Cytotoxicity induced by fine particulate matter (PM_{2.5}) via mitochondria-mediated apoptosis pathway in rat alveolar macrophages

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Abstract

Although positive associations exist between ambient particulate matter (PM_{2.5}; diameter ≤ 2.5 μm) and the morbidity and mortality rates for respiratory diseases, the biological mechanisms of the reported health effects are unclear. Considering that alveolar macrophages (AM) are the main cells responsible for phagocytic clearance of xenobiotic particles that reach the airspaces of the lungs, the purpose of this study was to investigate whether PM_{2.5} induced AM apoptosis, and investigate its possible mechanisms. Freshly isolated AM from Wistar rats were treated with extracted PM_{2.5} at concentrations of 33, 100, or 300 $\mu\text{g/mL}$ for 4 h; thereafter, the cytotoxic effects were evaluated. The results demonstrated that PM_{2.5} induced cytotoxicity by decreasing cell viability and increasing lactate dehydrogenase (LDH) levels in AMs. The levels of reactive oxygen species (ROS) and intracellular calcium cations (Ca^{2+}) markedly increased in higher PM_{2.5} concentration groups. Additionally, the apoptotic ratio increased, and the apoptosis-related proteins BCL2-associated X (Bax), caspase-3, and caspase-9 were upregulated, whereas B cell lymphoma-2 (Bcl-2) protein levels were downregulated following PM_{2.5} exposure. Cumulative findings showed that PM_{2.5} induced apoptosis in AMs through a mitochondrial-mediated pathway, which indicated that PM_{2.5} plays a significant role in lung injury diseases.

Keywords Particulate matter · Alveolar macrophage · Cytotoxicity · Apoptosis · Mitochondria-mediated apoptosis pathway · Caspase-3 · Caspase-9

Introduction

Hazy-fog episodes in world have recently become a major public health concern because it contains polluting primary or secondary particulate matter (PM) and, possibly, irritable chemicals and can have severe negative effects on the air quality and human health (Pérez-Díaz et al. 2017). PM_{2.5} is the primary particle of hazy fog, which is related to an elevated respiratory morbidity and mortality (Pope and Dockery 2006; Espitia-Perez et al. 2018; Zhu et al. 2020). According to Lelieveld et al. (2020), the number of premature deaths caused by air pollution in urban and rural areas was

approximately 8.4 million in 2020, which was about twice that reported by the World Health Organization in 2016 (WHO 2019). Pozzer et al. (2020) demonstrated that air pollution is an important co-factor increasing the risk of mortality from COVID-19. Inhaled PM_{2.5} can penetrate deeply into the lungs, deposit in the airways and alveoli, pass into the circulation, and damage not just in the respiratory system but also in the extrapulmonary organs (Gunasekar and Stanek 2011). Since the lung is one of the major targets of PM deposition, PM_{2.5} can trigger pulmonary inflammatory responses and impair lung function (Pinkerton et al. 2019; Wu et al. 2013). A growing number of publications have demonstrated that PM_{2.5} causes respiratory diseases including asthma, airway irritation chronic obstructive pulmonary disease (COPD), and lung cancer (Zhao et al. 2020; Tian et al. 2020; Wu et al. 2020; Wei et al. 2020).

Alveolar macrophage (AM) are the most abundant innate immune cells present in all mammalian organs, and play an important role in tissue homeostasis, host defense, clearance

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of surfactant and cell debris, pathogen recognition, initiation, and resolution of lung inflammation (Joshi et al. 2018; Zhang et al. 2012; Hu and Christman, 2019). In the lung, AMs are the first line of defense responsible for phagocytic clearance of xenobiotic particles that have reached the air spaces of the lungs; this is important as these cells have been considered as a critical participant in allergic lung diseases (Byrne et al. 2016). Bronchoalveolar lavage (BAL) has proven to be useful for detecting an inflammatory response in the lungs of animals exposed to toxicology studies; there is also optimism about the use of BAL analysis as an early predictor of late-occurring pulmonary diseases (Steinberg et al. 1992). Therefore, AM activity analysis is frequently used as an important indicator for evaluating responses in the lungs (Morio et al. 2001).

Reactive oxygen species (ROS) are involved in transmission stress of signaling in physiological and pathological processes; these are recognized widely as playing important roles in PM_{2.5}-mediated cytotoxicity (Huang et al. 2018). PM_{2.5} exposure induces the elevation of cellular ROS, and then causes an imbalance in cell homeostasis associated with damage to nucleic acids and proteins, lipids, membranes, and organelles, which results in apoptosis (Reyes-Zarate et al. 2016; Yang et al. 2018). Besides, cations (Ca²⁺) involved in multiple biological responses, they are considered as signaling molecules (Huang et al. 2018). Several studies have shown that in cancer cells, Ca²⁺ can induce apoptosis by destroying mitochondrial function (Hsieh et al. 2018).

Cellular apoptosis can occur via two major pathways—the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Galluzzi et al. 2018; Reed 2000). It has been reported that PM_{2.5} induces mitochondria ultrastructure damage including mitochondrial swelling, cristae disorder, and even vacuolation (Wei et al. 2019). The present study aimed to investigate whether PM_{2.5} induced AM apoptosis via the mitochondria-mediated pathway. Primary AMs were collected from lung lavage of healthy male Wistar rats which had been exposed to Taiyuan PM_{2.5}, in doses of either 33, 100 or 300 µg/mL *in vitro*. Cytotoxicity, ROS levels, intracellular Ca²⁺ concentrations, and mitochondria-mediated apoptosis pathway were evaluated.

Materials and methods

PM_{2.5} sampling and suspension preparation

PM_{2.5} sampling and suspension preparation were carried out as described previously by Wei et al. (2019). The sampling site was located at Shanxi University campus, Taiyuan, China (37° 47' N, 112° 34' E), and on the roof of the school of environmental science and resources (about 25 m above the ground level). During the severe haze period from December 28, 2011 to January 1, 2012, a PM_{2.5} high capacity air sampler

(Thermo Anderson, USA) with a pump flow of 1.13 m³/min was used to collect PM_{2.5} for 24 h/day. The PM_{2.5} sample was collected on a quartz filter membrane (Whatman QMA, UK; 0.3 µm DOP rejection efficiency > 99.995%; withstand high temperature was 500–900 °C and pore size was 2.2 µm). The quartz filters were preheated at 450 °C for 6 h prior to sampling and the dry weights of the quartz filter membranes were recorded before and after sampling.

After that, the quartz filter was cut into strips, soaked in Milli-Q water (18.2 MΩ-cm, Thermo Anderson, USA), and treated with bath sonication for 1 h. The filtered extract was transferred to a lyophilized bottle using a 0.2 µm syringe filter (Thermo Anderson, USA) and frozen at –80 °C. Before use, the samples were diluted to 10 mg/mL with normal saline (0.9% w/v NaCl, pH = 7).

Animal treatment and cell isolation

All of the animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the Ministry of Health of the People's Republic of China. The protocol was approved by the Shanxi University's Institutional Animal Care and Use Committee (Approved Animal Use Protocol Number: HZ20180503). A total of 15 healthy male Wistar rats weighing between 180 and 220 g were purchased from the Animal Center at Hebei Medical University. Prior to PM_{2.5} exposure, the animals were fed in the laboratory for a week with a 12-h light-dark cycle, at 24 ± 2 °C with a 50 ± 5% humidity. After that, the rats were anesthetized via a single intraperitoneal injection of pentobarbital sodium (2%, 0.2 mL/100 g body weight, Solarbio, China), and the whole lung was cannulated and lavaged with phosphate-buffered saline (PBS pH = 7.4, without Ca²⁺, Mg²⁺, and sterilized). The bronchoalveolar lavage fluid (BALF) obtained was centrifuged at 3000 rpm for 10 min at 4 °C (Geng et al. 2006).

As described by previous study (Wei et al. 2019), prior to resuspension, the cells recovered from the lavage process were measured using 0.04% trypan blue dye (Sigma-Aldrich, USA) exclusion to ensure that the cell viability was greater than 95% (Strober 2015). Then, the cells were plated at 10⁵ cells/35 mm in cell petri dishes and incubated in a CO₂ incubator for 2 h at 37 °C in 5% CO₂ and greater than 95% humidity conditions to enable adherence of AMs (Wei et al. 2019). Afterward, cells were washed twice with 2 mL sterilized saline, and exposed to PM_{2.5} suspended in fresh RPMI 1640 medium (Hyclone, USA) at a series of concentrations (final concentrations were 0, 33, 100, or 300 µg/mL) for another 4 h. Prior to treatment, the PM_{2.5} sample underwent ultrasonic treatment for 5 min. The control group was treated with RPMI 1640 at the same volume as test samples. Five replicate wells were conducted for each group.

Assessment of cytotoxicity

The MTT test

Cell viability, following exposure to PM_{2.5} at the differing concentrations for 4 h, was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test as described by Geng et al. (2005). The cells were seeded in 96-well plates at a density of approximately 2.0×10^5 cells/well. After 4 h, the cells were exposed to normal saline (0.9% w/v NaCl, pH = 7) containing different concentrations of PM_{2.5} (33, 100 or 300 µg/mL); Triton-X 100 (25 µg/mL) was used as a positive control (Fuentes-Mattei et al. 2010), and normal saline (0.9% w/v NaCl, pH = 7) served as negative controls. After the removal of the culture medium, cells were washed three times in PBS. Then, 20 µL of MTT solution (Sigma-Aldrich, USA) was applied and the cells were cultured for 4 h. The medium was then removed. Further, 150 µL 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA), the purification of DMSO was 99%, was added to each well in order to dissolve any formamide salts which had formed. After 10 min, the absorbance was measured for each well at a wavelength of at 570 nm using a Microplate Reader (Bio-Rad Model 550, USA). This experiment was performed in triplicates. The relative viability of cells was calculated according to the following formula:

Relative viability of cells (%)

$$= (\text{treated cells OD} / \text{untreated cells OD}) \times 100\% \quad (1)$$

The lactate dehydrogenase test

The reduction of intracellular LDH and its release into the extracellular medium is a sensitive indicator of nonreversible cell death due to the damaged caused to the cell membrane caused by cell apoptosis or necrosis (Yang et al. 2018). One hundred microliters of cell culture medium supernatant was collected to determine LDH activity. The test was performed according to the LDH assay kit manufacturer's instructions (Nanjing Jiancheng, China). As described above, after AMs were treated for 4 h with PM_{2.5} (at 0, 33, 100, or 300 µg/mL), a 100 µL aliquot of cell culture medium supernatant was collected from each dish (10^5 cells) in order to test extracellular LDH activity. Briefly, 250 µL of the reconstituted substrate mix and 50 µL Coenzyme I solution (Nanjing Jiancheng LDH assay kit) were added to each sample; following incubation in a 37 °C water bath for 15 min, the enzymatic reaction was stopped with 25 µL of 2,4-dinitrohydrazine (Nanjing Jiancheng LDH assay kit). The mixture was incubated again for 15 min in a 37 °C water bath and 2.5 mL NaOH (0.4

mol/L) was added. The absorbance was then measured at 440 nm using an ultraviolet (UV)-visible spectrophotometer (Beckman DU-640B, USA). All experiments were performed in triplicates.

Intracellular ROS measurement

Intracellular ROS generation induced by PM_{2.5} was detected via flow cytometry using a DCFH-DA dye (Wang et al. 2013). DCFH-DA is a dye which passively enters cells and reacts with the ROS within the cell, and produces a highly fluorescent compound called dichlorofluorescein (DCF) (Sheikh et al. 2017). The ROS Assay Kit (Jiancheng Biology Engineering Institute, Nanjing, China) was used as previously described (LeBel et al. 1992). Briefly, 2 mL working solution of DCFH-DA was added to the AMs (1×10^5 cells/35 mm petri dish) previously exposed to PM_{2.5} and incubated at 37 °C for 30 min in dark conditions. Next, the DCFH-DA was removed and the cells were washed three times in PBS (pH = 7.4, without Ca²⁺, Mg²⁺, sterilized), then resuspended in order to measure fluorescence intensity which was measured by flow cytometry (Becton-Dickison, USA) at excitation/emission wavelengths of 485/530 nm. ROS inhibitor (NAC) was added to the control and 100 µg/mL treated group to explore whether PM_{2.5} directly resulted in ROS generation. The results were expressed by calculating the relative fluorescence intensities in comparison to the control group. For each well, more than 1×10^5 cells were counted, and all experiments were performed in triplicates.

Cytosolic Ca²⁺ measurement

Intracellular ROS may affect intracellular Ca²⁺ homeostasis, induce lipid peroxidation, and DNA damage (Li et al. 2008). To quantify cytosolic Ca²⁺ in living cells, a cell-permeant probe, Fluo-4/acetoxymethyl ester (Fluo-4/AM, Beyotime Biotechnology), was used in accordance with previously described methods (Gramdordy et al. 1988). Briefly, AMs treated with PM_{2.5} were washed with three times with PBS, resuspended in fresh RPMI 1640 culture medium containing 4 µM Fluo-4/AM, and incubated for 45 min at 37 °C in the dark. The Fluo-4 AM was then removed, the AMs were washed three times, and resuspended in 500 µL PBS (pH = 7.4, without Ca²⁺, Mg²⁺ and sterilized). Cell fluorescence intensity (F) was measured at excitation/emission wavelengths of 485/530 nm using flow cytometry (Thermo Scientific Varioskan Flash, USA). Then, 0.1% Triton-x 100 was added to get the maximum fluorescence value (F_{Max}) and 0.05 mol/L EDTA was added to get the minimum fluorescence value (F_{Min}). The concentration was calculated according to the following formula:

$$[\text{Ca}^{2+}] = K_d (F - F_{\text{Min}}) / (F_{\text{Max}} - F) \quad (2)$$

K_d represents the dissociation constant, the value of which is 450 nmol/L (Gramdordy et al. 1988). All experiments were performed in triplicates.

AM apoptosis detection

The apoptotic rate of AMs was detected using the Annexin V-FITC/PI (propidium iodide) apoptosis measurement assay kit (Yang et al. 2018). Cells were treated with the different doses of PM_{2.5} for 4 h, then centrifuged at 1200 rpm for 3 min. Cells were then washed twice in pre-cooled PBS (pH to 7.4, without Ca²⁺, Mg²⁺, sterilized), and resuspended in 100 µL annexin-binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl₂, pH = 7.4). Then 5 µL of Annexin V-FITC and 1 µL propidium iodide (PI) were added (Sigma-Aldrich, USA), and the solution was incubated at 4 °C in the dark for 15 min. The apoptotic and necrotic cells were detected using flow cytometry at excitation/emission wavelengths of 488/525 nm (Millipore, USA) (Napieriska et al. 2009). For each exposure group, more than 1×10^5 cells were counted for analysis. The cytogram was divided into four quadrants: the upper left quadrant represents cell fragments, the lower left quadrant represents live cells, the upper right quadrant represents late apoptotic cells, and the lower right quadrant represents early apoptotic cells.

Western blot analysis

The expression of apoptosis-related proteins, including Bcl-2, Bax, caspase-3, and caspase-9, in AMs were determined by Western blot analysis. The protein were extracted from the AMs, previously exposed to PM_{2.5}, using a protein extraction kit (Sigma, USA). Briefly, the cell culture medium was removed, the cells were washed three times with pre-chilled PBS (pH = 7.4, without Ca²⁺, Mg²⁺, sterilized), and lysed in ice-cold Radio Immunoprecipitation. Assay buffer containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS and sodium orthovanadate, sodium fluoride, EDTA, leupeptin, and PMSF (pH = 7.4). Next, the cells were transferred to EP tubes and centrifuged at 13,000 rpm for 15 min at 4 °C. The concentrations of the proteins were detected using a BCA protein quantification kit (Sigma, USA). A standard curve was prepared according to the Bradford method, using bovine serum albumin (BSA) as the standard protein (Bradford 1976). Each protein was quantified to 30–50 µg.

The protein samples were loaded onto SDS-polyacrylamide gels (12% separation gels; Sigma, USA) and underwent electrophoresis at 110 V for 90 min. Next, the target protein was transferred to the nitrocellulose membrane for 45 min (350 mA) in an ice bath. Then the membranes were incubated in the blocking solution with PBS containing 3% bovine albumin (Solarbio, China) for 1 h. After blocking, the membranes were incubated in rabbit polyclonal antibodies

against Bax, or Bcl-2 (1:200, Biosynthesis, China), or rabbit monoclonal antibodies against caspase-3, caspase-9, or β -actin (1:1000, Cell signaling Technology, USA) overnight at 4 °C. The membranes were then washed three times with PBS for 10 min each time, and incubated with fluorescent labeled secondary antibodies (1:5000, LICOR Biosciences Corporation, USA) at room temperature for 1 h. The nitrocellulose membranes were then washed three times (10 min each) with PBS. The membranes were washed again with PBS and were then analyzed with the LI-COR Odyssey (LI-COR, USA) infrared scanning fluorescence detection system (Chen et al. 2017). The results were expressed by the ratio of optical density of target protein to β -actin protein.

Statistical analysis

The data were expressed as mean $\pm$ standard deviation (mean $\pm$ SD). All cell experiments were repeated three times. One-way analysis of variance (ANOVA) and post hoc Tukey's test were performed to determine differences due to treatment after confirming homoscedasticity and normality for data. Statistically significant differences were recognized at a level of $p < 0.05$.

Results

Assessment of cytotoxicity

As shown in Fig. 1a, MTT assay results suggested that compared with the unexposed control AMs, no statistically significant impacts of PM_{2.5} were detected at the concentration of 33 µg/mL exposure group. However, a dose-dependent decrease of AMs viability was observed in both 100 and 300 µg/mL exposure groups compared to control (Fig. 1a; $p < 0.05$ and $p < 0.01$, respectively).

The levels of LDH released from the PM_{2.5} exposure AMs significantly increased within the 100 µg/mL or 300 µg/mL PM_{2.5} exposure groups when compared to the control AMs (Fig. 1b; $p < 0.01$ and $p < 0.001$, respectively). The results indicated that the amount of LDH released from the PM_{2.5} induced AMs was related to the cell ability.

Intracellular ROS generation detection

In order to confirm whether ROS participated in PM_{2.5}-induced AM apoptosis, intracellular ROS was measured using the cell permeable probe DCFH-DA. Following a 4-h exposure to PM_{2.5}, fluorescence intensity significantly increased in the 33, 100, and 300 µg/mL treated groups (Fig. 2a; $p < 0.05$, $p < 0.001$, and $p < 0.001$, respectively). Fluorescence intensity in 100 µg/mL and 300 µg/mL treated groups which were almost 1.5- and 1.6-folds higher respectively than that of the

Fig. 1 Cytotoxic effects of PM_{2.5} extracts in rat alveolar macrophages (AMs). Panel **a** shows the arithmetic means $\pm$ SD of group data from MTT cell viability; panel **b** shows the arithmetic means $\pm$ SD of group data from lactate dehydrogenase activity assays. Both assay types were performed in AMs were exposed for 4 h to PM_{2.5} extracts at 0, 33, 100, or 300 µg/mL. ^a $p < 0.05$, ^{aa} $p < 0.01$, and ^{aaa} $p < 0.001$ vs. control; ^b $p < 0.05$, ^{bb} $p < 0.01$, ^{bbb} $p < 0.001$ vs. PM_{2.5} 33 µg/mL; ^c $p < 0.05$, ^{cc} $p < 0.01$ vs. PM_{2.5} 100 µg/mL

control group ($p < 0.001$ and $p < 0.001$). Results revealed that generation of intracellular ROS was caused by PM_{2.5} in a dose-dependent way. NAC protected the PM_{2.5}-induced ROS generation effectively in AMs (Fig. 2b).

Cytosolic Ca²⁺ measurement

To determine the correlation between activation of ROS and Ca²⁺ overload in cytoplasm, the levels of cytosolic Ca²⁺ were detected using a cell-permeant probe Fluo-4/AM. Results from the flow cytometry assay showed that AM exposure to PM_{2.5} at concentrations of either 33, 100, or 300 µg/mL induced intracellular Ca²⁺ accumulation in a concentration-dependent manner, with significant increases ranging from

Fig. 2 Effects of PM_{2.5} extracts on intracellular levels of reactive oxygen species (ROS) in rat alveolar macrophages (AMs). Panel **a** shows the arithmetic means $\pm$ SD of group data from DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) ROS quantification assays performed after AMs were exposed for 4 h to PM_{2.5} extracts at 0, 33, 100, or 300 µg/mL; panel **b** shows NAC effectively protect the PM_{2.5}-induced ROS generation in AMs. ^a $p < 0.05$, and ^{aaa} $p < 0.001$ vs. Control; ^b $p < 0.05$, ^{bb} $p < 0.01$ vs. PM_{2.5} 33 µg/mL

1.6 to 3.5 times the levels found in controls (Fig. 3; $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

Apoptosis ratio detection

AMs were exposed to different concentrations of PM_{2.5} (33, 100, or 300 µg/mL) for 4 h; the apoptotic ratio in AMs was then analyzed by flow cytometric Annexin V-FITC/PI analysis (Fig. 4a). As shown in Fig. 4b, compared to control, the apoptotic rate was obviously elevated at concentrations of 100 and 300 µg/mL, but not at 33 µg/mL ($p < 0.05$, $p < 0.001$, and $p > 0.05$, respectively). The data suggested that PM_{2.5}-induced AM apoptosis increased in severity following rising concentrations of PM_{2.5}.

Fig. 3 Effects of PM_{2.5} extracts on cytosolic Ca²⁺ concentrations in rat alveolar macrophages (AMs). This figure shows the arithmetic means $\pm$ standard deviations of group data from Fluo-3 assays performed after AMs were exposed for 4 h to PM_{2.5} extracts at 0, 33, 100, or 300 µg/mL. ^a $p < 0.05$, ^{aa} $p < 0.01$, and ^{aaa} $p < 0.001$ vs. control; ^{bb} $p < 0.01$ vs. PM_{2.5} 33 µg/mL; ^c $p < 0.05$ vs. PM_{2.5} 100 µg/mL

Changes of mitochondria-mediated apoptosis-related protein expression

The expression of the apoptotic-related proteins, Bax, Bcl-2, caspase-3, and caspase-9, were determined by western blot (Fig. 5a). The data showed that Bcl-2 was downregulated and Bax was upregulated following treatment with PM_{2.5} at concentrations of 33, 100, or 300 µg/mL. Moreover, caspase-3 and caspase-9 were significantly activated (Fig. 5b; $p < 0.05$). In the 300 µg/mL exposure group, the expression of caspase-3 and caspase-9 were 1.52- and 1.60-folds higher than the control group ($p < 0.001$). The ratio of Bcl-2 to Bax was significantly decreased in both the 100 and 300 µg/mL treated groups (Fig. 5c; $p < 0.01$).

Discussion

Previous studies have shown that PM_{2.5} pollution produces premature death globally and is the largest environmental cause of diseases (Yang et al. 2018). Results of a large population-based cohort study of approximately 1.1 million people over a period of 10 years showed a causal relationship demonstrating a positive association between PM_{2.5} and lung injuries (Weichenthal et al. 2017), as well as an increased risk of respiratory morbidity and mortality (Pun et al. 2017; Wang et al. 2020). The PM used in this study was < 2.5 µm in diameter, and able to penetrate into the alveolar regions of the lung to damage the AMs (Deng et al. 2013; Draijer and Peters-Golden 2017). A number of toxic effects in AMs caused by PM_{2.5} exposure have been reported (Chu et al. 2016; Zhao et al. 2016). However, the cellular biological

mechanism of PM_{2.5}-induced AM apoptosis is not completely understood.

To obtain better insights into PM_{2.5}-induced AM cytotoxicity, several AM cytotoxicity factors were determined following PM_{2.5} exposure. The cell viability assay is an important step in measuring cellular responses to toxicants (Smith et al. 2003). LDH is also a marker for common injuries of cell membrane damage (Aung et al. 2011). Firstly, we examined cell viability and LDH release; the results showed that PM_{2.5} exposure led to a decrease in AM viability and LDH release in a dose-dependent manner (Fig. 1). Similar research previously reported that PM_{2.5} exposure increased LDH level in rat AMs in a dose-dependent manner (Geng et al. 2006; Yang et al. 2018).

It has been reported that PM_{2.5} may induce generation of ROS (Wang et al. 2019), and excessive ROS could regulate cellular redox states (Shang et al. 2013), induce lipid peroxidation and DNA strand breaks, cause severe damage to RNA and proteins, and provoke cell death (Gunasekar and Stanek 2011; Morio et al. 2001; Liu et al. 2020). In the present study, the ROS levels showed that PM_{2.5} induced high levels of ROS in AMs in a dose-dependent manner (Fig. 2). Our previous study showed that PM_{2.5} reduced the activities of antioxidant enzymes such as CAT and GSH-PX in AM, and increased the content of MDA (Liu et al. 2018a). Similar results were reported in other cells (Liu et al. 2018b). The oxidative stress induced by PM_{2.5} has been regarded as a significant cytotoxicity response (Yang et al. 2018; Xu et al. 2020). We have determined the chemical composition of PM_{2.5} used in this study, and the results showed both organic and inorganic heavy metal elements including PAHs and Cd, Pb, Cr, Zn, etc. (Xia et al. 2010). These chemical components may partially affect oxidative stress responses induced by PM_{2.5}. Increased ROS is reportedly a typical phenomenon during mitochondria-dependent apoptosis (Sheikh et al. 2017; Vaux and Korsmeyer 1999). Therefore, it is also possible that ROS can induce AM cytotoxicity and apoptosis.

Mitochondria are the regulatory centers of apoptosis (Espitia-Perez et al. 2018). Excessive ROS can cause oxidative damage to mitochondria, lipid peroxidation of mitochondrial inner membranes, and damage mitochondrial membrane permeability (Yang et al. 2018). Severe mitochondrial damage will cause mitochondria to dysfunction and lead to apoptosis (Zhou et al. 2017). Previous studies showed that PM_{2.5} damaged the mitochondrial ultrastructure and caused cristae disorder, mitochondrial swelling, and even vacuolation (Qi et al. 2019; Wei et al. 2019). Therefore, we speculate that the damage caused to mitochondrial function will result in apoptosis. In addition, mitochondrion is the most important Ca²⁺ pool in cells, which is very important for the balance of Ca²⁺ concentration in the cytoplasm. Ca²⁺ has long been known to be critically involved in both the initiation and effectuation of cell death (Orrenius et al. 2015). Mitochondrial membrane

Fig. 4 Apoptotic effects of PM_{2.5} extracts on rat alveolar macrophages (AMs). Panel **a** shows cell apoptosis from Annexin V FITC assays. Panel **b** shows the arithmetic means $\pm$ standard deviations of the ratio of cell apoptotic rate. The assay was performed after AMs were exposed for 4 h to PM_{2.5} extracts at 0, 33, 100, or 300 $\mu\text{g/mL}$. ^a $p < 0.05$, ^{aaa} $p < 0.001$ vs. control; ^b $p < 0.05$ vs. PM_{2.5} 33 $\mu\text{g/mL}$

lipids are attacked by free radicals, lipid peroxidation occurs, which leads to a decrease in Ca^{2+} uptake by the mitochondria and therefore a cellular increase of Ca^{2+} . Evidence has shown that the release of Ca^{2+} from mitochondria is closely related to apoptosis (Huang et al. 2018). On the one hand, intracellular overload of Ca^{2+} can activate Ca^{2+} -dependence phosphatase and decrease intracellular ATP concentration, which causes release of cytochrome C (Cyt C) in mitochondria. Ca^{2+} can also activate endonuclease and degrade nuclear DNA. On the other hand, Ca^{2+} overload within the cytoplasm may cause mitochondrial swelling, outer membrane rupture (Redza-Dutordoir and Averill-Bates 2016; Ha et al. 2019), and even fragmentation under special conditions, which in turn can induce cellular apoptosis (Li et al. 2015; Watanabe et al. 2014). In the present study, intracellular ROS and cytoplasmic Ca^{2+}

in the PM_{2.5} exposed cells significantly increased (Fig. 2 and Fig. 3). These findings suggested that PM_{2.5} activated the ROS production initially and then destroyed the ultrastructure of mitochondria, induced the release of Ca^{2+} from mitochondria into cytoplasm, which lead to AM apoptosis.

In this study, the apoptotic ratio increased in a dose-dependent manner as a reaction to PM_{2.5} (Fig. 4). The results were in accordance with previous studies which suggested that PM_{2.5} and PM₁₀ can induce apoptosis in macrophages (Huang et al. 2004; Obot et al. 2002). To understand the mechanism of PM_{2.5}-induced apoptosis in AMs, the mitochondria-mediated apoptosis pathway was investigated. The proteins related to mitochondria-mediated apoptosis including caspase-3, caspase-9, Bax, and Bcl-2 were detected by western blot following 4-h exposure to PM_{2.5}. The results

Fig. 5 Effects of PM_{2.5} on proteins related to the mitochondrial-mediated apoptosis pathway. Panel **a** shows proteins related to the mitochondrial-mediated apoptosis pathway determined by western blot; panel **b** shows the ratio of caspase-3, caspase-9, Bcl-2, Bax protein expression; panel **c** shows the ratio of Bax to Bcl-2. The assay was performed after AMs were exposed for 4 h to PM_{2.5} extracts at 0, 33, 100, or 300 μg/mL. ^a*p* < 0.05, ^{aa}*p* < 0.01, and ^{aaa}*p* < 0.001 vs. control; ^b*p* < 0.05, ^{bb}*p* < 0.01 vs. PM_{2.5} 33 μg/mL; ^{cc}*p* < 0.01 vs. PM_{2.5} 100 μg/mL

showed that the pro-apoptotic proteins caspase-3, caspase-9, and Bax were upregulated and the anti-apoptotic protein Bcl-2 was downregulated. The ratio of Bcl-2 and Bax also declined. Caspase-3 and caspase-9 are important proteins involved in the mitochondria-mediated pathway (Fig. 5). ROS increase, Ca²⁺ influx, and severe mitochondrial dysfunction could lead to Cyt C release from mitochondria into the cytoplasm (Liu et al. 2018a; Liu et al. 2019). Then, Cyt C and caspase-9 form apoptotic bodies, activate caspase-3, thereby leading to cellular apoptosis (Scorrano and Korsmeyer 2003). Bcl-2 and Bax are located in the mitochondrial membrane; the ratio of Bcl-2

and Bax in the cell determines the formation of mitochondrial outer membrane pores (Huang et al. 2018; Zhang et al. 2010). The decline in the ratio of Bcl-2 and Bax is a sign that the mitochondrial-mediated apoptosis pathway was activated (Chen et al. 2017; Liu et al. 2019). Consequently, the results suggested that PM_{2.5} triggered apoptosis in AMs by activating the mitochondrial-mediated apoptosis pathway.

Conclusions

In summary, PM_{2.5} exposure caused increases in intracellular ROS, Ca²⁺ expression, and the apoptotic ratio of AMs. In addition, apoptotic-related proteins including caspase-3, caspase-9, Bax, and Bcl-2 were upregulated. Therefore, we concluded from these cumulative results that mitochondrial-mediated apoptosis in AMs was a key pathway in PM_{2.5}-triggered cytotoxicity.

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Authors' contributions The experiment was conceived and supervised by HW. HW and WY wrote the manuscript. WY and HY conducted the animal experiments and other biological experiment analysis. HG analyzed the experimental results. HW and HY contributed to the analysis of the data. All authors read and approved the final manuscript.

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Data availability Not applicable.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethics approval and consent to participate Current study which involves animal subjects has been reviewed and approved by Shanxi University's Institutional Animal Care and Use Committee (Approved Animal Use Protocol Number: HZ20180503).

Consent for publication Not applicable.

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