Caspian Journal of Environmental Sciences
Online ISSN: 1735-3866
Print ISSN: 1735-3033

Effects of the air pollution on the decreased P53, Nrf2 and HO-1 protein levels along with tissue damage caused by oxidative stress in the lung of rat as an animal model

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ABSTRACT

Air pollution is associated with respiratory pathologies, and exposure to airborne particles with an aerodynamic diameter of 2.5 microns ($PM_{2.5}$) is a significant risk factor for respiratory patients. This study aimed to investigate the effects of $PM_{2.5}$ exposures on lung tissue pathology and the function of antioxidant enzymes in a rat animal model. The animals were exposed to $PM_{2.5}$ for two different periods: 3 and 6 months, with a frequency of 4 days per week and a duration of 5 hours (from 9.00 am to 14.00 pm) per day. The activity of antioxidant enzymes, as well as lung histology and the protein expression of p53, Nrf2, and HO-1, were assessed in the lung tissue of the exposed rats. The results revealed a significant increase in malondialdehyde (MDA) levels and a significant decrease in antioxidant enzymes (CAT, SOD, and GPx) in the animals exposed to $PM_{2.5}$. Moreover, a noticeable reduction in the protein levels of p53, Nrf2, and HO-1 was observed in the lung tissues of the rats after $PM_{2.5}$ exposure, which was accompanied by pathological changes. In conclusion, $PM_{2.5}$ exposure leads to severe damage to lung tissue by reducing the activity of antioxidant enzymes and inducing oxidative stress through the activation of Nrf2 and P53 signalling pathways.

Keywords: Air pollution, P53, Nrf2, HO-1, Oxidative stress, Lung damage.
Article type: Research Article.

INTRODUCTION

Air pollution in big cities across Asia is a major concern and a significant health issue. Monitoring pollution and as a result, the presence of harmful chemicals and heavy metals have impact on people's health and quality of life in these areas is of utmost importance (Ghorani-Azam *et al*. 2016; Mehrandish *et al*. 2019; Oloyede *et al*. 2020; Manouchehri *et al*. 2021; Rao, 2023). Particulate matter (PM) is the most significant contributor to air pollution, comprising a mixture of solid and liquid particles. These particles may consist of materials such as shells, metals, dust, pollens, smoke, soot, as well as suspended droplets and particles in the air (Gemenetzis *et al*. 2006; Asadifard & Masoudi 2018; Masoudi *et al.* 2019). PM is categorized into different groups based on their aerodynamic diameter, including $PM_{0.1}$, PM_{1}, $PM_{2.5}$, and PM_{10}, representing particles with a diameter of less than 0.1, 1, 2.5, and 10 micrometers, respectively (Hassanvand *et al.* 2014). While particles of various sizes and shapes pose risks to human health, there is evidence suggesting that fine particles pose the greatest health risks (Riediker *et al.* 2019). Fine particles can penetrate deep into the lungs and even enter the bloodstream, causing significant damage to the heart and lungs (Bernstein *et al*. 2004). Long-term exposure to these particles increases hospital admissions, leads to school and business closures, and imposes travel restrictions, particularly for older individuals, children,

and those with pre-existing heart and lung conditions (Chauhan *et al*. 2003). Although coarse particles such as $PM_{5\text{-}10}$ are less concerning than particles with diameters of $PM_{0.1\text{-}2.5}$, they can still cause harm to the eyes, throat, and nose (Duncan 2006). The entry of $PM_{2.5}$ into the lung pores triggers inflammation and compromises the biological function of the lungs (Xing *et al*. 2016). The precise mechanisms by which $PM_{2.5}$ particles cause serious damage to cells, tissues, and organs are not fully understood. Secondary particles generated by nitrogen oxides or sulfur dioxide can induce oxidative stress within cells (Cho *et al*. 2018; Donaldson & Beswick 1996). Previous studies have also indicated oxidative damage in the lungs due to the presence of organic components within $PM_{2.5}$ (Donaldson & Beswick 1996). Small particles, especially water-soluble particles, can generate reactive oxygen species (ROS), leading to metal activation and the production of hydroxyl radicals (•OH). This process is associated with irreversible damage to DNA, including mutations, carcinogenesis, teratogenesis, and other forms of damage (Mehta *et al*. 2008). At low doses, PM exposure may not significantly affect lung structure and function; however, at high doses, it can induce severe expression of P53, inflammation, DNA damage, and altered expression of genes associated with epithelial cells and fibroblasts in lung tissue (Oren 2003; Zhu *et al*. 2010). P53 is a tumour suppressor that regulates the expression of genes involved in cell regulation, DNA repair, and apoptosis. The effective and detrimental dose of $PM_{2.5}$ on lung function is still a topic of debate. Understanding the precise mechanisms through which $PM_{2.5}$ affect lung tissue structure is crucial for identifying effective therapeutic strategies. The objective of this study was to investigate the effects of $PM_{2.5}$ exposure on lung tissue and explore potential changes in P53 expression and oxidative stress enzymes in rats following the exposure.

MATERIAL AND METHODS

Animal

A total of 24 male Wistar rats with an average weight of 250 g were used in this study. Rats were treated in accordance with the published guideline of The Care and Use of Laboratory Animals. All stages of this study, including work on laboratory animals, were approved by the Animal Care Committee based at Shahid Beheshti University.

Exposure site

Exposure was conducted on the roof of the University located in northern Tehran (35.7991° N, 51.3947° E) at an altitude of ~ 20 meters higher than the ground level.

Exposure chambers

Three groups were defined for the exposure of rats; exposure group 1 (control), exposure group 2 ($PM_{2.5}$) and exposure group 3 (clean air). The volume of the exposure chamber was 2.16 m^3 (1.2 m× 1.2 m×1.5 m). In exposure group 2, air flow with 12 L min^{-1} flow rate was introduced into the chamber by an Echo PM (Tecora Echo PM – TCR Tecora Italy) without a filter (TCR). To remove PM, EPA filter model H13 (SungJin Co., Ltd., Korea) was located in the inlet valve of the vacuum pump. As HEPA filters are capable of removing all PM with 99.97% efficiency (Holý *et al*. 2012). In exposure group 3, air with 12 L min^{-1} flow rate was introduced into the chamber via the vacuum pump with an active carbon air filter and a HEPA filter. Environmental parameters of room exposure such as temperature (20-25 °C) and relative humidity (40-60%) were controlled in both cold and warm seasons. The data concentrations of $PM_{2.5}$ in the ambient air were obtained by Air Quality Monitoring Centre (AQMC; Zarandi *et al*. 2019).

Period of exposure

Exposures were performed in 3 and 6 month periods. Exposure frequency was 4 days per week with 5 h (9.00 am to 14.00 pm) per day.

Determination of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and malondialdehyde (MDA) levels in the lung tissue

The amounts of SOD, catalase, GPx, and MDA in the lung tissue of rats exposed with and without $PM_{2.5}$ for 3- and 6- month periods were measured using special SOD (cat number: NS-15033), catalase (cat number: NS-15054), GPx (cat numer: NS-15082), and MDA (cat number: NS-15023) measuring kits respectively, according to the manufacturer's instructions (Navand Salamat, Iran). In brief, 10 mg lung tissues were dissected and washed

with chilled 1X PBS to remove any blood. The tissues were then transferred to a homogenizer and 500 µL lysis buffers were added for 30 min. Finally, tissues were centrifuged at 10,000 g for 20 min at 4 °C to pellet cell debris, and the supernatants were used to analyse SOD, catalase, GPx, and MDA according to the manufacturer's instructions. The activities of antioxidant enzymes were measured in the lung tissue of rats by special Kits. Also, lung histology and the expression of the P53, Nrf2, and HO-1 protein was performed in the lung tissue of rats exposed to $PM_{2.5}$.

Lung Histology

The right lung of rats was removed and then washed using PBS. Then, extracted tissues were placed in 4% formalin for two weeks. Tissue processing was performed with Autotechnicon. Tissue sections were cut at a thickness of 4 µm and stained with Haematoxylin and Eosin (H & E). The total lung damage score (LIS) was determined based on the mean damage to the bronchial, alveolar, and vascular structures based on Smith's analysis: 0: no injury to the lung tissue; (i): local lesions, necrosis or infiltration of inflammatory or hyperemia up to 25% in lung tissue; (ii): localized lesions, necrosis or penetration of inflammatory or hyperemia between 25 and 50% in lung tissue; (iii): localized lesions, necrosis or penetration of inflammatory cells or extensive hyperemia but focal of lung tissue; (iv): damage, necrosis or penetration of inflammatory cells or extensive hyperemia in lung tissue

Immunohistochemical staining

Immunohistochemical staining was performed as previously described with some modification. The expression levels of p53 were measured immunohistochemically in the paraffin-embedded lung sections. The slides were immersed in a 10 mM citrate buffer solution with pH 6.0. We immersed the slides in water for 5 min in 3% H_2O_2 and washed with PBS in order to quench endogenous peroxidase activity. Then the slides were blocked with 10% bovine serum albumin (BSA) at 37 °C for 45 min. Afterward, the slides were covered and incubated with 100 µL rabbit anti-rat p53 (Cat number: ab131442, abcam) antibody for 2 h at room temperature (RT), then washed and covered with goat anti-rabbit IgG H & L (HRP; Cat number: ab205718, abcam) secondary antibody and incubated for 30 min at RT. After washing with PBS, the slides were covered and incubated with 200 µL DAB substrate solution for 15 min. Finally, slides were visualized under an Olympus microscope.

Western blot analysis

After 3 and 6 months exposing, to evaluate the expression level of nuclear factor erythroid 2–related factor 2 (Nrf2) and Heme oxygenase 1 (HO-1) in the lung tissue of rats exposed with or without to $PM_{2.5}$, western blot analysis was performed as previously described with some modifications (Babaei *et al.* 2018; Jabarpour *et al.* 2018; Vafaei *et al.* 2017). In brief, rat lung tissues were lysed using RIPA lysis buffer (Cat number: R0278, Sigma). A total of 20 µg lysed tissue were mixed with equal volume of 2× laemmli sample buffer. 15 µg from lysate tissue was then subjected to SDS-PAGE and transferred to a 0.2 µm immune-Blot™ polyvinylidene difluoride (PVDF) membrane (Cat No: 1620174; Bio-Rad Laboratories, CA, USA). The membranes were then blocked with 5% BSA for 45 min and incubated with following primary antibodies including, anti-beta actin-loading control (Cat No: ab8227; Abcam), rabbit anti-Nrf2 (Cat No: ab92946, Abcam), rabbit anti-HO-1 (Cat No: ab13243, Abcam) antibodies for 2 h at RT. The membranes were then washed thrice with TBST and incubated with goat anti-rabbit IgG H & L (HRP; 1:10000; Cat No: ab97051; Abcam) antibody for 45 min at RT. To visualize bands, the membranes were incubated with enhanced chemiluminescence (ECL) for 1-2 min. We used β-actin as an internal control and protein expression was normalized. Densitometry of protein bands was performed using the Gel Analyzer 2010a software, such that, the percentage area under the curve of each band was divided by the percentage area under the curve of its corresponding β-actin band, and then calculated values were compared between groups as described previously (Keshavarz *et al*. 2019; Siavashi *et al*. 2019).

Statistical analysis

Data analyses were performed by GraphPad Prism 8.4.2 (GraphPad Software). The data were entered into the Prism software and the results were expressed as mean ± standard deviation (SD). The significance of differences between groups was performed using One-Way ANOVA. Tukey test was used as a post-hoc test. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

MDA and antioxidant enzyme activity levels in rats exposed to $PM_{2.5}$ were investigated in this study. MDA levels in lung tissue were significantly higher in rats exposed to $PM_{2.5}$ for both 3-month and 6-month periods compared to the control and gas groups (Figs. 1A-B). The activities of total SOD, catalase, and GPx in lung tissue were significantly lower in the $PM_{2.5}$-exposed rats for both 3-month and 6-month periods compared to the control and gas groups (Figs. 1A-B).

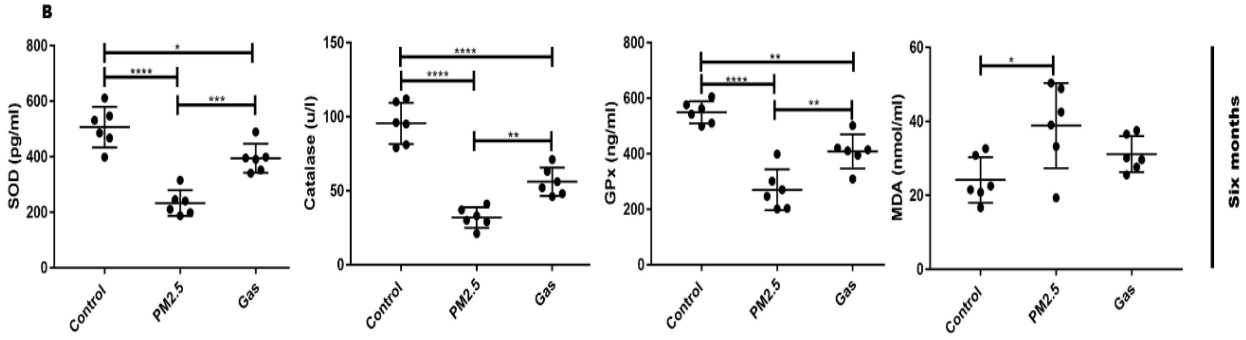

Fig. 1. The level of oxidative stress in lung tissue of rats exposed to $PM_{2.5}$ for 6 months. The levels of SOD, catalase, GPx and MDA (B) 6 months after exposure in the tissue of rats in each group. Data are expressed as mean ± SD (n = 5, each group). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ***p < 0.001 (One-Way ANOVA with Tukey post-hoc test).

Inflammatory cell infiltration was frequently observed in the lung tissue of rats exposed to $PM_{2.5}$. After 3 and 6 months of $PM_{2.5}$ exposure, inflammatory cell infiltration was observed in the pulmonary interstitium. Hyperplasia of goblet cells, hyperemia, and pneumocyte necrosis were observed in the $PM_{2.5}$-exposed group and to a lesser extent in the gas group (Fig. 2). The control animals exhibited a normal alveolar sac structure with uniform gas exchange, while the $PM_{2.5}$-exposed animals showed relatively dense alveolar sac structures (Fig. 2).

P53 expression was inhibited in lung tissue of rats exposed to $PM_{2.5}$. Immunohistochemistry was performed to investigate the effects of $PM_{2.5}$ on P53 expression. The results demonstrated a significant decrease in P53 expression in the $PM_{2.5}$ group compared to the other groups (Fig. 3). The reduction in P53 expression was further enhanced with increasing pollution levels. The expression of Nrf2 and HO-1 was reduced by $PM_{2.5}$. Western blotting was conducted to assess the potential impact of $PM_{2.5}$ on the Nrf2 pathway (Fig. 4A). Our findings revealed a significant decrease in Nrf2 and HO-1 expression in the lung tissue of rats exposed to $PM_{2.5}$ after a 3-month exposure period ($p < 0.05$) (Figs. 4B-C). Additionally, the expression levels of HO-1 protein in the gas group were significantly decreased compared to the control group after a 3-month exposure ($p < 0.05$). There was no significant difference in Nrf2 expression between the control and gas groups after a 3-month exposure ($p > 0.05$). After a 6-month exposure, the expression levels of both HO-1 and Nrf2 proteins significantly decreased in the lung tissue of rats exposed to $PM_{2.5}$ compared to the other groups ($p < 0.05$). Similarly, the expression levels of HO-1 and Nrf2 proteins in the gas group were significantly decreased compared to the control group ($p<0.05$; Fig. 4).

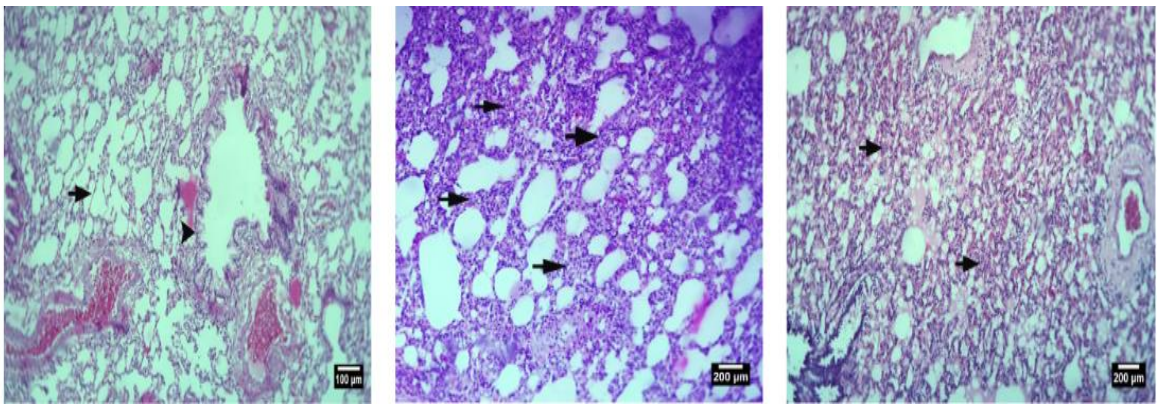

Fig. 2. Pathogenic effects of $PM_{2.5}$ on the lung tissue of rats exposed to $PM_{2.5}$. (B) 6 months after exposure in each group (n = 5, each group).

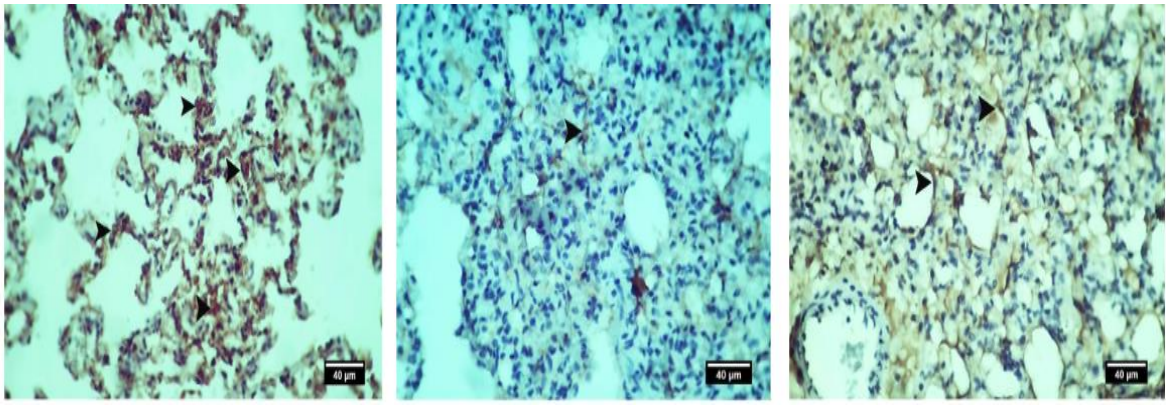

Fig. 3. Immunohistochemical staining for P53 of lung sections of rats exposed to $PM_{2.5}$ 6 months after exposure in each group (n = 5, each group).

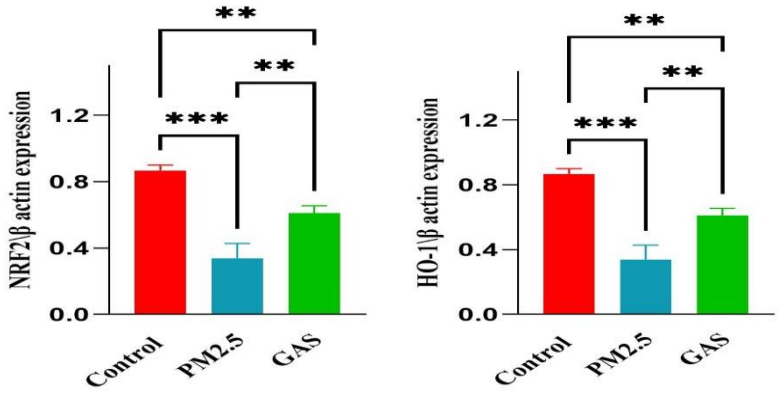

Fig. 4. Western blot expression of Nrf2 and HO-1 in lung tissues of rats exposed to $PM_{2.5}$. Western blot images and relative expression of Nrf2 (B) and HO-1. The percentage area under the curve of each protein band was divided by the percentage area under the curve of the corresponding β-actin band, and the normalized data were statistically compared between groups. Data were expressed as mean ± SD (three independent experiments were performed in triplicate). **p < 0.01, **p < 0.001(One-Way ANOVA with Tukey post-hoc test).

DISCUSSION

In this study, we evaluated the destructive effects of $PM_{2.5}$ on lung tissue through various analytical methods including histological analysis, immunohistochemistry, immunoblotting, and assessment of oxidative stress. The impact of $PM_{2.5}$ on lung function has been a subject of controversy, and understanding its detrimental mechanisms can aid in the development of effective therapeutic strategies. Airborne particles, including $PM_{2.5}$, contribute to numerous lung diseases that result in thousands of deaths worldwide each year. However, a comprehensive investigation into the effects of $PM_{2.5}$ on lung function has not been conducted. Our findings demonstrate that $PM_{2.5}$ causes injury to lung tissue, reduces the activity of antioxidant enzymes such as SOD, catalase, and GPx, and increases oxidative stress indicators like MDA. The latter (MDA), a marker of lipid peroxidation, is a sensitive test for assessing oxidative damage. In our study, the levels of MDA in the lung tissue of rats exposed to $PM_{2.5}$ were higher than those in the healthy control group. Several studies have reported elevated MDA levels in response to air pollution exposure (Lodovici *et al*. 2011; Zhang *et al*. 2020). This increase in MDA levels can be particularly harmful, leading to complications in lung function, chronic respiratory tract inflammation, and impaired clearance ability (Sint *et al*. 2008). Research on patients with chronic obstructive pulmonary disease exposed to $PM_{2.5}$ has shown a significant increase in oxidative-antioxidant imbalance, reflected by upraised MDA levels and reduced SOD activity (Wang *et al.* 2020). Air pollution exposure has been widely associated with oxidative stress-induced chronic obstructive pulmonary disease (Marginean *et al*. 2018), cardiovascular disease (Fiorito *et al*. 2018), autoimmune diseases (Gawda *et al.* 2017), and central nervous system disorders (Block & Calderón-Garcidueñas 2009). Histologically, our study revealed inflammatory cell infiltrations in the pulmonary interstitium, along with hyperplasia of goblet cells and pneumocyte necrosis in the $PM_{2.5}$-exposed group, albeit to a lesser extent in the gas-exposed group. These findings indicate that air pollution can cause histological damage to the respiratory tract. Recent studies have shown that long-term exposure to air pollution is associated with an

increased risk of lung histological conditions, such as lung adenocarcinoma (Moon *et al*. 2020). The histological features observed in our study can be attributed to reactive oxygen species (ROS) induction. Immunohistochemical and immunoblotting analyses revealed a significant reduction in the expression of P53, Nrf2, and HO-1 proteins in the lung tissues of rats exposed to air pollution for three and six months. A recent study reported increased levels of p53 in individuals exposed to air pollution compared to healthy controls (Jalali-Mashayekhi *et al*. 2020), which is inconsistent with our findings. However, this discrepancy may be attributed to the duration of exposure. Nevertheless, increased p53 levels have been observed in humans. P53 regulates various genes involved in cellular senescence, DNA repair, cell cycle arrest, and apoptosis. In genetically unstable cells, p53 can induce apoptosis by interacting with various anti-apoptotic and pro-apoptotic factors (Feroz & Sheikh 2020). It has been observed that increased ROS levels activate Nrf2, a transcription factor that controls the expression of numerous antioxidant enzymes, including HO-1 and SOD. Failure of the antioxidant response mediated by Nrf2 can result in more detrimental effects, including inflammation and programmed cell death (apoptosis), leading to pulmonary diseases (Kooter *et al*. 2008). Moreover, most antioxidants are regulated by the Nrf2 signalling pathway and its downstream factor, HO-1 (Kaspar *et al*. 2009). In our study, the levels of Nrf2 and HO-1 proteins in the lung tissue of $PM_{2.5}$-exposed rats were significantly lower compared to the other groups. NRF has been shown to protect lung cells against oxidative damage following PM exposure (Pardo *et al*. 2019). Numerous studies have confirmed that when cells are exposed to $PM_{2.5}$, Nrf2 acts as a signalling mediator for mitochondrial function (Ge *et al*. 2020; Pardo *et al*. 2019). Nrf2 deficiency exacerbates $PM_{2.5}$-induced production of pro-inflammatory cytokines and neurotoxicity in microglia of the olfactory bulb (Chen *et al*. 2018). The increased oxidative stress caused by $PM_{2.5}$ can stimulate airway epithelial cells and lung surface macrophages, leading to the release of inflammatory mediators such as IL-1β, TNF-α, GM-CSF, IL-6, and IL-4, thereby contributing to chronic lung inflammation (Jimenez *et al*. 2002; Song *et al*. 2017). The histopathological analysis in our study also revealed interstitial pneumonia in rats with long-term exposure to $PM_{2.5}$. The respiratory damage caused by $PM_{2.5}$ is a complex pathological process in which $PM_{2.5}$ particles enter the lung tissue and interact with macrophages and lung epithelial cells in the alveoli.

CONCLUSION

In conclusion, exposure to fine particulate matter ($PM_{2.5}$) leads to significant detrimental effects on pulmonary tissue. According to our findings, $PM_{2.5}$ diminished the functionality of antioxidant enzymes by triggering the Nrf2 and P53 signalling pathways, consequently leading to an escalation in oxidative damage.

Conflict of interest

The authors have no conflict of interest.

Author's contributions

Pejman Mortazavi and Saeed Motesaddi Zarandi designed the study. Mahdi Farhadi and Akram Eidi contributed to collecting the data. Mahdi Farhadi wrote the manuscript. All authors read and approved the manuscript.

Ethical approval

The Ethical Committee of Islamic Azad University approved the study.

Consent to participate

Not applicable.

Consent for publication

None

FUNDING

None.

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