventricle was collected and cut into the myocardial tissue block of 0.5 cm<sup>3</sup>. The tissue block was fixed by formaldehyde, dehydrated, embedded routinely with paraffin, cut into 5µm thick sections and stained with HE (hematoxylin-eosin) for light microscopy. Some sections were used for apoptotic assessment with a terminal deoxynucleotidyl transferase (TDT)-mediated dUTP nick-end

labeling (TUNEL) assay. The morphological changes of myocardial tissue should be observed by optical microscopy, and the diagnosis of pathological changes should be confirmed by two pathologists.

### Western blotting

The heart tissue should be homogenized with RIPA lysis buffer (Applygen Technologies Inc, China) in ice-bath, from which the total protein should be extracted and quantified by the kit for detection of protein content with Lowry method (KeyGEN Biotechnology Co, China). The total protein was separated by electrophoresis on 12% sodium dodecyl sulfate sodium salt polyacrylamide gelelectrophoresis (SDS-PAGE), then transferred onto polyvinylidene fluoride (PVDF) membrane of 0.2 µm aperture (Immobilon™ Millipore, USA). The membranes were incubated with anti-Bax, anti-Bcl-2, anti-phospho-p53, anti-phospho-JNK (all at 1:1000), (Cell Signaling Technology, Inc, USA) antibodies overnight at 4°C and the corresponding secondary antibodies for 1 h at room temperature. Finally, the membranes should be treated with SuperSignal West Femto Substrate (Thermo Scientific, USA) for developing in dark room, and the result was analyzed using the housekeeping gene GAPDH as the internal reference (Cwbiotech, Inc, China).

### Statistical analysis

Statistical analysis of all data was performed by using SPSS 13.0. Differences between means were assessed by using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. The symbol\* and \*\* were used to show the statistically significant difference ( $P < 0.05$ ) and very significant difference ( $P < 0.01$ ). All the data were shown as mean±SD and the obeyed data to be checked by t-test.

## Results

### Assessment of the model of rats with hyperlipidemia and analysis of myocardial injury

Before being fed a high fat diet, the total cholesterol (TC), triglyceride (TG), high density lipoprotein(HDL), low density lipoprotein(LDL) and cTnI levels in rat serum did not show a significant difference between the two groups ( $P > 0.05$ ; Figure 1A-1E). After being fed different diets for 24 weeks, the levels of serum TC, LDL, TG and cTnI in the high fat diet group significantly increased ( $P < 0.05$ ; Figure 1A, 1B, 1D, 1E). However, the HDL level was not significantly changed ( $P > 0.05$ ; Figure 1C). Meanwhile, after 24 weeks of high fat diet, the number of myocardial nuclei in these rats was higher than that in

the normal diet group ( $P < 0.05$ ; Figure 1F). The results showed that rats fed with a high fat diet for 24 weeks presented symptoms of hyperlipidemia, with mild myocardial injury, which made them a susceptible animal model for the evaluation of toxic effects of  $PM_{2.5}$  exposure on the cardiovascular system.

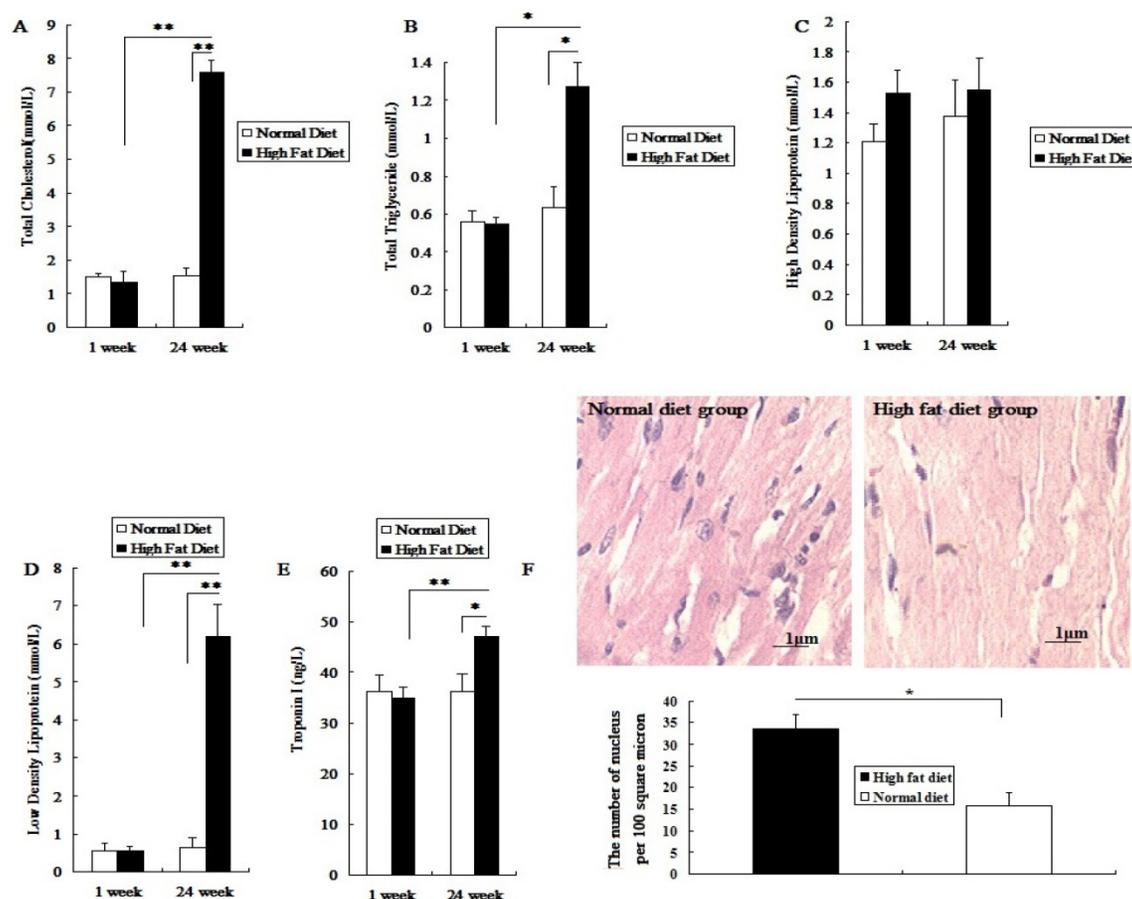
### Effects of $PM_{2.5}$ exposure on the coagulation system

With increasing exposure doses of  $PM_{2.5}$ , the coagulation status index which included FIB, PT and APTT in hyperlipidemic rats were changed obviously, while the changes of coagulation indexes could be observed only at dose of 40mg/kg weight body in normal rats ( $P < 0.05$ ; Figure 2A, 2B, 2C). Besides that, there were significant differences in coagulation status index between two different concentration of  $PM_{2.5}$  in hyperlipidemic rats ( $P < 0.05$ ; Figure 2A, 2B, 2C). In both groups, pairwise comparison between treatments of same dose also showed significant difference ( $P < 0.05$ ; Figure 2A, 2B, 2C). No vascular pathological abnormalities were found in rats fed with normal diet at different  $PM_{2.5}$  exposure concentrations (Figure 2D1, Figure 2D2, Figure 2D3),

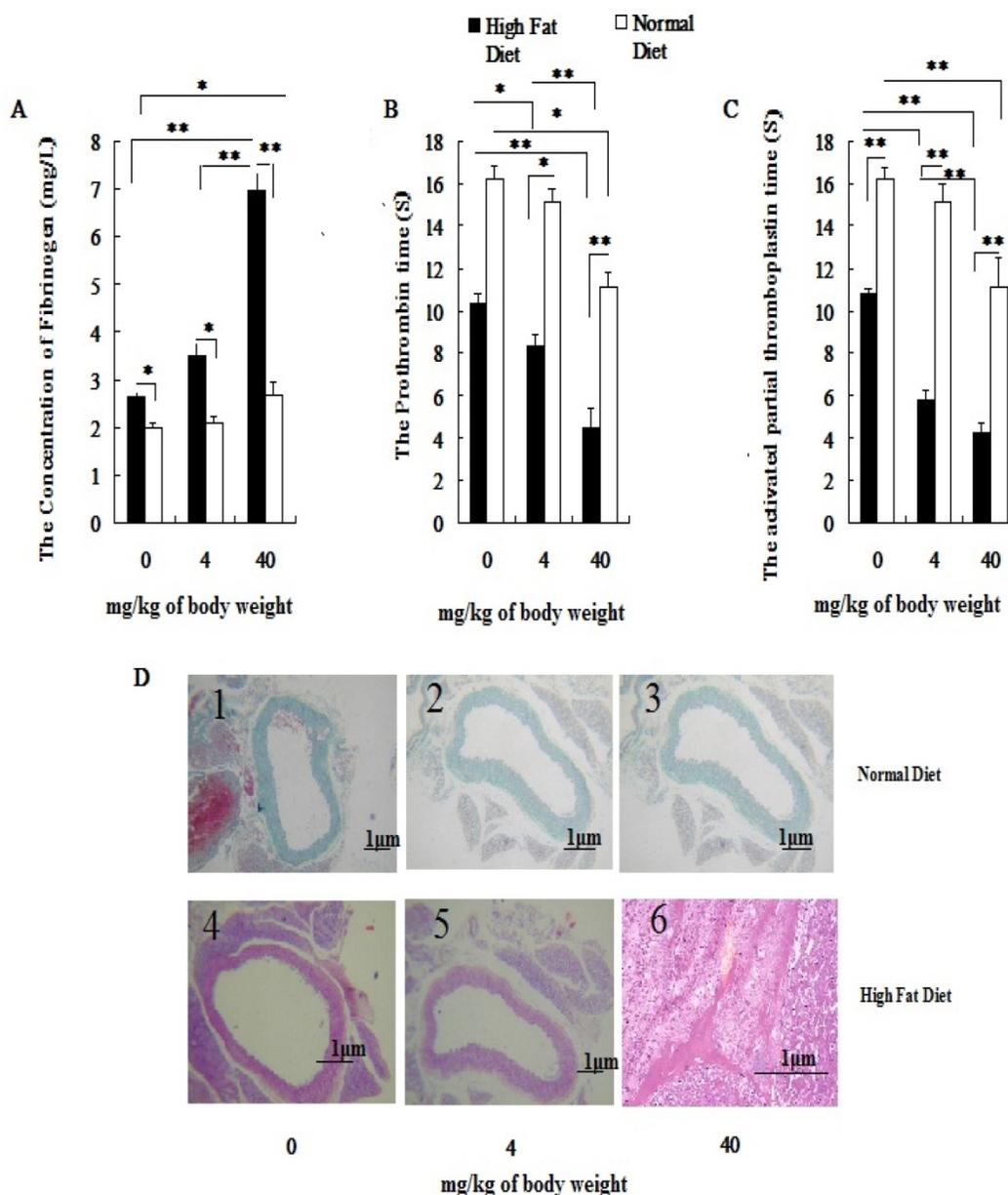
while thrombosis was found in rats fed with high-fat diet when they were exposed to  $PM_{2.5}$  at dose of 40 mg/kg body weight (Figure 2D6).

### Effects of $PM_{2.5}$ exposure on the blood pressure and heart rate of hyperlipidemic rats

Both the blood pressure (BP) and the heart rate (HR) of hyperlipidemic rats were significantly higher than those of the normal rats before  $PM_{2.5}$  exposure (Figure 3A, 3B). After  $PM_{2.5}$  exposure, with an increase of its concentration, the blood pressure, heart rate of hyperlipidemic rats significantly increased and standard deviation of NN intervals (SDNN) decreased greatly, and all indexes were significantly different from the control rats ( $P < 0.01$ ). Pairwise comparison between the two different doses in hyperlipidemic rats also showed significant differences ( $P < 0.01$ ). Changes in BP, HR and SDNN only happened at dose of 40 mg/kg body weight in normal rats. In hyperlipidemic rats, blood pressure and heart rate showed a more obvious raise with the increasing doses of  $PM_{2.5}$ , while the heart rate variability index, represented by the SDNN showed a significant reduction (Figure 3C).



**Figure 1. Establishment and assessment of hyperlipidemic rat model.** The serum was collected after high-fat diet for 24 weeks. A, the value of TC in serum; B, the value of TG in serum; C, the value of HDL-C in serum; D, the value of LDL-C in serum; E, the value of cTn-I in serum; F, The HE staining of myocardial tissue. The data are expressed as mean  $\pm$  SD ( $n=7$ ), \*  $P < 0.05$  and \*\*  $P < 0.01$  vs the normal diet group. The pathological tissue block was sampled from the rat's left ventricle, and the sampling site for each rat should be same.



**Figure 2. Influence of exposure to PM<sub>2.5</sub> on the rat's blood coagulation system under the different physiological status.** The plasma was collected from the rat fed on high fat diet at the time point of 24 h after the last dripping of PM<sub>2.5</sub>. A, the value of FIB in plasma; B, the value of PT in plasma; C, the value of APTT in plasma; D, HE staining of rat blood vessels. The data are shown as mean  $\pm$  SD (n=7), \*  $P < 0.05$  indicating statistically significant difference; \*\*  $P < 0.01$  indicating very significant difference. The blood vessels were taken from the aortic arch.

### Effects of PM<sub>2.5</sub> exposure on myocardial enzymes, oxidative stress, inflammation

Before PM<sub>2.5</sub> exposure, hs-CRP, CK, CK-MB in normal rats were significantly lower than those of hyperlipidemic rats. After the PM<sub>2.5</sub> exposure, the hs-CRP, CK, CK-MB, LDH and cTnI levels significantly increased in hyperlipidemic rats at different doses of PM<sub>2.5</sub>, while, all indexes were changed greatly only at dose of 40 mg/kg weight body ( $P < 0.01$ ) in normal rats. In addition, under the same exposure concentration, the serum hs-CRP, cTnI, CK, CK-MB and LDH levels of hyperlipidemic

rats were significantly higher than those of the normal rats ( $P < 0.01$ ; Figures 4A-4D, 4F), whereas the SOD level was significantly lowered ( $P < 0.01$ ; Figure 4E).

### Cardiomyocyte apoptosis caused by PM<sub>2.5</sub> exposure

Clinical evidences have proved that PM<sub>2.5</sub> exposure can initiate susceptible population myocardial infarction. In our study, we found that instillation with saline and low concentrations of PM<sub>2.5</sub> did not significantly affect the structure of myocardial tissues in the normal rats, and their myocardial fibers were well organized with no

pathological changes, such as myocardial fibrosis or necrosis. Only treated with PM<sub>2.5</sub> at 40 mg/kg weight body, mild injury was observed in cardiomyocytes (Figure 5A). In hyperlipidemic rats instilled with saline, the myocardial tissue showed normality (Figure 5A). However, when instilled with low concentrations of PM<sub>2.5</sub>, they showed some pathological changes in the myocardial tissue (Figure 5A), while with high concentrations of PM<sub>2.5</sub>, extensive myocardial fibrosis, necrosis, telangiectasia and myocardial interstitial edema were observed (Figure 5A). TUNEL analysis indicated that with the increase of PM<sub>2.5</sub> concentration, the apoptotic cardiomyocytes increased correspondingly. Moreover, the number of TUNEL-positive cells of hyperlipidemic group were higher than that of the normal group (Figure 5B).

### PM<sub>2.5</sub> exposure activated the JNK/P53 pathway

We have found that PM<sub>2.5</sub> exposure could induce cardiomyocyte apoptosis, but the mechanism is unclear. Our further study found that the phosphorylation levels of JNK and P53 in the myocardial tissue increased significantly in hyperlipidemic rats or in normal rats after PM<sub>2.5</sub> exposure ( $P < 0.01$ ; Figure 6 AB), with a corresponding increase in the pro-apoptotic Bax levels. At the same exposure concentration, the expression levels of p-JNK, p-p53 and Bax in the cardiomyocytes of hyperlipidemic rats were far higher than those of the control rats ( $P < 0.01$ ). However, the Bcl-2 expression level showed the opposite trend (Figure 6 AB). To understand how PM<sub>2.5</sub> triggered cardiomyocyte apoptosis via JNK or P53, we further detected the change of its downstream effective protein to evaluate which pathways are important. The result showed that both JNK and P53 expression were increased with the concentration of PM<sub>2.5</sub> exposure, and also found that the expression of

caspase2 mediated by P53 was not significantly changed while the expression of caspase3 regulated by JNK were increased with the concentration of PM<sub>2.5</sub> exposure (Figure 6 F).

### Characteristics of PM<sub>2.5</sub> components and mass concentration in winter from Beijing urban area

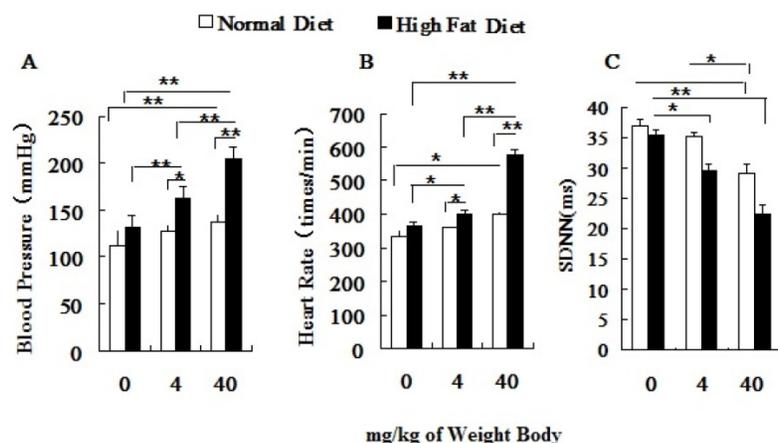
Table 1 showed the concentration range of PM<sub>2.5</sub>, its adsorbed metal elements and polycyclic aromatic hydrocarbons (PAHs) from Oct. to Dec. 2012 in Beijing.

**Table 1.** Air quality monitoring parameters

Component	Low concentration ( $\mu\text{g}/\text{m}^3$ )	High concentration ( $\mu\text{g}/\text{m}^3$ )
particle	81.800	173.800
Al	2.050	2.160
Ca	7.800	13.600
Fe	1.510	1.820
Mg	4.800	5.600
Na	3.800	4.400
As	0.009	0.0230
Zn	0.270	3.130
Cu	0.0460	0.160
Cd	0.003	0.0230
Pb	0.093	0.350
PAH(4)	0.072	0.2010
PAH(5,6)	0.082	0.2920

## Discussion

PM<sub>2.5</sub> exposure causes great harm to human health, resulting in various diseases such as asthma, bronchitis and cardiovascular diseases [4,6]. Epidemiological survey and toxicological experiments show that prolonged exposure to PM<sub>2.5</sub> may induce oxidative stress and inflammatory response in lung and whole body, increasing the risk of death caused by atherosclerosis (AS) and ischemic heart disease [12-13]. Short term exposure to the high concentrations of PM<sub>2.5</sub> may trigger cardiovascular complications, such as vulnerable plaque rupture, thrombosis and acute ischemic heart and cerebrovascular events [31]. Epidemiological data also suggest that people with obesity and hyperlipidemia, children, elderly, people with heart and lung diseases, as well as immunocompromised patients are more susceptible to PM<sub>2.5</sub> exposure than healthy people [32-33]. Hyperlipidemia, a type of metabolic disorder, has had a high prevalence rate in recent years, as well as being a high risk factor for other cardiovascular diseases. In Jilin Province, northeast China, the prevalence of hyperlipidemia among participants

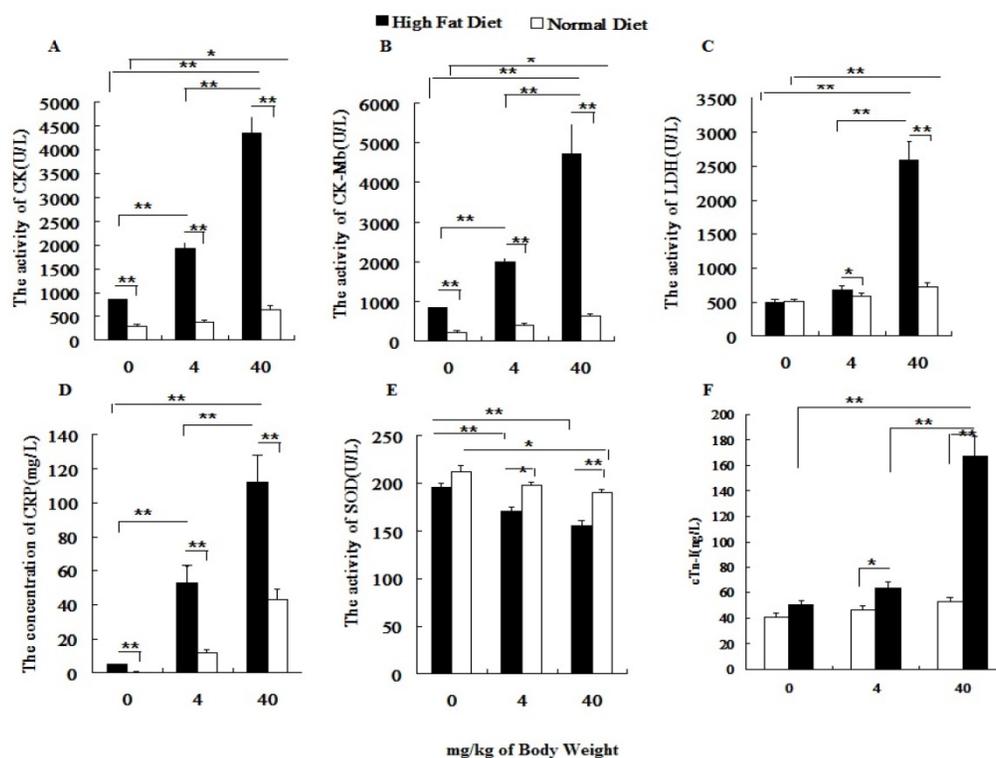


**Figure 3.** Influence of PM<sub>2.5</sub> on the variation of blood pressure and heart rate. ECG was measured at 24h after the last dripping. A, blood pressure; B, heart rate; C, SDNN; The data are presented as mean  $\pm$  SD (n=7); \*  $P < 0.05$  indicating statistically significant difference; \*\*  $P < 0.01$  indicating very significant difference.

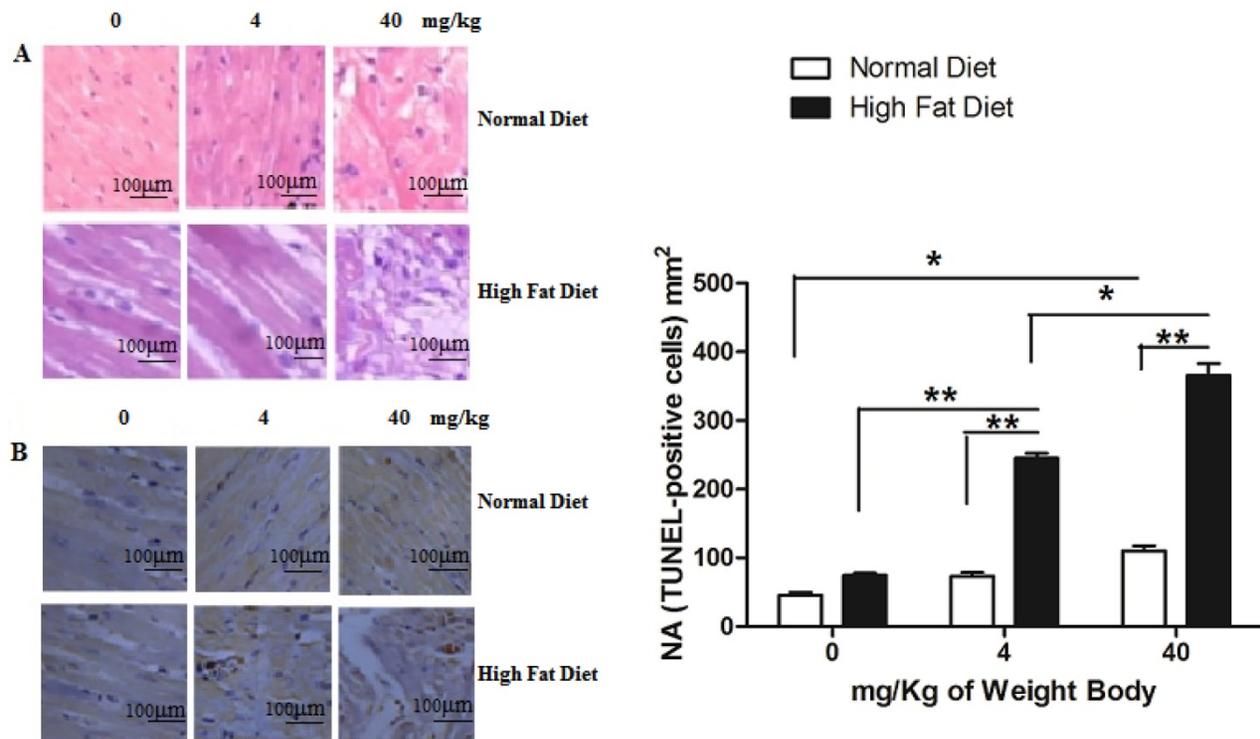
reached 51.09% [34], and the prevalence of hyperlipidemia among subjects from the southwestern China was 49.3% [35], and these people were easy suffered from CAD when PM<sub>2.5</sub> pollution happened. Rats induced with high fat diet present typical symptoms, making them suitable for studying the toxic effects of PM<sub>2.5</sub> exposure in animals that are susceptible to cardiovascular diseases. And the cardiovascular events resulting from PM<sub>2.5</sub> exposure can be diagnosed by ECG and related physiological and biochemical indexes [36]. Besides that, studies found that SD rats inhaled concentrated ambient particles, their myocardial LDH level increased by 5%, while their serum LDH level increased by 80%, and CK, CK-MB and CTnI levels also showed different degrees of elevation [37-38]. In this study, we employed these indexes to detect the effect of PM<sub>2.5</sub> exposure on hyperlipidemic rats. In addition, the damage to human health due to PM<sub>2.5</sub> is associated not only with its own characteristics and pollution levels, but also with the tolerance of the exposed population [39-41]. Beijing is typical PM<sub>2.5</sub> pollution region for susceptible population, so we utilized PM<sub>2.5</sub> collected from Beijing to study its impact on hyperlipidemic rats.

This study showed that exposure to high concentrations of PM<sub>2.5</sub> resulted in significant change in ECG and blood pressure in hyperlipidemic rats. Their heart rates and blood pressures increased with the increase of PM<sub>2.5</sub> concentration, indicating a dose

effect relationship. Simultaneously, their blood biochemical indexes, such as myocardial enzymes, acute-phase proteins and coagulation parameters also changed obviously. We also verified that after PM<sub>2.5</sub> exposure, the CK, CK-MB, LDH, cTnI, CRP, SOD, PT, APTT and FIB levels were significantly different from the corresponding control groups in both groups of rats under different pathological states. In particular, hyperlipidemic rats showed more drastic changes compared to normal rats at the same exposure concentration, causing remarkable changes in the myocardial enzyme profile. The pathological assay also showed that the degree of myocardial injury in hyperlipidemic rats exposed to PM<sub>2.5</sub> was significantly higher than that in normal rats which did not show significant changes in the cardiac structure and function after exposure to the high concentrations of PM<sub>2.5</sub>. In hyperlipidemic rats, exposure to low PM<sub>2.5</sub> concentration did not result in severe consequences, but exposure to high concentration of PM<sub>2.5</sub> caused thrombosis and blockage of blood vessels in some rats (Fig.2). We also found that PM<sub>2.5</sub> exposure could induce more severe reactions of acute inflammation and oxidative stress in hyperlipidemic rats, which could lead to changes in the blood composition and coagulation system, indicating that the toxicological effects caused by PM<sub>2.5</sub> exposure could vary under different pathological conditions.



**Figure 4. Influence of PM<sub>2.5</sub> exposure on serum biochemical indexes at different status.** Blood sample was collected from abdominal aorta at 24h after the last exposure to PM<sub>2.5</sub>. A, the activity of CK in serum; B, the activity of CK-MB in serum; C, the activity of LDH in serum; D, the concentration of CRP in serum; E, the activity of SOD in serum; F, the concentration of cTn-I in serum; The data are presented as mean ± SD (n=7)



**Figure 5. Pathological changes of myocardial tissue after PM<sub>2.5</sub> exposure.** A, HE staining of rat myocardial tissue; B, The TUNEL staining of rat myocardial tissue. The data are shown as mean  $\pm$  SD (n=7), \* $P$ <0.05 indicating a statistically significant difference, and \*\* $P$ <0.01 indicating a very significant difference. Pathological samples were obtained from the rat's left ventricle, and the sampling site for each rat was the same.

During myocardial ischemia, a large amount of cardiomyocyte apoptosis occurs. With Bcl-2 and Bax being the main genes regulating cardiomyocyte apoptosis, both of them are regulated by the JNK and P53 signaling pathway [26-28]. We also found that the phosphorylation levels of JNK and P53 raised with the increase in concentration of PM<sub>2.5</sub> exposure. The expression level of Bax was much higher in hyperlipidemic rats than that in normal rats, while Bcl-2 was significantly reduced, which may be the reason for the high occurrence of cardiomyocyte apoptosis in hyperlipidemic rats. Caspases are executors of apoptosis, and are mediated by different signal molecules. In the process of apoptosis, the caspase2 can be regulated by P53 only to execute the death of cell, and caspase3 can be adjusted by JNK/P53 or JNK to achieve the cell death. In this study, we found that PM<sub>2.5</sub> exposure could not influence the expression of caspase2, but caspase3 increased with the concentration of PM<sub>2.5</sub> exposure. These results indicated that PM<sub>2.5</sub> exposure could easily affect JNK/P53/Caspase3 or JNK/Caspase3 to induce the cardiomyocyte apoptosis, which may be the main reason for cardiovascular diseases induced by PM<sub>2.5</sub> exposure.

In addition, the toxic effects of PM<sub>2.5</sub> exposure are closely associated with its composition and concentration. Beijing, as the representative metropo-

lis in developing countries, has serious PM<sub>2.5</sub> pollution. From 2010 to 2012, its daily average PM<sub>2.5</sub> value was 96.2  $\mu\text{g}/\text{m}^3$ , which is 3-7 times of the WHO standard [42]. Its highest value exceeds 600  $\mu\text{g}/\text{m}^3$  on a haze day. As the minute tidal volume of rat is about 0.16 L [43], the PM<sub>2.5</sub> exposure dose for rat in the haze day could be about 1.7 mg/d, which is equivalent to the PM<sub>2.5</sub> exposure dose (4 mg/kg) used in our study. We selected the exposure concentrations of 4-40 mg/kg, in order to better illustrate the impact of PM<sub>2.5</sub> exposure on the cardiovascular system. The possible differences in the impact of PM<sub>2.5</sub> collected from other regions and the toxic effects of exposure at other concentration levels on cardiomyocyte apoptosis need to be further studied.

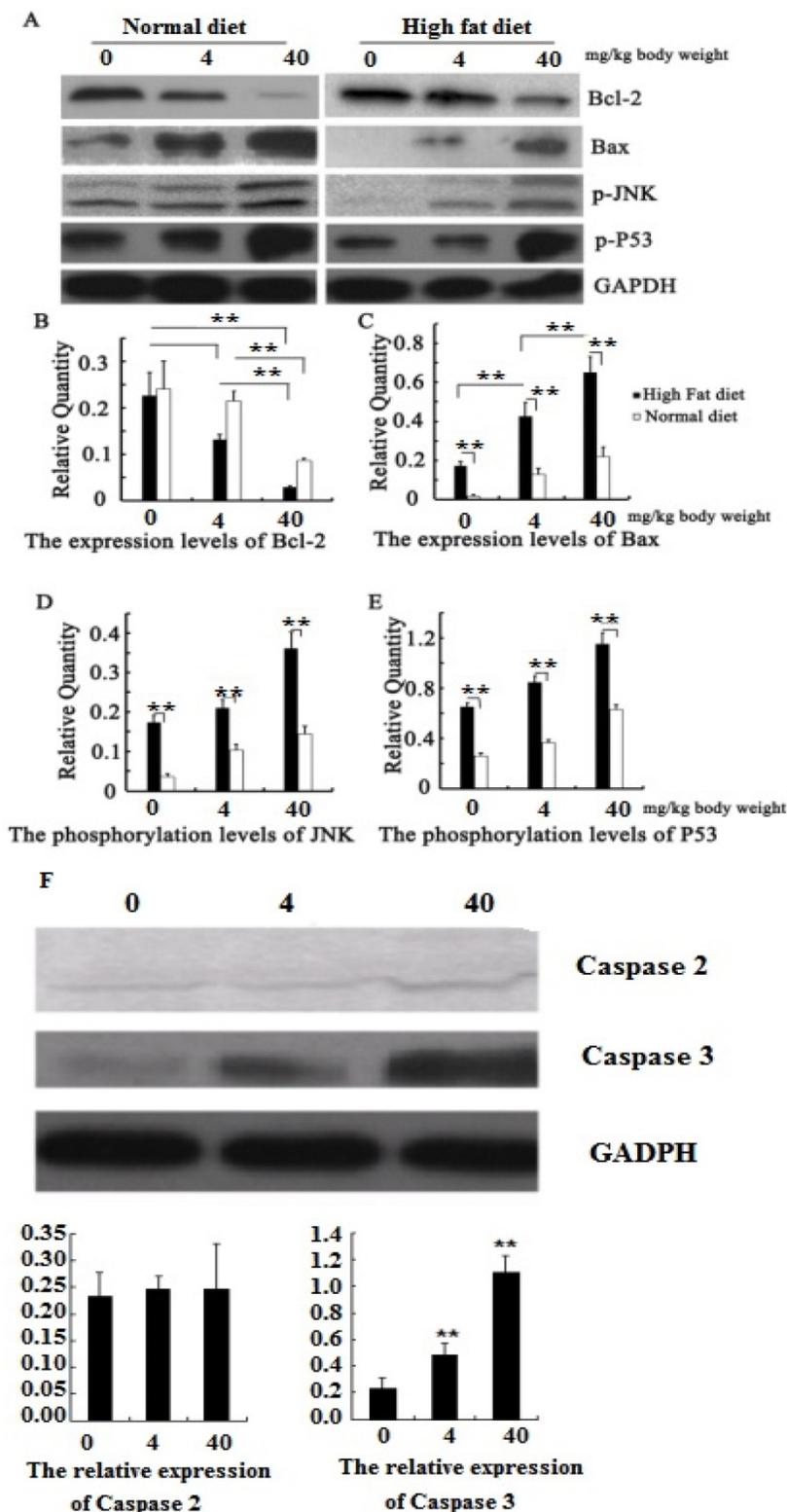
Furthermore, we found that PM<sub>2.5</sub> exposure could more easily induce oxidative stress and inflammation in hyperlipidemic rats, promoted a hypercoagulable state, which may affect the cardiac function.

## Conclusions

This study first identified that JNK or JNK/p53 was involved in the cardiomyocyte apoptosis induced by PM<sub>2.5</sub> exposure, which regulated its downstream effector genes, Bcl-2 and Bax, and then increased the expression of caspase3 leading to the myocardial injury. PM<sub>2.5</sub> exposure had bigger effect on JNK/p53

pathway in hyperlipidemic rats than that in normal rats, which may be the mechanism for hyperlipidemic rats being more susceptible to PM<sub>2.5</sub> exposure. It provides the basis for further exploring the toxic

effects of PM<sub>2.5</sub> exposure on the cardiovascular system and the mechanism of acute cardiovascular event induced in susceptible populations.



**Figure 6. Quantitative analysis of the effects of PM<sub>2.5</sub> on p53 / JNK and its downstream proteins.** A, the expression of proteins by Western blotting; B, the protein expression levels of Bcl-2; C, the protein expression levels of Bax; D, the phosphorylation levels of JNK; E, the phosphorylation levels of P53; F, the expression of caspase 2 and 3 after PM<sub>2.5</sub> exposure in hyperlipidemic rats. The data are presented as mean ± SD (n=5); \* P<0.05 indicating a statistically significant difference, and \*\* P<0.01 indicating a very significant difference.

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## Competing Interests

The authors have declared that no competing interest exists.

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