

Effect of refloated soil dust on oxidative stress and apoptosis in rat lung

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Abstract. Little study has illustrated the associations between inhalation of refloated soil dust and the morbidity of respiratory disease in animals. In this study, soil dust samples in arid and semiarid areas of China were collected for investigating the underlying biotoxicity and mechanisms of oxidative stress and apoptosis in rat lung induced by inhalation of refloated soil dust. The soil dust samples were collected from the arid and semiarid areas in Zhangjiakou city, located close to Inner Mongolia Plateau, China. After intratracheal instillation of soil dust, superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) activities in rat lung were declined in a dose-dependent relationship, and pathology changes in lung tissue were also enhanced along with increased levels of soil dust. Meanwhile, apoptotic cells were scattered in the pulmonary epithelial and distributed in the mesenchyma, and the apoptotic index was significantly increased in rat lung instilled by soil dust. Besides, soil dust promoted Bax expression and inhibited Bcl2 expression, i.e., increased Bax/Bcl2 ratio in a concentration-dependent manner. In summary, short-term exposure to soil dust caused oxidative damage and induced apoptosis in rat lung through the regulation of Bax and Bcl2 expression.

Keywords: Refloated soil dust; Lung; Oxidative stress; Apoptosis; Intratracheal instillation.

1. Introduction

Warming climate and land use lead to the degradation of the vegetation ecosystem in arid and semiarid areas in China [1]. Especially in areas including Taklimakan Desert, Inner Mongolia Plateau, and Zhangbei Plateau, dust storm frequently occurs and deteriorates the air quality of downwind areas in East Asia due to the refloated dust on the surface of soil [2-4]. To date, the origin, transmission, and chemical characteristics of refloated dust have been intensively investigated [2-4]. Yet, the study on the negative impacts of refloated dust on animals living in this area is limited.

Extensive studies have carried out to investigate the mechanism of ambient particles resulting in the injury of lung function [5-8]. The size of the ambient particles varies greatly and determines the deposited location in lung. There is an opposite relationship between size and the degree of lung penetration. PM10 can penetrate into the lower respiratory system, while PM2.5 can penetrate into the gas-exchange region of the lung [8]. PM2.5 and PM10 have indicated the ability to induce reactive oxygen species (ROS) production, which led to oxidative damage [9, 10]. Furthermore, compared with PM10, PM2.5 induced more malondialdehyde (MDA) formation, oxidative DNA damage repair enzyme Mth1 mRNA expression, and anti-oxidative stress enzyme Nrf2 protein expression in rat lung epithelial cell. In vitro study also demonstrated that the organic extracts of PM2.5 (crude extract) collected in the Suwon traffic area generated significant DNA breakage and micronucleus formation in BEAS-2B cells in a dose-dependent manner, and these genotoxic effects were significantly blocked by scavenging agents (superoxide dismutase (SOD), sodium selenite (SS), mannitol (M), catalase (CAT)). In vivo study showed that SOD and GSH-Px activities in the lung were reduced in PM2.5-exposed rats. These findings suggest that oxidative DNA damage arising from PM2.5 may be mediated by oxidative stress initiated by ROS. In addition, experimental studies have demonstrated that exposure to PM2.5 and PM10 could increase pro-inflammatory cytokine (IL-1 β , IL-6, IL-8, TNF-

α , MIP-1 α and MIP-2) content in human alveolar epithelial cells (A549), human alveolar macrophages cell, primary rat alveolar type 2 cells, and in human circulating blood and rat bronchoalveolar lavage fluids [12, 13-15]. Previous references have showed that both ROS production and inflammatory cytokines secretion induced by PM were associated with apoptosis [9, 15]. PM causes alveolar epithelial cell (A549) DNA damage and apoptosis by mechanisms that involve the mitochondria-regulated death pathway and the generation of iron-derived free radicals . It is noted that apoptosis is regulated by B cell lymphoma-2 (Bcl2) family members containing antiapoptotic (such as Bcl2 and Bcl-xl) and proapoptotic proteins (such as Bad, Bax or Bid). Once apoptotic stimulus are activated, proapoptotic Bcl2 proteins Bax and Bak will initiate apoptosis by the release of cytochrome c and activation of the caspase cascade, whereas antiapoptotic Bcl2 proteins Bcl2 and Bcl-xl will block the activation of the caspases . The balance between antiapoptotic and proapoptotic proteins of the Bcl2 family plays a key role in the mitochondrial response to apoptotic stimuli [16, 17].

The above studies adopted ambient particles as an external exposure source, which is emitted from various kinds of anthropogenic and secondary sources. In contrast, the detailed mechanisms how refloated dust-induced apoptosis remain largely unclear. Thus, our study collected soil dust in Angulinuo Playa Lake, which is located in arid and semiarid areas near Zhangjiakou city, Hebei Province. This area is identified as one of the potential source regions of Asian dust storms influencing the air quality in Beijing and surrounding areas every spring season[2-4, 17]. This study aims to: (1) evaluate the biotoxicity of refloated soil dust on rat lung; and (3) explore the underlying mechanism of refloated dust-induced apoptosis in rat lung. The findings should be of assistance to better understand the negative effects of refloated dust induced on animals living in arid and semiarid areas.

2. Materials and methods

2.1 Particle collection and Preparation

Sixteen sites were chosen as sampling sites surrounding the Angulinuo Playa Lake (N41.316°, E114.350°) near Zhangjiakou city, Hebei province, China. All sampling sites were about more than 200 km away from Beijing and very near each other. One 0~5 cm surface soil was collected per sampling site in April 2012. Meteorological data from the Website (<https://lishi.tianqi.com/chongqing/201204.html>) for the sampling period were recorded in the range of 14-22°C with the mean level of 16°C, respectively, and little precipitation was observed during the entire month. All particle preparation and analyses were conducted in Beijing under environmental conditions similar to those of the field study area. Samples were sealed in plastic bags so that condensation and dissolution did not occur. Sixteen equal weights of soil dust samples were mixed, air dried, and filtered through a 200-mesh sieve. Five grams of the filtered mixture were dissolved with 15 mL deionized water and extracted in a sonicator for 60 min. 5 g of the filtered mixture were dissolved and diluted with saline as 1.5 mg/mL, 7.5 mg/mL, and 37.5 mg/mL solutions for acute intratracheal instillation exposure experiments.

2.2 Animal and tissue preparations

The present study conformed to the Guide for Laboratory animal Guideline for ethical review of animal welfare, China (GB/T 35892-2018). A total of 32 male Wistar rats (7 weeks of age, 200±20 g) were purchased from Beijing HFK Bioscience Co. Ltd, Beijing, China. Rats were housed under conventional conditions (at a temperature of 22±1 °C, a relative humidity of 40-60%) with a regular 12-h light: 12-h dark cycle (with lights on at 7:00 a.m.), and allowed ad libitum access to water and food. All rats were randomly assigned to four groups (control group (CG), low dose group (LG) with 1.5 mg/mL dust, middle dose group (MG) with 7.5 mg/mL dust, high dose group (HG) with 37.5 mg/mL dust) (n=8 in each group). After a week acclimation, the rats in LG, MG, and HG group was

assigned to the intratracheal instillation where rats were anesthetized and fixed to make the pharynx exposed. An intratracheal instillation was performed as follows: the tube was placed into the trachea, and the rats were intratracheally instilled with 0.2 mL of different concentrations of soil saline-alkali dust solution, respectively. The control group did not intratracheal instillation of any concentrations of soil saline-alkali dust solution. After treatment for 24 h, all rats were sacrificed and the lung tissues were collected. Half tissues were fixed in 4% paraformaldehyde for 48 h and then embedded in paraffin wax for histological analysis. The remaining lung samples were quickly frozen in liquid nitrogen, then prepared using a lung homogenate for analysis of antioxidation activity and western blot.

2.3 Measurements of oxidative stress-related enzymes

The lung samples in each group were homogenized in a 0.9% saline solution (1:9), then centrifugated (1000 g, 20 min) to get the supernatant for analysis of the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) using commercial kits (A001-3, A005, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All detection procedures were performed according to the manufacturer's instructions. The activities of SOD and GSH-Px were expressed as U/mg protein.

2.4 Histology

The lung was routinely processed into 5 μm paraffin sections (25 μm intervals between cross-sections) for histology and in situ apoptosis detection. To observe the difference of the structure and morphology of the rat lung between different treatments, hematoxylin and eosin staining (H & E staining) were performed.

2.5 Lung cell apoptosis detection by TUNEL assay

The lung samples in each group were homogenized in a 0.9% saline solution (1:9), then centrifugated (1000 g, 20 min) to get the supernatant for analysis of the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) using commercial kits (A001-3, A005, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All detection procedures were performed according to the manufacturer's instructions. The activities of SOD and GSH-Px were expressed as U/mg protein.

2.6 Western blot

Lung tissues were lysed with RIPA lysis buffer (CW2333, CWBIO, Beijing, China) to determine the expression of Bcl2 and Bax. Total protein for each sample was electrophoresed in 12% SDS-PAGE and then electrotransferred to PDVF membrane (Millipore, Billerica, MA USA). After blocking in block buffer (TBST containing 0.05% Tween-20 and 5% fat-free dry milk) for 1 h at room temperature, the membrane was incubated overnight at 4°C with primary antibodies at a 1:200 dilution (Bcl2, Bax and β -actin, 12789-1-AP, 50599-2-Ig, 20536-1-AP, PTG, USA). After washing with TBST, they were incubated with HRP-conjugated goat anti-rabbit (1:2000, SA00001-2, PTG, USA) Ig-G for 1 h. The signal was detected by an ECL kit (B500022, PTG, USA) using an imaging system (Tanon, Shanghai, China). The bands were analyzed using an analyzer software provided by Tanon and shown as the integral optical density (IOD). The data for the Bcl2 and Bax protein levels was expressed as the IOD of the Bcl2 and Bax bands versus the IOD of the corresponding β -actin bands.

2.7 Data analysis

The data are presented as the mean and standard error (mean \pm SE) for each sample. Statistical differences between samples were tested with one-way ANOVA and post hoc comparisons performed with Dunnett's method using the SPSS 22 software (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

3. Results

3.1 Oxidative stress

Statistical analysis showed that the activity levels of antioxidant enzymes SOD and GSH-PX in the rat lung were influenced by soil saline-alkali dust particles and had a dose-dependent relationship. As shown in Figure 1A, the lung activities of SOD in the rats of MG and HG groups showed statistically significant decreases by 25.34% ($P < 0.05$) and 43.33% ($P < 0.05$) compared with the CG group, respectively. Although SOD activity in the LG group was 17.37% higher than that of CG group, there was no significant difference in the activity of SOD between the CG and LG groups ($P > 0.05$). Likewise, the GSH-PX activity in the HG group was 42.96% ($P < 0.05$) lower than that of CG group (Figure 1B). No significant difference in the activity of GSH-PX was observed between the CG and LG or MG groups ($P > 0.05$).

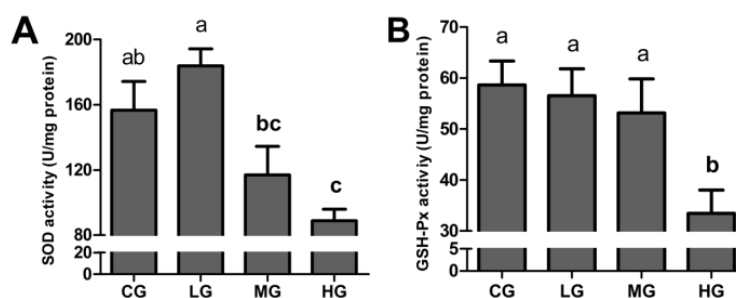


Figure 1. Effect of soil dust on the activities of SOD (A) and GSH-PX (B) in rat lung. Values were expressed as the mean \pm SEM. CG, control group; LG, low dose group; MG, middle dose group; HG, high dose group. $p < 0.05$ were used to denote the significance compared with the control group.

3.2 Pathology changes in lung tissue

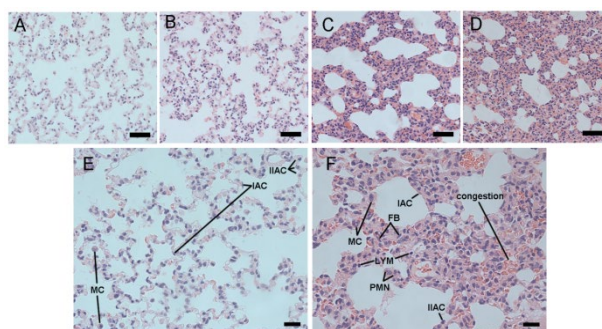


Figure 2. Effect of soil dust on lung damage of rat. A, B, C and D represents control group (CG), low dose group (LG), middle dose group (MG) and high dose group (HG), respectively. Alveolar septa thickening, the shrinkage of alveolar space, and the congestion of capillaries in the alveolar septum was found in MG and HG groups. E and F showed the inflammatory cells of CG and HG, respectively. Macrophage with some projections of surface, neutrophil and lymphocyte were observed in the lung of HG. IAC: Type I alveolar cell; IIAC: Type II alveolar cell; MC: macrophage; PMN: neutrophil; LYM: lymphocyte; FB: fibroblast. Bars = 50 μ m for A-D, bars = 20 μ m for E and F.

Morphological changes of the lung in rats were examined by H & E staining as shown in Figures 2A-F. The lung structure did not clearly change between CG and LG groups, while the lung damage (pathological changes) was increasingly noticeable from MG to HG groups. Compared with the CG group, alveolar septa thickening, shrinkage of the alveolar space, and the congestion of capillaries in the alveolar septum were found in MG and HG groups (Figures 2A-D). Inflammatory cells in the lung, such as lymphocytes, neutrophils, and macrophages, were observed in the MG and HG groups (Figure 2F). Furthermore, the surface of macrophages exhibits some projections in HG groups, while

macrophages in CG group are almost round with a smooth surface. These pathological characters were enhanced along with increasing concentrations of the soil saline-alkali dust solution.

3.3 Apoptosis in the lung

In the present study, Figure 3 (A-D) presented the immunolabeling data (i.e., yellow-brown staining in the nucleus) for TUNEL-positive cells in rats after exposure to soil saline-alkali dust via intratracheal instillation. Apoptotic cells were intensely scattered in the pulmonary epithelial and distributed in the mesenchyma. Negative controls showed no specific immunostaining in the lung of the rat (Figure 3E). Statistical analysis showed that soil saline-alkali dust significantly increased the apoptotic index (Figure 4F). The apoptotic index in the CG group was significantly lower than the LG group (36.05%, $P < 0.05$), the MG group (60.41%, $P < 0.05$) and the HG group (66.79%, $P < 0.05$).

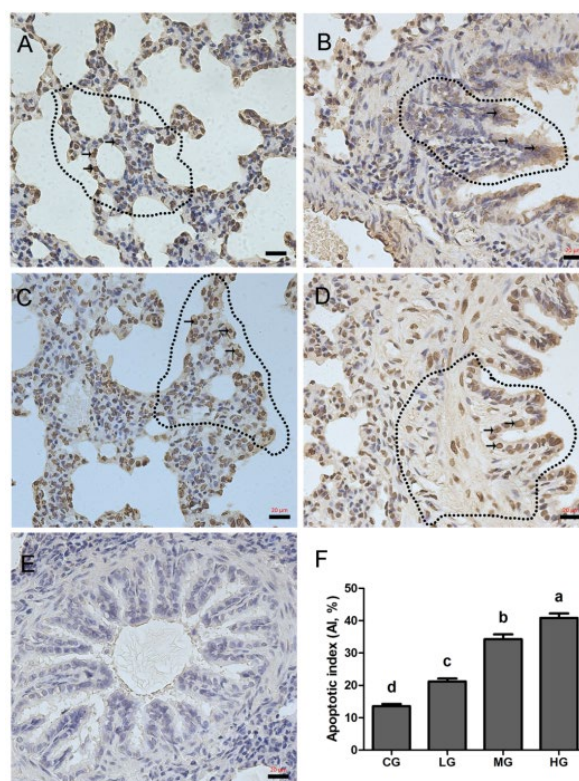


Figure 3. Effect of soil dust on apoptosis in the lung of rat. A, B, C, D and E represents control group (CG), low dose group (LG), middle dose group (MG), high dose group (HG) and Negative controls, respectively. F represents apoptotic index. Values were expressed as the mean \pm SEM. Apoptotic cells were presented as a yellow-brown staining in the nucleus. The ranges of values for each group are shown as dotted line. The arrows represent apoptotic cells. $p < 0.05$ were used to denote the significance compared with the control group. Bars = 20 μ m for A-F

3.4 Bax and Bcl2 expression and the ratio of Bax/Bcl2

As shown in Figures 4A, B, the protein expression of Bax in the HG and MG groups was 36.40% ($P < 0.05$) and 29.73% ($P < 0.05$) higher than that of the CG group. No statistically significant differences of Bax protein expression were found between the CG and LG groups ($P > 0.05$). However, Bcl2 protein expression was significantly decreased by soil saline-alkali dust treatment. Compared with that of the CG group, Bcl2 expression in the LG, MG, and HG groups was diminished by 21.40% ($P < 0.05$), 30.95% ($P < 0.05$) and 33.93% ($P < 0.05$), respectively (Figures 4A, C). Similar to Bax protein expression, the changes in the ratio of Bax/Bcl2 showed a saline-alkali dust concentration-dependent increase in rat lung. Bax/Bcl2 ratio in the CG group was lower than that of the LG group (53.62%, $P < 0.05$), MG group (91.30%, $P < 0.05$) and HG group (105.80%, $P < 0.05$) (Figures 4A, D).

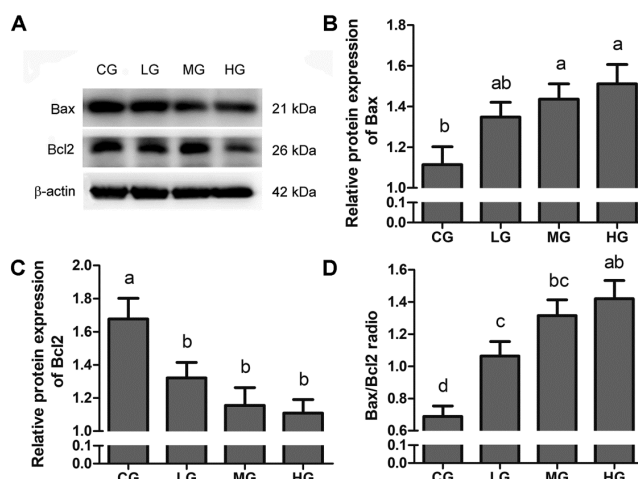


Figure 4. Effect of soil dust on the expression of Bax (B) and Bcl2 (C) and the ratio of Bax/Bcl2 (D) in rat lung. The lungs in each group were processed for protein to analyze the expression of Bax (A, B) and Bcl2 (A, C) by western blot. β -actin was used as an internal control. Values were expressed as the mean \pm SEM. CG, control group; LG, low group; MG, middle group; HG, high group. $p < 0.05$ were used to denote the significance compared with the control group.

4. Discussion

In the present study, our data demonstrated that the lung activities of antioxidant enzymes, SOD and GSH-PX, in rats were remarkably reduced after intratracheal instillation of high concentrations of soil dust, suggesting SOD and GSH-PX induced antioxidant effects on oxidative stress generated by soil dust. Soil dust has potential toxicity to rat lung. The similar result was also found in PM_{2.5}-exposed rats. Moreover, scavenging agents (SOD, SS, M, CAT) blocked the DNA breakage and micronucleus formation induced by organic extract of PM_{2.5} in BEAS-2B cells. These data confirm that the balance between oxidation and antioxidation is greatly influenced by refloated soil dust, and agree with the growing awareness that oxidative stress is induced by refloated dust accompanying inflammation and causes tissue or cell injury [10, 13, 18, 19]. Morphological observations also showed that soil dust induced lung pathological changes in rats, and these results were enlarged with increasing concentration of soil dust. Alveolar septa thickening was the typical characteristic after intratracheal instillation of middle and high concentrations of soil dust. This further supports that refloated dust induces pathological characters in rat lungs and oxidative damage in A549 cells through alteration of antioxidant enzyme activity [20, 21].

Studies showed that refloated dust induced the oxidative stress initiated by ROS 16, although the toxicological mechanisms of refloated dust have not been well understood. Several evidences revealed an association between oxidative stress and apoptosis [9, 14, 22]. Herein, this study shows that refloated dust also induced apoptosis in rat lung as accessed by TUNEL-stained nuclear morphology, and the apoptotic index increased with the concentration of refloated dust. Furthermore, apoptotic cells were located in the pulmonary epithelial and dispersed in the mesenchyma after intratracheal instillation of refloated dust [15, 16, 23, 24].

It is well known that apoptosis is a highly complicated mechanism and includes death receptor pathway and mitochondrial pathway. Dagher et al. reported that in vitro short-term exposure to particles induced apoptosis mechanisms that involve iron-derived free radicals, mitochondrial pathway and TNF- α -induced pathway. Apoptotic signals including cell stress, free radical damage, and proapoptotic proteins of Bcl2 family (Bax and Bak) are activated, which in turn lead to the release of mitochondrial cytochrome c and caspase activation, resulting in apoptosis propagation. Bcl2, anti-apoptotic protein of Bcl2 family, has been shown to suppress apoptosis and prolong cell survival. The Bax/Bcl2 ratio is known to determine cell fate to apoptosis. Western blot results indicated that refloated dust promoted Bax expression and inhibited Bcl2 expression, and the ratio of Bax/Bcl2 was

also increased in rat lung in a re-floated dust concentration-dependent manner, which agrees with Dagher et al. studies in human epithelial lung cells (L132). These findings were consistent with the apoptotic index in a re-floated dust concentration-dependent increase in TUNEL stain, suggesting the imbalance between the expression of Bax and Bcl2 in rat lung caused by re-floated dust is important to cell fate.

5. Conclusion

Our study confirmed that inhalations of re-floated dust could lead to the decrease of SOD and GSH-PX in rats. The reposes of SOD and GSH-PX were associated with increased levels of soil dust in a dose-dependent relationship. The enhanced changes of pathology rate in lung tissue were also observed with increased levels of re-floated dust in a dose-dependent relationship. In addition, the re-floated dust could promote Bax expression and inhibit Bcl2 expression, i.e., increased Bax/Bcl2 ratio in a concentration-dependent manner. Our findings indicated that effective measures for reducing the exposure risks of re-floated dust should be formulated for protecting wild animals in arid and semiarid areas

Institutional Review Board Statement

This study was approved by Beijing Milu Ecological Research Center. No animals were sacrificed in this study. All experiments were performed under guaranteed animal welfare.

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