



Cytotoxicity, Oxidative Stress, and Autophagy in Human Alveolar Epithelial Cell Line (A549 Cells) Exposed to Standardized Urban Dust

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Abstract

Exposure to urban airborne particulate matter (PM) associates with adverse health effects, but the exact mechanisms remain unclear. In this study, we focused on cytotoxicity (MTT), oxidative stress (DCF/FC), DNA damage (PI/FC), necrosis/apoptosis (FC), and autophagy (LC3 expression; WB/FC) triggered by urban dust (UD) in naïve human alveolar epithelial A549 cells and in the cells with reduced glutathione (GSH). The A549 cells were grown in F12K/FCS media supplemented with coarse carbon black (CB; Huber990; 260 nm diameter; 200 $\mu\text{g}\cdot\text{ml}^{-1}$) or urban dust (UD; Standard Reference Materials; 200 $\mu\text{g}\cdot\text{ml}^{-1}$) for 24 h. To deplete intracellular glutathione (GSH), l-buthionine-(S,R)-sulfoximine (BSO; 100 mM; 24 h) was used. Pre-treatment with BSO depleted the cellular GSH by about 30%. A similar effect was noticed after UD. The CB was without any effects on the parameters tested, except for LC3 expression (autophagy) which increased by about twofold. However, UD decreased cell viability by about 27%, decreased cell proliferation in BSO pre-treated

cells, increased ROS production, and increased both Hsp70 and LC3 proteins by about twofold, but most changes were unrelated to ROS-mediated GSH depletion. We conclude that urban dust-induced oxidative stress is important in PM toxicity, but other as yet unrecognized mechanisms are also involved.

Keywords

A549 cells · Alveolar cells · Apoptosis · Autophagy · Cytotoxicity · Oxidative stress · Particulate matter · Urban dust

1 Introduction

Exposure to air pollutants causes many health problems including asthma, COPD, cardiopulmonary diseases, lung cancer, and birth defects (Li et al. 2019; Ali et al. 2018). The consequences of exposure are highly unpredictable, but the exposure brings about persistent inflammation of the airways (Hankey and Marshall 2017; Hüls et al. 2017). Short-term exposures to air particulate matter (PM) usually exacerbate pre-existing diseases, especially in respiratory and cardiovascular system, depending on the individual age and health status, and increase hospital admissions. Long-term exposures, on the other side, increase

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the rate of disease progression and significantly reduce life expectancy (Wong et al. 2016). Due to high inconsistency of clinical and experimental data in this field, it is crucial to establish some biomarkers for PM exposure, which may unravel the complexity of relationship between exposure and respiratory health. There are two main protective systems in the upper and lower respiratory tracts, i.e., physical and biological. Lung epithelial cells can be considered mostly as a physical barrier, but low molecular PM can also penetrate into interstitial spaces and induce inflammatory response (Li et al. 2010). The respiratory epithelium consists of various cell types responsible for gas exchange, surfactant synthesis, and immunological responses. Most of the air particles damage epithelial cells (Barker et al. 2014). It is established that PM-induced toxicity is strongly related to oxidative stress (Münzel and Daiber 2018). PM is capable of directly producing reactive oxygen species (ROS), causing tissue damage, inflammation, and eventually cell death (Shang et al. 2017). Experimental data show that ROS are increased in the human alveolar epithelial cell line – A549 cells, exposed to PM (Peixoto et al. 2017). Another interesting feature of PM toxicity is altered autophagy (Pesonen and Vähäkangas 2019), which is a self-digestion lysosome-related process aimed at reprocessing damaged organelles and other cellular constituents accumulated in a double-membrane vesicles called autophagosomes (Ramesh et al. 2019). In healthy cells, damaged cellular components are degraded in lysosomal pathways, but in pathology, altered autophagy may promote inflammation and further cell damage. It has been shown that autophagy can be stimulated by redox imbalance, including ROS, but also by nutrient deprivation and infections (Abounit et al. 2012; Azad et al. 2009). In recent years, evidence accumulates in support of a notion that PM-induced ROS are important to autophagy (Levonen et al. 2014; Filomeni et al. 2010), but it is still unclear of how ROS and inflammation drives autophagy in PM-exposed cells. Therefore, in this study we seek to explore the mechanisms of PM-induced ROS and autophagy using

standardized urban dust (UD) and the human alveolar epithelial A549 cells.

2 Methods

2.1 Cell Culture

A549 (ATCC® CCL185™) cells grown in Dulbecco's Modified Eagle's Medium (F12K/FCS) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ were used in this study. For particular experiments, the cells were plated out onto 6- or 24-well plates and were grown in culture media supplemented with coarse carbon black (CB) or with urban dust (UD) for 24 h (both 200 µg·ml⁻¹).

2.2 Cell Treatment

The conditioned media were prepared using a commercial standardized UD, purchased from the National Institute of Standards and Technology (Gaithersburg, US) and a CB, Huber 990, a primary particle diameter of 260 nm, purchased from H. Haeffner and Co. Ltd. (Chepstow, UK). The latter was used as a reference substance. According to the Certificate of Analysis of Standard Reference Material 1649b, the particle size of UD is within a range of 0.2–110 µm, with the mean particle size of about 10 µm. For experiments, particles were suspended in the cell culture medium at a concentration of 200 µg/ml and were sonicated in a Bandelin Sonopuls ultrasonic homogenizer (Berlin, Germany) for 30 s prior to use. The UD and CB-conditioned media were used within 5 min of preparation. In some experiments, A549 cells were pre-treated overnight with 100 µM buthionine sulfoximine (BSO) to deplete intracellular glutathione (GSH) levels. Cell-free controls were included to each experiment in order to assess the interference of particles with each assay.

2.3 Cell Viability and Proliferation

Cell viability was assessed with 3-[4,5-dimethylthiazolyl-2] 2,5-diphenyltetrazolium bromide (MTT) assay (Niks and Otto 1990) and by flow cytometry estimation of proliferating cell numbers. The MTT and cell cycle assays were performed after 24 h of cell treatment with UD or CB. In the MTT test, changes in absorbance due to formazone production in viable cells were measured using a double beam Perkin Elmer spectrophotometer (Waltham, MA) at 570 nm, with a 630 nm reference wavelength. Cell viability was estimated as a percentage of the control that was considered 100% viable. To quantify alterations in growth rates, cells were stained for 30 min with propidium iodide (PI; 50 µg per ml) in TRIS buffer (100 mM; pH 7.5), containing 0.1% potassium cyanide, 0.01% NP-40 detergent, 40 µg per ml Type III-A RNase, and 0.1% NaN₃. The DNA profiles in particular cells and cell cycle analysis were performed in the aligned FACSCanto II, flow cytometer (BD Biosciences Systems; San Jose, CA), equipped with an argon laser operating at 488 nm with adjusted forward angle and side light scatter. PI fluorescence was measured in 5,000–10,000 cells, and DNA fluorescence histograms were analyzed by cell cycle software (Flowing Software v2.5; Turku, Finland). The cells were quantified by their relative distribution in the S-phase (DNA synthesis) and G2/M (post DNA synthesis/mitosis) phases of the cell cycle and were assigned as proliferating cell fractions.

2.4 Oxidative Stress

Intracellular generation of reactive oxygen intermediates was quantified in control cells and in cells treated with UD and CB using dichlorodihydrofluorescein diacetate (H₂DCFDA) (Sigma-Aldrich; St. Louis, MO) according to Ubezio and Civoli (1994). The cells were loaded with 5 µM H₂DCFDA for 30 min, washed, resuspended in phosphate-buffered saline, and

assayed by flow cytometry. Green dichlorofluorescein (DCF) fluorescence was captured on FL1 channel and registered as histograms of fluorescence distribution.

2.5 Autophagy and Heat Shock Protein 70

Microtubule-associated protein 1A/1B-light chain 3 protein (LC3) and heat shock protein 70 (Hsp70) were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis – immunoblotting and by flow cytometry using appropriate rabbit monoclonal antibodies recognizing human Hsp70 (Abcam; Cambridge, UK) or LC3 proteins (Cell Signaling Inc.; Danvers, MA) and positive and negative controls. For Western blots, 10 µg (for Hsp70) or 20 µg (for LC3) of cell homogenate proteins were separated in reducing conditions by 10% or 20% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE), respectively. The proteins were transferred onto polyvinylidene difluoride membranes and incubated with specific secondary antibodies linked to alkaline phosphatase. The bound complexes were detected using BCIP (Sigma Chemical Co., Poznan, Poland) and quantified using Image Quant software (BioRad; Warsaw, Poland). A constitutively expressed protein, β-actin, served as a loading control, and the data were quantified in respect to β-actin expression. Samples were run in duplicate on each gel, and the mean values ±SD were expressed as 100 relative units to compare the data from different experiments. For flow cytometry analysis, cells were fixed in 0.01% formaldehyde for 10 min, permeabilized with 0.01% NP-40 in phosphate-buffered saline (PBS), washed with PBS, stained with specific monoclonal antibodies against LC3 (LC3A/B rabbit mAb, Alexa Fluor 488 Conjugate; Cell Signaling Inc., Danvers, MA) or with Hsp70-specific antibody bound to FITC (Abcam, Cambridge, UK), and incubated for 30 min at 4 °C. The cells were then washed, centrifuged, and resuspended in 500 µl of ice-cold

PBS containing 10% fetal calf serum (FCS) and 0.1% NaN_3 . Samples were analyzed with a FACSCanto II flow cytometer (BD Biosciences Systems; San Jose, CA) equipped with a standard filter setup.

2.6 Apoptosis

A fluorescein isothiocyanate (FITC)-conjugated annexin V (Clontech Labs, Takara BioEurope; Saint-Germain-en-Laye, France) was used to detect apoptotic cells (Demchenko 2012). The A549 cells were harvested, washed twice with PBS pH 7.4, incubated in annexin V-labeling solution (final annexin V concentration, 0.5 $\mu\text{g}/\text{ml}$), washed again, and analyzed with a FACSCanto II flow cytometer (BD Biosciences Systems; San Jose, CA). Green FITC fluorescence was captured on FL1 channel through a 530 nm, 30 nm bandwidth band-pass filter. All analyses were performed at a low rate settings, with <1,000 events/s. Experimental data were plotted as fluorescence histograms and were analyzed using the Flowing Software v2.5 (Turku, Finland).

2.7 Proteins and Glutathione (GSH) Levels

Homogenate proteins were measured using the bicinchoninic acid (BCA) kit (Sigma-Aldrich; Poznan, Poland). GSH was quantified in deproteinated samples using an assay kit (Cayman Chemical; Ann Arbor, CA) that contains glutathione reductase and 5,5'-dithio-bis-2-nitrobenzoic acid and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB) measured at 405 nm.

2.8 Data Analysis

Data were expressed as means \pm SD of 6–10 assays. Statistical differences were assessed using one-way or two-way analysis of variance

ANOVA, followed by the Bonferroni post hoc test for selected pairs of data. A p -value < 0.05 defined statistically significant changes. Statistical analysis was performed using a commercial Statistica v6.0 package (StatSoft; Tulsa, OK).

3 Results

Table 1 and Fig. 1 show the effect of CB and UD on cell viability (MTT test), proliferation (S + G2/M cells), oxidative stress (DCF fluorescence), apoptosis (annexin binding), Hsp70 expression, and autophagy (LC3 expression) in A549 cells treated for 24 h with 200 $\mu\text{g}\cdot\text{ml}^{-1}$ CB or UD. Moreover, experiments with A549 cells pre-treated for 24 h with BSO to deplete GSH also are included. In the cells pre-treated with BSO, GSH levels decreased by about 30%, and a similar decrease was observed in UD-treated cells (results not shown). The UD, but not CB, decreased cell viability by about 27% (Fig. 1, panel a) ($p < 0.01$), and a similar effect was observed in the cells pre-treated with BSO ($p < 0.01$). Cell proliferation was affected neither by CB nor UD (Fig. 1; panel b). However, when the A549 cells were pre-treated with BSO and then treated with UD, a significant reduction by about 15% ($p < 0.05$) of S + G2/M cells was noticed. Both BSO and UD induced oxidative stress in A549 cells, but these effects were not additive when both compounds were applied sequentially to the cells (Fig. 1; panel c). Concerning the induction of apoptosis, a slight increase of annexin binding to the cell membrane was detected only in the cells pre-treated with BSO and then treated with UD (Fig. 1; panel d). The maximum number of apoptotic cells was about 9% ($p < 0.05$). BSO increased both Hsp70 (by about 33%; $p < 0.05$) and autophagy (about sixfold; $p < 0.01$), while UD induced comparable increases (by more than two-fold) in both groups. The effects of BSO and UD on autophagy, but not on Hsp70, were additive ($p < 0.01$), which was also noticed in the Western blotting, while CB partly normalized increased autophagy induced by BSO ($p < 0.01$).

Table 1 The effects of urban dust (UD) and carbon black (CB) on cell viability (MTT test), proliferation (S + G2/M cells), oxidative stress (dichlorofluorescein fluorescence), apoptosis (annexin binding), Hsp70 (flow cytometry), and autophagy (LC3 expression; flow cytometry) in human alveolar epithelial cell line A549. Naïve or buthionine sulfoximine-pre-treated cells (glutathione depleting agent; 24 h; 100 $\mu\text{g}\cdot\text{ml}^{-1}$) were grown for 24 h in medium

supplemented with 200 $\mu\text{g}/\text{ml}$ UD or CB. The results of MTT were quantified photometrically; all other data were quantified in flow cytometry and are shown as fractions (%) of positive or negative cells (proliferation, apoptosis). Median fluorescence intensities were also calculated (oxidative stress, Hsp70, and autophagy) and were shown as means \pm SD of 3–5 experiments

	Cell viability (%)	Proliferation (%)	Oxidative stress (relative units)	Apoptosis (%)	Hsp70 (relative units)	Autophagy (relative units)
Control	100 \pm 8	55 \pm 7	100 \pm 38	5 \pm 1	100 \pm 11	100 \pm 45
BSO	102 \pm 10	51 \pm 6	130 \pm 41*	4 \pm 2	133 \pm 9*	600 \pm 223**
CB	94 \pm 7	56 \pm 5	111 \pm 61	6 \pm 2	105 \pm 13	132 \pm 54
UD	73 \pm 6**	54 \pm 5	182 \pm 65**	5 \pm 2	222 \pm 18***^^	252 \pm 112***
BSO + CB	88 \pm 8	56 \pm 6	116 \pm 63	4 \pm 1	80 \pm 21*^	334 \pm 126***^^
BSO + UD	8 \pm 6**	47 \pm 4*	173 \pm 58***	9 \pm 2 *** ^	266 \pm 32***^^	1,122 \pm 478***^^

* $p < 0.05$; ** $p < 0.01$ for comparisons to the corresponding control cells;

$p < 0.05$; ## $p < 0.01$ for comparisons to the corresponding BSO-treated cells;

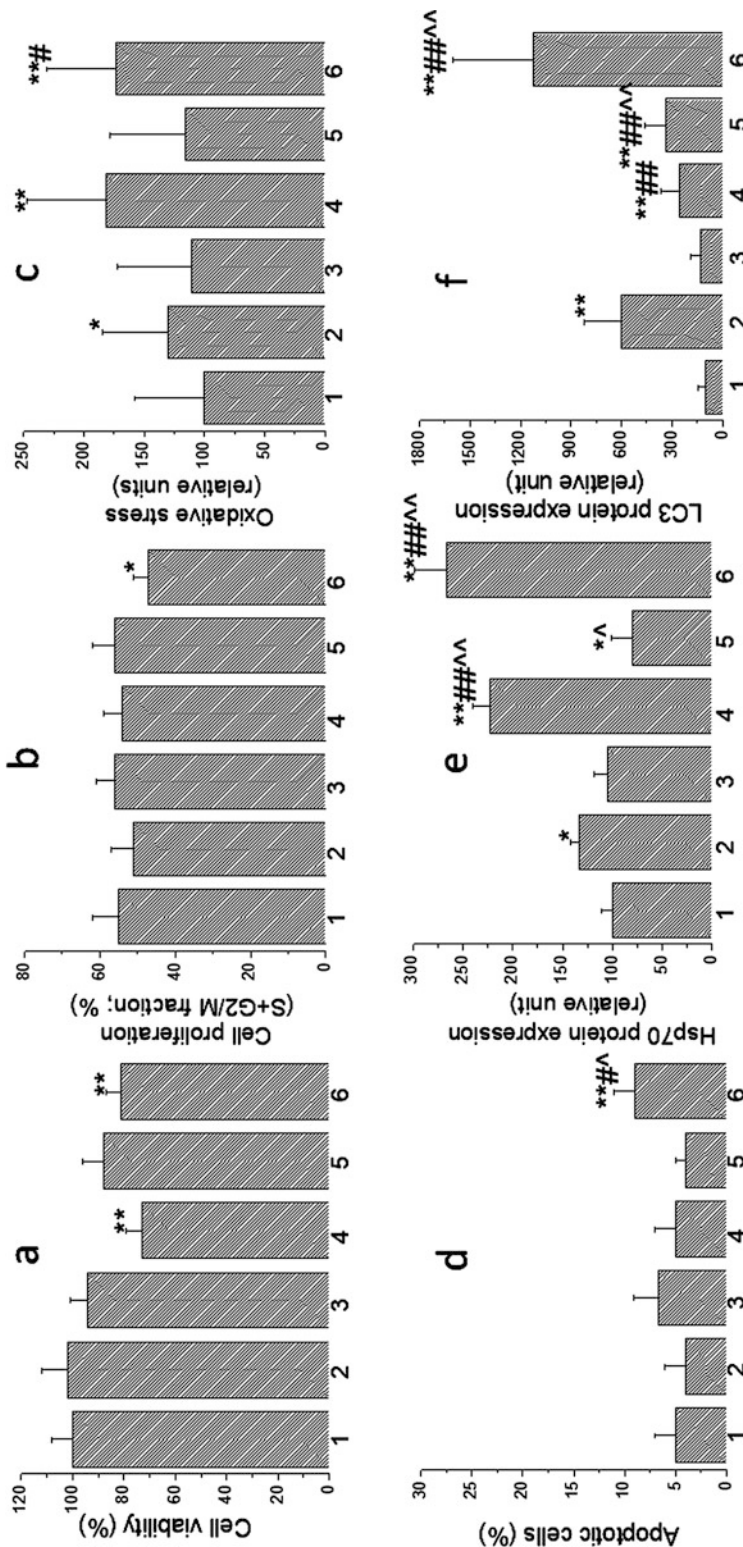
^ $p < 0.05$; ^^ $p < 0.01$ for comparisons to CB or UD, respectively

4 Discussion

Airborne PM are now a major public health concern. Contaminated air may contain a plethora of reactive compounds including hydrocarbons, metals, and fine and ultrafine particles which, binding to cell membranes, are capable of producing ROS, alter cell maturation and growth, induce apoptosis or necrosis, affect autophagy, and produce inflammation and cancer (Ali et al. 2018; Münzel and Daiber 2018; Santibáñez-Andrade et al. 2017). Consequences of exposure to PM are highly variable, but increasing evidence supports the notion that PM-induced ROS increase and a resulting redox imbalance may have highly erosive, time-dependent effects. Oxidative stress has been detected not only in experimental models of airborne toxicity but also in clinical samples acquired from exposed individuals (Tao et al. 2003). It has been shown that ROS may affect autophagy (Levonen et al. 2014; Filomeni et al. 2010), which is relevant to the homeostatic balance of cells exposed to stress. Consequences of acute and not severe toxic stress can usually be repaired, while extended stress usually exceeds cellular adaptive capacity and causes cell death. Consequently, autophagy may be equally involved in cell protection as in cell

death. The role of autophagy in toxicology has been described, and its importance in PM-induced respiratory diseases is now recognized (Ryter and Choi 2010). However, the exact mechanisms through which PM triggers biochemical and functional responses and activates chronic inflammation or cancer remain elusive.

In this study we sought to explore the mechanisms of PM-induced ROS, redox imbalance, and autophagy using standardized UD and human alveolar A549 cell line. The cells pre-treated with BSO had a lower level of GSH by about 30%. A similar decrease in GSH was noticed in the UD-treated cells, but not in the cells grown with a coarse CB which is considered a chemically neutral and nontoxic substance (Megido et al. 2016). Similar effects have been earlier described in alveolar macrophages by Geng et al. (2005) and Zhang et al. (2015), where $\text{PM}_{2.5}$ produce a dose-dependent decline of GSH and increase oxidative stress. In those studies 300 $\mu\text{g}/\text{ml}$ of $\text{PM}_{2.5}$ decreased GSH by about 33%, while a lipid peroxidation product, malondialdehyde, increased by about 47%. In the present experimental model, UD decreased intracellular GSH by about 30% and diminished cell viability by about 27%. However, a similar



1- Control; 2- BSO; 3- CB; 4- UD; 5- BSO+CB; 6- BSO+UD

Fig. 1 The effects of urban dust (UD) and carbon black (CB) on cell viability (a), proliferation (b), oxidative stress (c), apoptosis (d), Hsp70 (e), and autophagy (f) in human alveolar epithelial cell line A549. Cells were treated for 24 h with 200 µg/ml UD or CB. In some experiments, the cells were pre-treated for 24 h with 100 µg/ml buthionine sulfoximine (BSO) to deplete their glutathione. Except for MTT test, all data were quantified in flow cytometry and are shown as fractions (%) of positive or negative cells (proliferation, apoptosis), Median fluorescence intensities were calculated (oxidative stress, Hsp70, and autophagy) and are shown as means ± SD of 3–5 experiments.

* $p < 0.05$; ** $p < 0.01$ for comparisons with the corresponding control cells; # $p < 0.05$; ## $p < 0.01$ for comparisons with the corresponding BSO-treated cells; ^ $p < 0.01$ for comparisons with CB or UD, respectively

decrease in GSH by BSO was without a significant effect on cell survival. Moreover, when both GSH-depleting compounds, BSO and UD, were sequentially applied to the cells, UD toxicity was not enhanced. It is, therefore, possible that GSH depletion is not a prerequisite for acute UD toxicity in A549 cells. Cell proliferation was unaffected by CB and UD. However, when A549 cells were pre-treated with BSO and then treated with UD, a significant reduction in the number of dividing cells was noticed. The experiments with low molecular size PM have indicated that its low concentration suffices to produce G2/M arrest in cultured epithelial cells (Gualtieri et al. 2010). In the present study, proliferation of A549 cells was inhibited by UD only when the cells were pre-treated with BSO. In the same group of cells, a slight increase in apoptotic cell numbers was found, but the maximum fraction of apoptotic cells was less than 10%, and it seems that the unspecific annexin binding cannot be excluded in that type of toxic stress. It is known that PM is able to induce oxidative stress (Münzel and Daiber 2018).

In the present study, pro-oxidative alterations were detected in cells treated with BSO, in cells grown with UD, and in cells treated successively with both compounds. However, there was no additive effect of BSO and UD on oxidative stress. An early marker of oxidative stress and unfolded protein response, Hsp70 protein, also increased in the groups of A549 cells with elevated ROS, which may indicate oxidative protein damage. The important role of oxidative stress in cellular responses is evidenced in the experiments with cell exposure to airborne pollutants and antioxidants which partly blocked PM-induced DNA damage and G2 arrest (Longhin et al. 2013). In the present study, BSO produced very high increase in LC3 expression, which can be indicative of increased autophagy. CB was without an effect on LC3, while UD stimulated autophagy by more than twofold. It is possible that enhanced autophagy may represent an adaptive reaction to stress. Recently, enhanced autophagic activity has been linked to reparation of damaged DNA. However, excessive activation of autophagy due to hypoxia-ischemia or

starvation has also been observed in cells undergoing cell death (Albrecht et al. 2019). We have previously shown that UD may induce DNA damage in A549 cells, including both single- and double-strand breaks that occurred as early as 1 h of cell exposure to UD and persisted for many hours in the presence of UD (Mroz et al. 2008). It is possible that cell exposure to UD may generate, to some extent, a genotoxic insult, followed by increased autophagy and subsequent protein translocation from the cytosol to the nucleus to repair and maintain genome integrity.

In summary, this study provides evidence for the role of redox imbalance in urban dust cytotoxicity and in autophagy in epithelial cells. Nevertheless, further studies are needed to clarify the relationship between redox-dependent and redox-independent aspects of urban dust toxicity and their relation to autophagy, which seems essential for the understanding of chronic inflammation and genomic stability in lung epithelial cells exposed to environmental pollutants.

Conflicts of Interest The authors had no conflicts of interest to declare in relation to this article.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors. The study has been approved by an institutional research ethics committee.

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