

African Dust Storms Reaching Puerto Rican Coast Stimulate the Secretion of IL-6 and IL-8 and Cause Cytotoxicity to Human Bronchial Epithelial Cells (BEAS-2B)

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ABSTRACT

African dust storm events (ADE) travel across the Atlantic Ocean (ADEAO) and reach the Puerto Rican coast (ADEPRC), potentially impacting air quality and human health. To what extent seasonal variations in atmospheric particulate matter (PM) size fractions, composition and sources trigger respiratory-adverse effects to Puerto Ricans is still unclear. In the present study, we investigated the pro-inflammatory and cytotoxic effects of PM samples harvested during ADEAO (PM₁₀), ADEPRC (PM_{2.5} and PM₁₀) and Non-ADE (Pre- and Post-ADEAO and Non-ADEPRC), using BEAS-2B cells. Endotoxins (ENX) in PM_{2.5} and PM₁₀ extracts and traces of metals (TMET) in PM_{2.5} extracts were also examined. IL-6 and IL-8 secretion and cytotoxicity were used as endpoints. ADEAO and ADEPRC extracts were found to be more cytotoxic than Non-ADE and ADEAO were more toxic than ADEPRC extracts. PM₁₀ extracts from ADEAO and Post-ADEAO caused significant secretion of IL-8. IL-6 and IL-8 secretion was higher following treatment with PM₁₀ and PM_{2.5} ADEPRC than with Non-ADEPRC extracts. ENX levels were found to be higher in PM₁₀ ADEAO than in the rest of the samples tested. TMET levels were higher in PM_{2.5} ADEPRC than in Non-ADEPRC extracts. Deferoxamine significantly reduced cytotoxicity and IL-6 and IL-8 secretion whereas Polymyxin B did not. TMET in PM_{2.5} fractions is a major determinant in

ADEPRC-induced toxicity and work in conjunction with ENX to cause toxicity to lung cells *in vitro*. ENX and TMET may be responsible, in part, for triggering PM-respiratory adverse responses in susceptible and predisposed individuals.

Keywords: Dust Storm; Particulate Matter; Endotoxins; Metals; BEAS-2B Cells

1. INTRODUCTION

African dust storm events (ADE) have increased sharply since early 1970s. These environmental episodes have been attributed mainly to a drought period in the Saharan/Sahel region caused by changes in the global distribution of sea surface temperature [1-3]. Several hundred million tons of African dust is transported across the Atlantic Ocean (ADEAO) annually, leaving a trail of atmospheric pollutants throughout the Caribbean, Central and North America [4-7]. The seasonal influx of African dust, reaching the Northeastern coast of Puerto Rico (ADEPRC) during spring and summer, transports particulate matter (PM) capable of causing health-adverse effects [8-12]. However, to what degree ADEAO and ADEPRC fractions and constituents may contribute to the pathogenesis of respiratory and systemic illnesses observed in certain individuals during and after the ADEPRC, still remains unclear. More detailed (epidemiological, *in vivo* and *in vitro*) investigations are warranted to better understand the environmental factors and PM characteristics that play a critical role in the ADEPRC-

induced respiratory related diseases.

Ambient PM is a complex mixture of solid and liquid particles of different sizes from various sources with distinct chemical compositions and constituents [13-18]. African dust carries large volumes of air masses characterized by a bimodal number distribution of particle sizes with a predominant mode near 0.6 μm but shifts slightly towards larger modes when it reaches the western Atlantic/Caribbean sites (1.2 - 2.5 μm in diameter) [19,20]. Airborne PMs with a median aerodynamic diameter of equal or less than 2.5 μm ($\text{PM}_{2.5}$) and equal or less than 10 μm (PM_{10}) are known as fine and inhalable coarse fractions, respectively. Airborne $\text{PM}_{2.5}$ arises from combustion processes or atmospheric transformation of combustion emissions whereas PM_{10} contains mainly mineral particles of crustal materials. $\text{PM}_{2.5}$ and PM_{10} fractions have shown to contain organic materials (e.g., bacterial endotoxins (ENX), fungi (spores), pollen fragments, polycyclic aromatic hydrocarbons (PAH), and carbonaceous elements), as well as inorganic materials (e.g., water soluble traces of metals-TMET), minerals (quartz, silicates), salts (ammonium-sulfates and nitrates), and soil dust particles [3,14,18,21-24]. Ambient PM-induced health adverse effects can be triggered by the particle itself or the materials adsorbed to the particle [25-26]. When inhaled, this complex mixture may cause or exacerbate allergies, asthma, cardiovascular diseases and, in extreme cases, lung cancer and mortality [13-14, 19,24,27-36]. The extent of detrimental response to ambient PM exposure depends on different factors such as environmental (e.g., PM size and chemical constituents, demographics sources, climate and temperature changes, ozone and anthropogenic influences) and inter-individual variations (e.g., age, ethnicity, sex, health conditions and genetic predisposition). These multifactorial characteristics make difficult to underline the exact mechanism of PM-induced respiratory adverse effects. However, several *in vitro* studies have suggested different cell mechanisms associated to certain PM constituents such as: 1) bacterial ENX may be responsible for the inflammatory response due to the activation of Toll-like receptor-4 (TLR4) and transcription factors (e.g., NF- κ B) and the release of pro-inflammatory cytokines (e.g., IL-6 and IL-8); 2) TMET [e.g., aluminum (Al), arsenic (As), iron (Fe), cadmium (Cd), copper (Cu), nickel (Ni), mercury (Hg), lead (Pb) and vanadium (V)] may cause cellular lipids, proteins and DNA damage, mutagenicity and carcinogenicity by generating reactive oxygen species (ROS); 3) PAH may cause oxidative stress, aberrant changes in cell cycling, gene expression and DNA functions by generating toxic metabolites due to the activation of aryl hydrocarbon (Ah) receptors and induction of Phase I and II metabolizing enzymes; and 4) air pollutants such as nitro-, sulfur- and oxygen-elements (e.g.,

NO_2 , SO_2 , H_2S , O_3) may cause lung epithelial cell damage, oxidative cellular stress and carcinogenicity [3, 25-26,29,34,37-47]. Based on these and other published studies (Aust *et al.*, 2002; Wang *et al.*, 2013), inflammatory injury and oxidative damage are considered as common mechanisms of PM-induced health adverse effects.

Respiratory illnesses, such as allergy and asthma, in the Caribbean region have increased over the past 30 years, where the pediatric population has been particularly the most affected [12,48-51]. The association between respiratory illnesses in children and the presence of atmospheric contaminants as well as the mechanisms by which these adverse effects occur are not well understood. However, there is a growing body of evidence indicating that African dust storms transporting $\text{PM}_{2.5}$ and PM_{10} fractions and contaminants, such as ENX and TMET, may play a major role in the pathogenesis of respiratory illness and contribute to the increase of emergency room (ER) visits and hospitalizations of children [48,52-54].

In the present study we investigated the pro-inflammatory and cytotoxic effects of ambient PM ($\text{PM}_{2.5}$ and PM_{10}) and its specific constituents (ENX and TMET) before, during and after dust storms (ADEAO and ADEPRC) using an *in vitro* model of BEAS-2B cells. The goal was to evaluate the hypothesis that ADE influx impacts the Puerto Rican coast air quality conditions and may be responsible, in part, for the respiratory-illnesses observed in susceptible and predisposed individuals.

2. MATERIALS AND METHODS

2.1. Sampling Selection

2.1.1. PM_{10} Collected Over the Atlantic Ocean (AO)

The ADEAO PM_{10} samples used in this study were collected in 2004 during the Aerosol and Oceanographic Science Expedition (AEROSE). Details of the AEROSE science plan, mission and sampling collection have already been reported elsewhere [10,20]. Briefly, the AEROSE project consisted of a series of intensive field experiments conducted aboard the U.S. National Oceanic and Atmospheric Administration (NOAA) ship *Ronald H. Brown* during the North Hemispheric spring (March 2004) and summer (June-July 2007) expedition. PM_{10} samples were collected during the AEROSE-I, which started on February 29, 2004 in Bridgetown, Barbados and ended in San Juan, Puerto Rico on March 27. March PM_{10} samples were selected for this investigation to correlate them with samples gathered during a severe dust storm event that reached the coast of Puerto Rico during this time period. In addition, airborne dust samples present before (Pre-ADEAO) and after (Post-ADEAO)

ADEAO were also collected during the AEROSE-I expedition and analyzed for comparison purposes.

2.1.2. PM_{2.5} and PM₁₀ Collected at Fajardo, Puerto Rican Coast (ADEPRC)

A total of 28 ADEPRC were monitored during 2004. The identification of 5 of these ADEPRC in March allowed the harvest and preparation of a composite sample mixture containing sufficient material to obtain extract for this research. During spring and summer of the year studied, the PM mass concentration data showed a Puerto Rico seasonal variation pattern with high levels of PM_{2.5} and PM₁₀ fractions, which correlated with the temporal dust events pattern detected by the Total Ozone Mapping Spectrometer (TOMS). PM_{2.5} samples were collected on Teflon filters while PM₁₀ samples on quartz filters by the PR Environmental Quality Board (PR-EQB) air monitoring program. The air samplers were identified as EQB station 22, located at the northeastern coast of PR, close to a lighthouse in the municipality of Fajardo. This station is situated at a remote site (natural reserve) that minimizes possible anthropogenic PM contributions. In order to designate the days containing the PM_{2.5} and PM₁₀ events originating from the African continent, a combination of two data sources was used. One source was the daily satellite images gathered by TOMS [6] and the other was the onsite PM_{2.5} and PM₁₀ concentrations obtained by the PR-EQB (**Figure 1**). A relative increase above the lowest PM_{2.5} and PM₁₀ daily mean concentrations (2.8 $\mu\text{g}/\text{m}^3$ and 18.3 $\mu\text{g}/\text{m}^3$, respectively) at Fajardo location was established as criteria to suggest the possible occurrence of a dust event. Once it was identified by means of weight, the dates were evaluated further by the confirmation of clouds over the region using images from TOMS. The ADEPRC sample set included PM collected a day before and 1 - 2 days after the event since smaller particles from the event still remain in suspension after the main cloud front. The Non-ADE days were classified from the same period as the ADEAO. Samples harvested during the months of January, February, October, November and December of 2004, fulfilled the criteria of Non-ADE according to the guidelines mentioned above and were used as classification controls.

2.2. PM_{2.5} and PM₁₀ Extractions

All glassware used was acid washed using a modified cleanup procedure as described by Molinelli *et al.*, 2006 [22]. Briefly, all filters were extracted for a 48 h period in 150 - 200 ml of hexane/acetone (Fisher, H303SK4 and A9294) 1:1 using a Soxhlet extraction apparatus. The resulting extracts were reduced to a volume of 2 - 8 ml using a rotor evaporator under vacuum, subsequently dried under a gentle stream of pure nitrogen and their

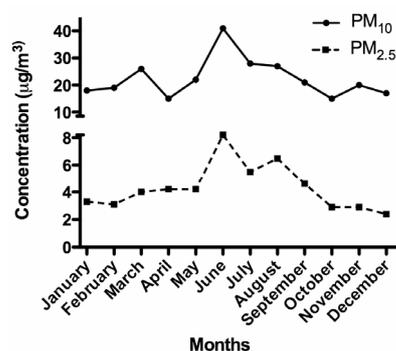


Figure 1. PM_{2.5} and PM₁₀ monthly mean concentrations reported in 2004 by PR-EQB for the rural coastal region of Fajardo, Puerto Rico.

masses determined gravimetrically. The organic extracts were dissolved in dimethylsulfoxide (DMSO, Sigma, Cat No D2650) to a concentration of 100 mg/ml. All extracts were stored at -20°C until further use. Blank filters were also extracted using the previous methodology for quality control purposes.

2.3. PM_{2.5} and PM₁₀ Endotoxin Quantification

2.3.1. Filters

ENX in PM₁₀ filters collected at sea during AEROSE were determined with the Kinetic *Limulus* Amebocyte Lysate assay (Pyrochrome, Associates of Cape Cod, East Falmouth, MA, USA) following the manufacturer's instructions.

2.3.2. Extracts

ENX concentrations in PM₁₀ and PM_{2.5} organic extracts were determined in duplicate using the Kinetic *Limulus* Amebocyte Lysate Assay (*Kinetic-QCL*, Lonza, Walkersville, MD, USA) as described by the manufacturer. A 1/1000 organic extract dilution was used to quantify ENX in the extracts. A Lipopolysaccharide (LPS, Sigma, Cat No L2630) positive control was included for each sample in order to guarantee the specificity of the assay. Absorbance readings at 37°C were recorded on an EL_x 808 micro-plate reader (Biotek Instruments Inc., Winooski, VT, USA) and data analysis was performed with the WinKQCL Software (Lonza, Walkersville, MD, USA).

2.4. PM_{2.5} Metal Analyses

PM_{2.5} organic extracts were diluted in 1 ml of 2% nitric acid and metals quantified by inductive coupled plasma mass spectrometry using an Agilent 7500 (ICP-MS). A modified Environmental Protection Agency 200.8 method was employed. The modification consisted of using helium or hydrogen to remove isobaric interferences through a reaction cell. Each sample was run in

triplicate and mean concentration determined for: aluminum (Al), cadmium (Cd), copper (Cu), iron (Fe), lead (Pb) and vanadium (V).

2.5. Cell Culture and Treatments

BEAS-2B cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; Cat No CRL-9609). Cells were cultured according to ATCC protocols, maintained in keratinocyte basal medium (KBM-2, Lonza, Walkersville, MD, USA Cat No CC 3103) and supplemented with keratinocyte growth medium (KGM-2 SingleQuots; Lonza, Walkersville, MD, USA; Cat No CC4152). Cells were used at passages 44 - 59 and maintained at 37°C in a humidified atmosphere of 5% CO₂. Cells were seeded at a density of 5×10^4 cells/well onto 96-well plates and incubated for 24 h. Extracts were diluted in cell media at final concentration of 0.1 % DMSO and used to expose cells to PM₁₀ (ADEAO and ADEPRC) and PM_{2.5} (ADEPRC) at concentrations ranging from 10 to 100 µg/ml for 24 h. Based on diesel exhaust particles studies [55], this extract concentration range is biologically relevant. Using this range it is possible to induce biologic effects and can be achieved *in vivo* after the appropriate adjustments (e.g. breathing patterns, airway anatomy, diseased airways, particle size and deposition). Three independent exposure experiments were performed. Two additional set of cells (n = 3 independent dishes) were also used to concurrently be treated for a 24 h period with: 1) ADEPRC PM₁₀ organic extract (10 and 25 µg/ml) pre-incubated with polymyxin B sulfate (PMB, Sigma Cat No P0972), an ENX inhibitor, at a final concentration 10 µg/ml; and 2) ADEPRC PM_{2.5} organic extract (50, 75 and 100 µg/ml) pre-incubated with deferoxamine mesylate (DF, Sigma, Cat No D9533), a metal chelator, at a final concentration of 50 µM. Appropriate positive (LPS at a final concentration of 10 µg/ml and LPS pre-treated with PMB) and negative controls (medium, DMSO and media with DF and PMB) were run simultaneously in each experiment. Cell supernatants were collected after each exposure and stored at -80°C until further cytokine analyses.

2.6. Cell Viability

The Neutral Red bioassay was employed to assess cell viability. Following 24 h treatment with PM extracts and inhibitors, BEAS-2B cells were incubated with Neutral Red dye (Sigma, Cat No N2889) at a final concentration of 100 µg/ml for 3 h. The dye was then removed; cells were fixed in 1% calcium chloride, 0.5% formaldehyde, rinsed with phosphate buffered saline (PBS) and lysed using 1% acetic acid and 50% ethanol. Cell viability was spectrophotometrically determined at 540 nm using an Ultramark microplate reader (Bio Rad, Richmond, CA,

USA). Three independent cytotoxicity experiments were performed. Triton-X (50 µg/ml) was used as a positive control for cell toxicity. Negative controls exhibited 90% or greater cell viability. A viability of less than 80% was considered cytotoxic, leaving a range of 20% for normal cell death.

2.7. Cytokine Secretion Measurements

The levels of IL-6 and IL-8, as well as other pro-inflammatory cytokines and proteins such as TNF- α , interleukin-1 beta (IL-1 β), monocyte chemoattractant protein-1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were analyzed using a multiplex bead assay (MAP Kit from Millipore, Billerica, MA, USA or Fluorokine Multi-analyte Profiling Kit from R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Cytokine concentrations were determined using the dual laser flow analyzer Luminex 100 (Luminex Corp, Austin, TX, USA). Standard curves for each cytokine were plotted employing a 5-parameter logistic fit (5-PL). Three independent cytokine secretion experiments were performed.

2.8. Statistical Analyses

To assess differences among groups ANOVA statistical assays were performed followed by the non-parametric Tukey test for multiple comparisons. To analyze differences between individual groups the unpaired Student's *t* Test was employed. The criterion for statistical significance was set at $p \leq 0.05$. Statistical analyses were performed using the GraphPad InStat 3 software. Analyses were based on 3 independent experiments per cell response.

3. RESULTS

3.1. PM₁₀ Fractions

3.1.1. ENX Levels and Cytotoxicity of PM₁₀ ADEAO and ADEPRC Extracts

ADEAO extracts were found to be more cytotoxic to BEAS-2B cells than extracts from Pre-ADEAO, but as toxic as Post-ADEAO (**Figure 2(a)**). Pre-ADEAO samples were not toxic to BEAS-2B cells at all. ADEAO samples were found to be as toxic as ADEPRC samples (LC₅₀ = 37 µg/ml and 38 µg/ml, respectively). ADEPRC extracts showed a dose-response cytotoxic effect and were highly toxic at 25µg/ml (**Figure 2(b)**). The ADEPRC and Non-ADEPRC extracts tested showed a non-significant increase in cell viability with the addition of PMB (**Figure 3**) at either dose of 10 or 25 µg/ml.

PM₁₀ ADEAO extracts contained approximately 5 times the amount of ENX found in the Pre-ADEAO or Post-ADEAO extracts (**Table 1**). ENX levels present in

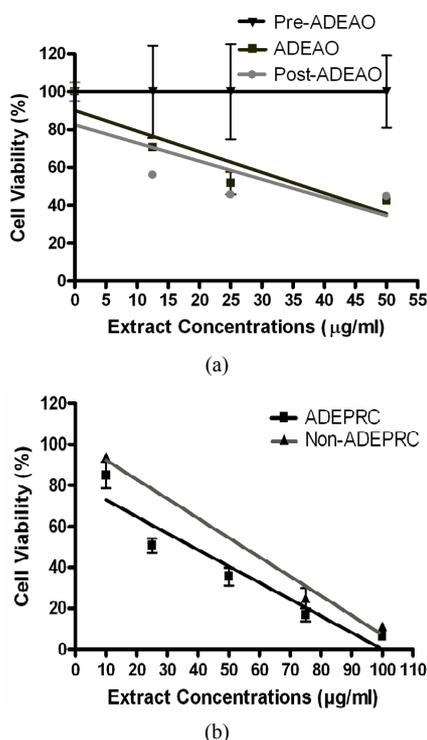


Figure 2. Cytotoxic effect of PM₁₀ extracts in BEAS-2B Cells. Cells were treated for 24 h with PM₁₀ extracts collected from the Atlantic Ocean (A: ADEAO) and Puerto Rican Coast (B: ADEPRC). LC₅₀ (lethal concentration of the PM extracts that kills 50% of the cells in 24 h) values were determined. ADEAO (LC₅₀=37 µg/ml; Post-ADEAO LC₅₀=34 µg/ml) and ADEPRC (LC₅₀=38 µg/ml; Non-ADEPRC LC₅₀=55 µg/ml) extracts were cytotoxic; however pre-ADEAO extracts were not. All extract concentrations were tested in 3 independent experiments.

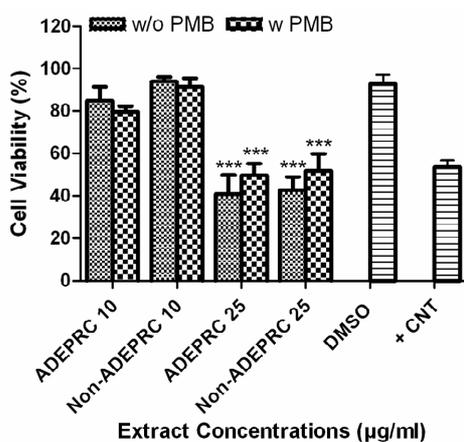


Figure 3. Effect of Polymyxin B (PMB, an ENX inhibitor) and PM₁₀ ADEPRC extracts on BEAS-2B cell viability. Cells were treated with PM₁₀ ADEPRC extracts in the presence (w PMB) and absence of PMB (w/o PMB) at 10 µg/ml for 24 h and cell viability was evaluated. Triton-X (+CNT) at 25 µg/ml was used as positive control. Bars represent mean cell viability ± SEM. Stars over the bars indicate comparison of a treatment with DMSO. All samples were tested in three independent experiments; *** p < 0.001

the PM₁₀ oceanic quartz filters were found to be as high as 6.41 EU/m³ during ADEAO as compared to 0.50 in Pre-ADEAO and 0.80 EU/m³ in Post-ADEAO. PM₁₀ ADEAO ENX levels were higher when compared to those found in PM₁₀ ADEPRC extracts, 511 and 114 EU/mg, respectively. The amount of ENX present in PM₁₀ organic extracts was also higher than that found in PM_{2.5} samples, <50 EU/mg (Table 1).

3.1.2. IL-6 and IL-8 Release by PM₁₀ ADEAO and ADEPRC Extracts

ADEAO and Post-ADEAO extracts at 25 µg/ml induced significantly IL-8 secretion (Figure 4) while Pre-ADEAO extracts did not. The IL-6 and IL-8 secretion was significantly higher in ADEPRC extracts than in Non-ADEPRC (Figures 5(a) and (b)). The use of PMB on ADEPRC and Non-ADEPRC extracts decreased the levels of IL-6, but not significantly. Conversely, PMB was found to increase significantly the secretion of IL-8 in the presence of the ADEPRC extracts at 10 µg/ml (Figure 5(b)). PMB and DMSO alone did not cause any

Table 1. Endotoxin concentration in PM_{2.5} and PM₁₀ extracts.

Particulate Matter	Sample	Endotoxins (EU ^a /mg)
PM ₁₀	Pre-ADEAO	155.0
	ADEAO	511.0
	Post-ADEAO	75.9
	ADEPRC	114
	Non-ADEPRC	111
PM _{2.5}	ADEPRC	<50
	Non-ADEPRC	<50

^aEndotoxin Units

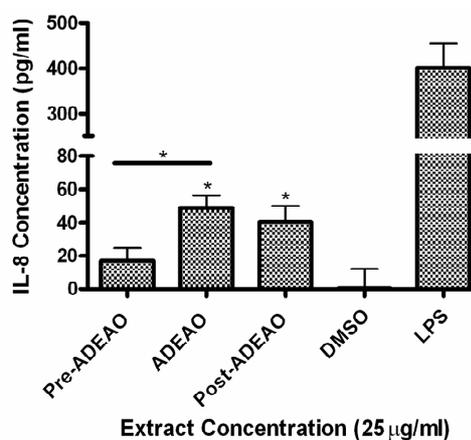


Figure 4. Induction of IL-8 in BEAS-2B exposed to PM₁₀ extracts collected before, during and after the Atlantic Ocean dust storm (ADEAO). LPS (10 µg/ml) was used as a positive control. Bars represent mean IL-8 concentrations ± SEM. Stars over the bars indicate comparison of a treatment with DMSO. Stars over the line indicate a comparison between two treatments. All samples were tested in three independent experiments; * p < 0.05.

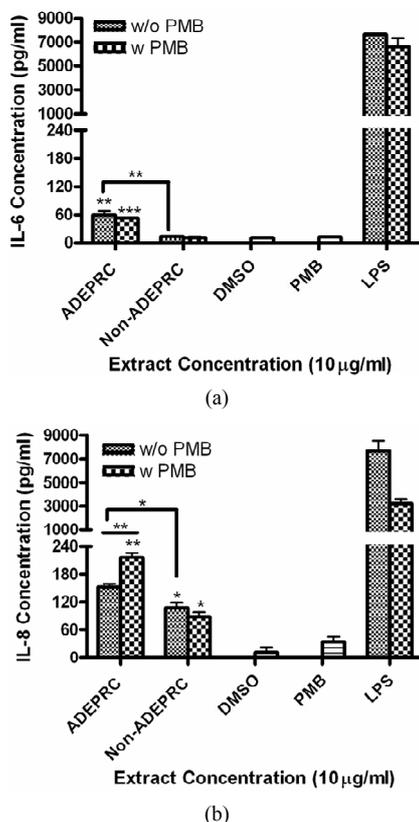


Figure 5. Effect of Polymyxin B (PMB, an ENX inhibitor) at 10 $\mu\text{g/ml}$ on the induction IL-6 (A) and IL-8 (B) in BEAS-2B exposed to PM_{10} (ADEPRC) extracts for 24 h. Cells were treated with PM_{10} ADEPRC extracts in the presence (w PMB) and absence of PMB (w/o PMB) and cytokine induction was measured. LPS (10 $\mu\text{g/ml}$) was used as a positive control. Bars represent mean IL-8 concentration \pm SEM. Stars over the bars indicate comparison of a treatment with DMSO. Stars over the line indicate a comparison between two treatments. All samples were tested in three independent experiments; *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

changes in IL-6 or IL-8 levels. Furthermore, extracts did not provoke secretion of any of the other pro-inflammatory mediators tested such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, GM-CSF and MCP-1 (data not shown).

3.2. $\text{PM}_{2.5}$ Fractions

3.2.1. Cytotoxicity of $\text{PM}_{2.5}$ ADEPRC Extracts

The dose-response exhibited an inverse relationship between cell viability and extract concentration (ADEPRC $R^2 = 0.96$ and Non-ADEPRC $R^2 = 0.81$). ADEPRC extracts were more toxic than Non-ADEPRC to BEAS-2B cells (Figure 6). ADEPRC extracts were concurrently tested with DF at various concentrations. DF increased cell viability at all concentrations tested and a significant effect was observed at 75 $\mu\text{g/ml}$ (Figure 7(a)). Non-ADEPRC (Figure 7(b)) incubated with DF did not significantly increase cell viability at 75 and

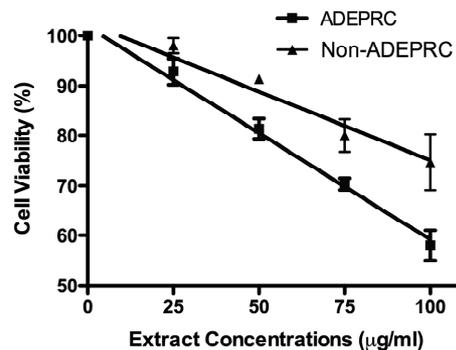


Figure 6. Cytotoxic effect of $\text{PM}_{2.5}$ extracts in BEAS-2B Cells. Cells were treated for 24 h with $\text{PM}_{2.5}$ extracts collected in the Puerto Rican coast during the African storm (ADEPRC) and non-storm (Non-ADEPRC) season. LC_{50} (lethal concentration of the PM extracts that kills 50% of the cells in 24 h) values were determined. ADEPRC ($\text{LC}_{50} = 122 \mu\text{g/ml}$ (extrapolated)) and Non-ADEPRC ($\text{LC}_{50} = 191 \mu\text{g/ml}$ (extrapolated)) were cytotoxic. All extract concentrations were tested in 3 independent experiments.

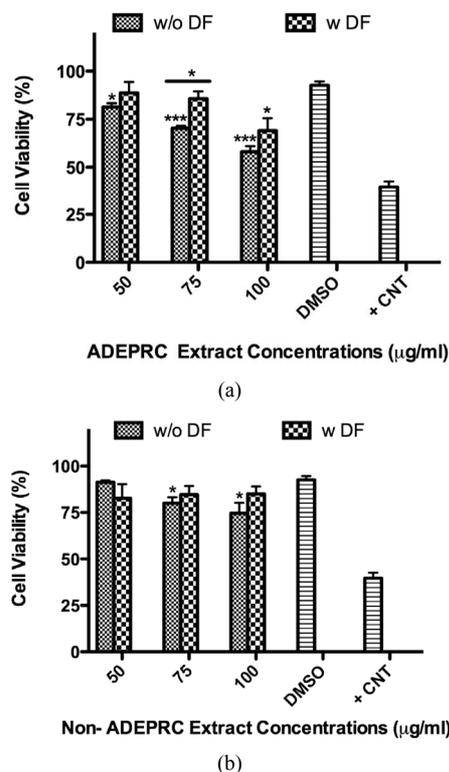


Figure 7. Effect of Deferoxamine (DF, a metal chelator) and $\text{PM}_{2.5}$ ADEPRC extracts on BEAS-2B cell viability. Cells were treated with $\text{PM}_{2.5}$ extracts collected during the dust storm (ADEPRC) and non-storm (Non-ADEPRC) season in the presence (w DF) and absence of DF (w/o DF) at 50 μM for 24 h and cell viability was evaluated. Triton-X (+CNT) at 25 $\mu\text{g/ml}$ was used as positive control. Bars represent mean cell viability \pm SEM. Stars over the bars indicate comparison of a treatment with DMSO. Stars over the line indicate a comparison between two treatments. All extract concentrations were tested in 3 independent experiments; *** $p < 0.001$, * $p < 0.05$.

100 µg/ml. Cytotoxicity caused by ADEPRC extracts correlated with the presence of high levels of metals, including Cu, Pb and V (Table 2).

3.2.2. IL-6 and IL-8 release by PM_{2.5} ADEPRC Extracts

Significant differences were observed on immunological responses between ADEPRC and Non-ADEPRC particularly on the induction of IL-6 and IL-8 secretion by BEAS-2B (Figures 8(a) and (b)). This effect follows that of a dose-response type. This induction was distinct at the non-toxic concentrations of PM extracts tested at 25 and 50 µg/ml. It is evident that the effect of ADEPRC extracts on cytokine secretion by BEAS-2B was far more prominent than in the Non-ADEPRC extracts. Cells simultaneously treated with ADEPRC extracts at 50 µg/ml and DF at 50 µM reduced IL-6 (Figure 9(a)) and significantly IL-8 (Figure 9(b)) secretions. This effect was also observed with the Non-ADEPRC extracts, but not significantly. PM_{2.5} extracts significantly decreased the secretion of MCP-1. No changes were observed with the use of DF (data not shown). PM_{2.5} extracts did not cause any change in other pro-inflammatory mediators such as TNF-α, IL-1β and GM-CSF (data not shown).

3.2.3. TMET Levels in PM_{2.5} ADEPRC Samples

Al, Cd, Cu, Fe, Pb, and V were measured to determine their contribution to the toxicity and pro-inflammatory characteristics on ADEPRC extracts (Table 2). The ADEPRC extracts contained relative amounts of TMET and were ranked as follows: Cd < V < Pb < Al < Cu < Fe. The Non-ADEPRC extracts were also ranked as follows: Cd < V < Pb < Cu < Al < Fe. The most abundant TMET in ADEPRC extracts were Cu, Pb and V and were higher than Non-ADEPRC. The total metal distribution in ADEPRC and Non-ADEPRC extracts (Table 2), which include both the organic and aqueous phase, shows the

Table 2. Metal Content in Organic Extracts vs Total Composition.

Trace Element	% Composition			
	Organic Extracts		Total	
	ADEPRC	Non-ADEPRC	ADEPRC	Non-ADEPRC
Al	16.4	33.3	63.5	54.7
Fe	39.9	40.4	16.8	13.3
Cd	0.4	0.3	0.1	0.1
Cu	30.7	14.2	5.1	3.9
Pb	9.3	7.3	2.3	1.5
V	3.4	1.1	0.8	11

Trace elements values for aqueous and organic extraction were combined and expressed as total % composition. Shaded in gray are crustal elements and Non-shaded are anthropogenic elements.

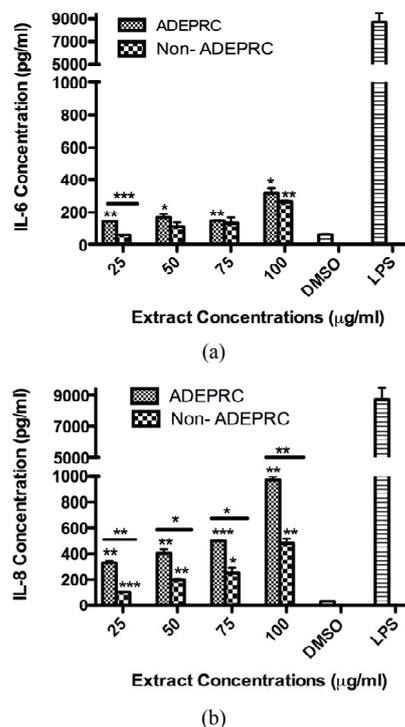


Figure 8. Induction of IL-6 (a) and IL-8 (b) in BEAS-2B exposed to Puerto Rican coast (ADEPRC) PM_{2.5} extracts for 24 h. LPS (10 µg/ml) was used as a positive control. Bars represent mean IL-6 and IL-8 concentrations ± SEM. Stars over the bars indicate comparison of a treatment with DMSO. Stars over the line indicate a comparison between two treatments. All samples were tested in three independent experiments; *** p < 0.001, ** p < 0.01, * p < 0.05.

relative abundance of Fe and Al and the contribution to local sources of these two heavy metals.

4. DISCUSSION

African dust transports persistent organic pollutants, traces of metals and viable microorganisms across the Atlantic Ocean to the Caribbean, affecting the air quality and impacting human health in the region. However, due to seasonal variations, global climate changes, human activity, PM complexity, and individual characteristics, the health impact and the local environmental factors responsible for triggering PM-adverse effects are difficult to predict and identify. Here, we provide data suggesting that ENX and TMET constituents in PM-ADEAO/-ADEPRC are responsible for triggering the secretion of pro-inflammatory cytokines and inducing cytotoxicity in human bronchial epithelial cells.

The presence of gram-negative bacteria, containing lipopolysaccharides (LPS) in their cell walls, has been previously reported in African dust clouds crossing the Atlantic Ocean [3]. LPS is an endotoxin (ENX) capable of causing toxicity and immunogenicity due to its lipid

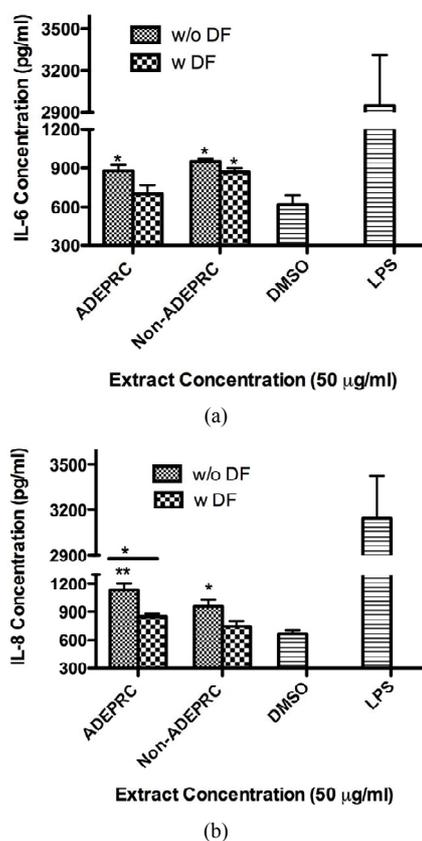


Figure 9. Effect of Deferoxamine (DF, a metal chelator) at 50 μ M on the induction IL-6 (a) and IL-8 (b) in BEAS-2B exposed to PM_{2.5} (ADEPRC) extracts for 24 h. Cells were treated with PM_{2.5} extracts collected during the dust storm (ADEPRC) and non-storm (Non-ADEPRC) season in the presence (w DF) and absence of DF (w/o DF) cytokine induction was measured. LPS (10 μ g/ml) was used as a positive control. Bars represent mean IL-6 and IL-8 concentrations \pm SEM. Stars over the bars indicate comparison of a treatment with DMSO. Stars over the line indicate a comparison between two treatments. All samples were tested in three independent experiments; ** $p < 0.01$, * $p < 0.05$.

and polysaccharide components. LPS elicits a variety of inflammatory responses by activating Toll-like receptor-4 (TLR4) and triggering the signaling cascade mediated by transcription factors such as NF- κ B to secrete pro-inflammatory cytokines (e.g., IL-6, IL-8, TNF- α); these cytokines at high concentrations may initiate health adverse conditions such as asthma [27,37-38,56-59]. In the present study, ENX was detected in all PM_{2.5} as well as in PM₁₀ extracts from the Atlantic Ocean and the Puerto Rican coast. The secretion of IL-6 and IL-8 was found to increase considerably following PM treatment to BEAS-2B cells. TNF- α , IL-1 β , GM-CSF, and MCP-1 were not induced by the PM extracts. More detailed investigations are needed to determine the mechanisms associated with this cellular response using BEAS-2B cells. PM_{2.5} and PM₁₀ extracts were significantly cytotoxic to BEAS-2B

cells; however, the degree of cytotoxicity was not found to be directly dependent on the levels of ENX present in the samples (See **Table 1** and **Figures 3** and **7**). The levels of ENX found in the PM₁₀ extracts collected during Pre-ADEAO (155 EU/mg) were comparable to those PM₁₀ samples collected during ADEPRC (114 EU/mg) and Non-ADEPRC (111 EU/mg); but Pre-ADEAO samples were not cytotoxic to BEAS-2B cells. In addition, PM₁₀ Post-ADEAO samples contained low levels of ENX (75.9 EU/mg) as compared to the rest of the samples collected but were as toxic as PM₁₀ ADEAO samples which contained the highest ENX concentration (511 EU/mg). In this study, we also found that the levels of ENX present in the PM₁₀ samples collected in the Atlantic Ocean and in the Puerto Rican coast were higher and more cytotoxic than those present in the PM_{2.5} fractions, which presented the lowest ENX concentrations (<50 EU/mg). These findings are in agreement with previous studies indicating that ENX content in fine PM fractions (<PM_{2.5}), reaching the Caribbean and Puerto Rican coast, is usually lower or negligible when compared to the content in the coarse (<PM₁₀) fractions [10,37,59]. Interestingly, PMB, a known LPS inhibitor, did not reduce cell damage caused by the PM₁₀ extracts. In summary, these findings suggest that: 1) ENX were initially deposited onto PM fractions (e.g., PM₁₀) during the dust storm originated in the African Continent and gradually lost during its course trajectory through the Caribbean and Puerto Rican coast; 2) ENX content in African dust plays a minor role in the cytotoxicity caused by PM₁₀; and 3) IL-6 and IL-8 were the predominant induced cytokines in this *in vitro* system, as compared to the rest of the cytokines tested (e.g., TNF- α , IL-1 β , GM-CSF, and MCP-1).

In contrast, the administration of DF (metal chelator) to BEAS-2B cells, treated with ADEPRC-PM_{2.5} extracts, significantly reduced the secretion of cytokines and cytotoxicity; supporting the hypothesis that TMET play a major role in causing lung epithelial cell injury *in vitro* [60]. Previous studies have demonstrated that human airway epithelial cells exposed to metals found in air particles release significant amounts of pro-inflammatory mediators and are toxic *in vitro* [61,62]. In the present study, the concentration of some metals such as Al, Cd, Cu, Fe, Pb and V were detected in the PM_{2.5} fractions during the Non-ADE season but most of these metals increased when ADEAO approached the Puerto Rican coast (**Table 2**); however, these findings are in disagreement with previous studies which suggested that the levels of TMET present in the ADEAO-PM_{2.5} fraction are expected to be lower [10].

The presence of TMET in the atmospheric air, as well as other atmospheric contaminants such as ENX, fluctu-

ates and is dependent on the source, the frequency and magnitude of each particular dust storm. Studies conducted by the Center for Environmental and Toxicological Research (CETR) at the University of Puerto Rico, Medical Sciences Campus (UPR-MSC) have shown that the influx of African dust affects considerably the air quality conditions in the Northeastern areas of Puerto Rico [12,22,62-67]. These studies have demonstrated that PM₁₀ fractions collected from Puerto Rican urban areas (e.g., Guaynabo, Salinas) contained higher concentrations of ENX and TMET, respectively (e.g., Cd, Cu, Fe, Ni, Hg, Pb and V) than extracts collected from rural coastal regions (e.g., Fajardo) and were significantly toxic to *in vitro* systems [12,22,62]. Subsequent work also demonstrated that PM_{2.5} fractions collected from indoor areas contained higher concentrations of these metals than PM samples obtained from outdoor areas, causing significant release of pro-inflammatory cytokines *in vitro* [64-68]. These results suggest that PM_{2.5} and PM₁₀ fractions and some of their constituents (e.g., ENX and TMET) present at different Puerto Rican locations (urban and rural areas) may play a key role in the exacerbation of certain respiratory diseases (e.g. asthma) in predisposed individuals exposed to African dust clouds [8,10,12]. Epidemiological studies by the U.S. CDC and others have reported a high prevalence of asthma, particularly in children [49,69,70]. Similar results have also been reported by other investigations performed in the Caribbean region [46,50,51,54,59,71-74].

Respiratory illnesses such as allergies and asthma are a major concern in Puerto Rico, which has one of the highest asthma prevalence rates in the world between other races/ethnicities, including other Hispanics [69,70,75]. According to CDC, Puerto Ricans are more likely to have respiratory illness, specifically asthma than white non-Hispanics and total Hispanics. Furthermore, epidemiological studies conducted by the island's health department during 2007 and 2010, indicated that an average of 25,000 cases were filed each year and more than 90,000 adults could not work or perform regular activities due to suffering of respiratory diseases. Despite of decades of research in this area, it is still uncertain why Puerto Ricans suffer so much from asthma; however health officials in the island suspect that environmental and individual characteristics are responsible for triggering respiratory diseases. PM_{2.5} and PM₁₀ are a major threat to children because of their higher exposure to ambient PM, mostly when involved in outdoor activities as compared to adults and because of their immature state of the lung and immune system function [72,76]. In addition to ENX and TMET, other particle pollutants such as PAH, NO₂, SO₂, and O₃ have also been associated with the pathogenesis of asthma and the increase of

ER visits and hospitalizations of children; inflammation and oxidative stress are considered the major factors responsible for such adverse conditions [48,53,77-78]. A recent report has suggested an epigenetic and genetic variant in ADCYAP1R1 associated with asthma in Puerto Rican children [79]. ADCYAP1R1, also known as PAC₁, is a gene encoding for adenylate cyclase activating polypeptide 1 (pituitary) receptor type I. PAC₁ is a membrane-associated protein that shares significant homology with members of the glucagon/secretin receptor family. This receptor is highly expressed in the central nervous system (CNS), but it has been found in considerable levels in other tissues (e.g., spinal cord, digestive system, liver, adrenal medulla, and smooth muscle). ADCYAP1R1 mediates diverse biological actions of adenylate cyclase. For example, it regulates stress response across species [80] and also has been linked to post-traumatic stress disorder in adult and anxiety in children.

Another area of intense investigation in recent years is the participation of transient receptor potential (TRP) cation channels and calcium transporters in regulating the acute toxicological effects of ambient PM. The mammalian TRP was initially identified as particle sensors, whose primary function was to regulate the cell membrane permeability to a variety of ions. Subsequently, it was demonstrated that, some of the TRP subfamilies were linked to many pathophysiological and specific diseases; and also capable of mediating airway injury, hyper-reactivity and cardiovascular toxicity of inhaled pollutants [81-88]. The mammalian TRP family consists of six main subfamilies referred to as TRPC, TRPV, TRPM, TRPP, TRPML and TRPA groups. TRPV1, TRPV4, TRPA1, and TRPM8 have been proposed to mediate health adverse effect following exposure to urban air pollution [88-90]. It has been hypothesized that these four TRP subfamily members trigger acute adverse respiratory and cardiovascular effects by mediating calcium influx, activating signal transduction pathways and matrix-metalloproteinases (MMP) [91]. The increased MMP secretion by lung epithelial cells exposed to ambient PM has been linked to respiratory diseases [92-94], including pediatric acute lung injury [95]. Recent work has also demonstrated that TRPV4 polymorphism is associated with COPD [96] and cough in individuals without asthma [97]; and that TRPV1 and TRPV4 loss of function by a genetic variant lower the risk of active childhood asthma [98]. In addition to TRPs and calcium transporters, humic-like substances (HLS) and cell iron equilibrium have also been associated with lung injury following exposure to ambient PM pollutants [99,100]. Studies analyzing function and expression of TRP cellular channels in the lung may enable us to better understand the development of diseases caused by ambient PM and develop new therapeutic strategies for respiratory illnesses

in the near future. We have briefly discussed the possible contributions and effects of PM constituents in human lung epithelial cells. A summary of the transport of dust storms across the Atlantic Ocean and the possible pathways of PM constituents that may lead to cytokine induction, cytotoxicity and respiratory disease are shown in **Figure 10**.

Therefore, it is of considerable importance to conduct more defined epidemiological and *in vivo* and *in vitro* studies to 1) better understand the precise environmental factors that play a critical role in the ADEPRC-induced

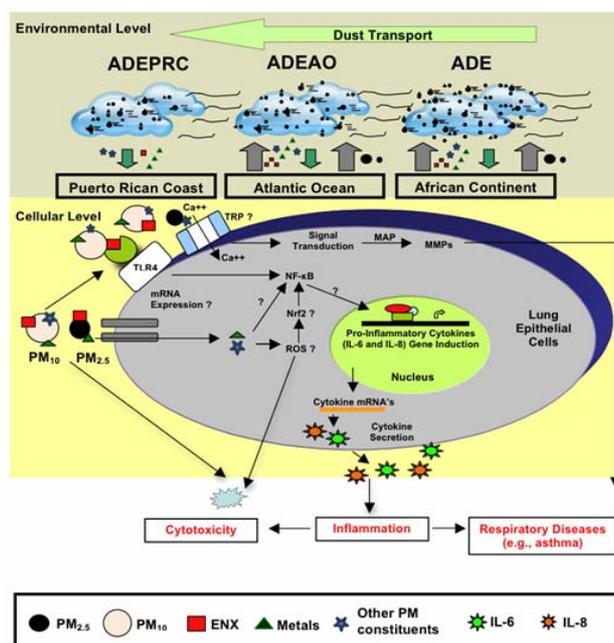


Figure 10. Schematic model explaining the movement of African dust through the Atlantic Ocean (ADEAO) reaching the Puerto Rican coast (ADEPRC) and the possible relationship between its constituents (e.g., PM_{2.5-10}, ENX, TMET, other PM constituents). Briefly, as African dust crosses the Atlantic Ocean, PM_{2.5} and PM₁₀ distribution along with their constituents are modified. Upon arrival to the Puerto Rican coast, ENX levels are reduced whereas the levels of TMET are increased. ENX and TMET are cytotoxic to human bronchial epithelial cells, and can lead to inflammation and oxidative stress. This detrimental response may be caused due to the release of pro-inflammatory cytokines (e.g., IL-6 and IL-8), activation of nuclear receptor pathways (e.g., NF-κB, Nrf2), and the formation of reactive oxygen species (ROS). Interrogative symbols (?) represent factors involved in the signal transduction mediating cytokine secretion that are currently under study. Transient receptor potential (TRP) has also been associated with health adverse effects of ambient PM. TRP mediate calcium influx, activate signal transduction pathways (e.g., MAP kinases) and modulate MMP (matrix metalloproteases) causing lung injury. Small circles represent PM_{2.5} and large circles PM₁₀; Endotoxins (ENX) are represented as red squares, traces of metals (TMET) as green triangles and other potential PM constituents as light blue stars. Cytokines are represented as dark blue and green stars.

respiratory related diseases; 2) identify the biological mechanisms that render one population being more susceptible than another to PM health adverse effects; and 3) develop new strategies, educational programs and treatments for the patients and ultimately reduce asthma hospitalization and morbidity rate on the island. Taken together, the present study indicates that ENX and TMET can cause toxic effects to lung cells and TMET is the main responsible for the toxicity seen in this *in vitro* study. We hypothesize that ENX triggers the release of pro-inflammatory cytokines, such as IL-6 and IL-8, and render the cells more susceptible to PM contaminants such as metals, causing epithelial lung injury. Our findings support previous studies indicating that PM_{2.5} and PM₁₀ may have the potential to trigger allergic and asthma episodes in susceptible and predisposed individuals, especially in children during ADEPRC season. This research also recognizes the importance of PM size fractions, composition and origin in causing potential lung adverse effects.

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