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Urban $PM_{2.5}$ induces ROS generation and RBC damage in COPD patients

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1. ABSTRACT

Particulate matters (PM) produce adverse effects on the respiratory system and cause COPD. These effects are thought to involve intrinsic generation of ROS which are present in ambient PM (transition metals and aromatic organic compounds). Here, we examined the chemical composition and ultra-microscopic structure of $PM_{2.5}$. The effect of this PM was studied in red blood cell (RBC) membranes (ghosts) from healthy volunteers (n = 11) and COPD patients (n = 43). These effects were compared with that produced by a Fenton metal-catalytic ROS generator. Oxidative biomarkers and cell damage were singificantly increased in presence of $PM_{2.5}$ or ROS generator in RBC of COPD patients as compared with those in cells from healthy volunteers. In contrast, total SH groups, band 3 phospho-tyrosine phosphatase (PTPase) and glucose-6 phosphate dehydrogenase (G6PD) activities were all diminished in cells from COPD patients. In conclusion, $PM_{2.5}$ increases damage to RBCs from COPD patients, decreases the activity of PTPase and G6PD, and alters the function of the anionic exchanger (AE1) and the antioxidant response by decreasing SH groups.

2. INTRODUCTION

According to a census carried out in the 2000, Metropolitan Area of Mexico City with 18 million inhabitants is one of the most densely populated cities in the world (1). This area is an elevated basin, approximately, 2240 meters above sea level. Since 23% less oxygen is available at this level, combustion is less efficient.

There are indications that when sulfur dioxide (SO_2) concentration is high, new particles are formed. The average atmospheric lifetime of sulfur emitted in Mexico City is 5.5 days, which is longer than the average lifetime of sulfur released in the rest of the world (3.9 days). Because of the altitude and the subtropical latitude of the Mexico City basin, the region receives intense solar radiation that promotes efficient photochemical formation of pollutants. This changes their chemical composition during air transportation and results in particulate materials with different chemical properties.

For example, in the southeast zone of the city (Iztapalapa), the organic fraction of fine particles ($PM_{2.5}$) at

the Centro Nacional de Investigación y Capacitación Ambiental (National Center for Environmental Research and Training, CENICA) site is estimated to represent 54.6% of the total mass, with the remainder being inorganic compounds (mainly ammonium nitrate and sulfate/ammonium salts), black carbon (BC) and soil (2). Air pollution is associated with respiratory and cardiac diseases, particularly in children, women during the first trimester of pregnancy and the elderly. Yet, the mechanism(s) of pollutant-mediated injury remains unknown. The diverse composition of particulate material, may contribute to the range of different results that have been reported (3). However, the prevailing opinion is that molecular mechanisms of pollutant-induced injury might be due to oxidative stress (4). Suspended air particles are potent oxidants, oxidizing important biological molecules either directly in the cellular compartments or indirectly through the activation of intracellular pathways (5). Experimental evidence has shown that the toxicity and carcinogenicity of particulate pollutants are independent of their chemical composition. Pollution toxicity is induced through ROS production in target cells, and the heterogeneity in chemical compounds in ambient particulate matter, including transition metals and aromatic organic compounds, may contribute to adverse effects through ROS (3-4, 6). Acute effects occur at relatively low pollutant concentration and are associated with particles of apparently innocuous composition (largely carbon, ammonium sulfate and nitrate) (5). Ultra-fine particles are contained in the fine fraction, and the soluble material may translocate to extrapulmonary sites for local cellular activation (7-8).

Patients with Chronic Obstructive Pulmonary Disease (COPD), are most susceptible to air pollution. Due to the predicted increase in the prevalence and mortality of COPD in future, a unified, international effort is required to reverse these trends. The Global Initiative for Chronic Obstructive Lung Disease outlines a simple classification system that divides the disease severity into four stages (9). According to this classification, the following groupds are defined: GOLD I-Mild; GOLD II-Moderate; GOLD III-Severe; and GOLD IV-Very severe. Oxidative stress has been implicated in the pathogenesis and progression of COPD (7). Oxidative stress causes structural changes to essential components of the lung, leading to irreversible damage to both the parenchyma and the airway wall. Moreover, the serious imbalance between ROS production and antioxidant defenses leads to the activation of transcription factors (such as nuclear factor NF-KB), inactivation of anti-proteases, increased sequestration of neutrophils in the pulmonary microvasculature, and oxidative tissue and cellular injury (8). ROS are generated, principally, from leukocytes in the blood/air spaces or inhaled in the form of environmental oxidant pollutants, including cigarette smoke, particulate matter, and smoke from wood (3).

An increase oxidative stress in RBC has been reported in many pathological conditions and diseases (10,11). Due to the ability to detect oxidative modifications in cells during the GOLD II stage and after experimental exposure to ROS and reactive nitrogen species (RNS) damage to RBCs has been proposed as a biosensor of COPD progression. Typically, these RBC modifications are studied by scanning electron microscopy and flow-cytometric analysis. RBCs show a moderate OE response that is characterized by a decrease in functional capacity of ion exchange. Affected exchanges include O_2 and CO_2 uptake from the lungs or peripheral tissues, and CO_2 extrusion as HCO_3^- through the band 3 anion exchanger (12). The band 3 anion exchanger forms a scaffold for the assembly of a protein complex that can transmit extracellular signals and modulate the transport and mechanical properties of the erythrocyte. These altered RBCs have a reduced transport capacity and a reduced peripheral release of O_2 (11).

In this study, we evaluated increases in oxidative damage that are normally observed in various stages of COPD. We incubated RBCs from COPD patients with $PM_{2.5}$ or a ROS generator system and measured membrane biomarkers of oxidative injury to assess the acceleration of COPD progression following ROS generation. We also quantified oxidative injury to the RBC membrane. Finally, changes in phospho-regulation between the different GOLD stages were evaluated by measuring the activities of phosphotyrosine phosphatase (PTPase) and glucose-6 phosphate dehydrogenase and relating these changes to RBC function. The results presented here, provide strong evidence that there is an increased vulnerability to oxidative damage in RBC of COPD patients due to the fact that their antioxidant system is compromised.

3. MATERIALS AND METHODS

Unless otherwise specified, all reagents used in this study were from Sigma Chemical Co. (St. Louis, MO).

3.1. Collection of particulate matter

Respirable particles [aerodynamic diameter < 10 μm (PM_{10})] and fine particles [< 2.5 μm ($PM_{2.5}$)] were collected at the Centro Nacional de Investigación y Capacitación Ambiental (National Center for Environmental Research and Training, CENICA). Fourteen PM_{10} and 13 $PM_{2.5}$ samples were obtained simultaneously over a 24-hour period from May 2006 to February 2007. The samples were collected with Andersen-Graseby high volume samplers onto quartz fiber filters (Whatman). The CENICA site is situated in southeast Mexico City (Iztapalapa zone) at the Autonomous Metropolitan University campus. It is the most populated area of the city and is less than 2 km from the most important food merchandise distribution center in the city. The samplers were located on the roof of a four-story building.

Before and after sample collection, the filters were conditioned at 22 ± 3°C and 40 ± 5% RH during a 24-hour period and weighed with an analytical balance (Sartorious, sensitivity 10^{-4} g). After weighing, a section of the PM_{10} filter was subjected to chemical analysis following the standard procedures of the USA EPA (1996 and 1998) by inductively coupled plasma atomic emission spectroscopy (Perkin Elmer, 3300 DV) and atomic

Table 1. Demographics and lung function characteristics

	HV, n=11	**Moderate,** n=26	**Severe,** n=17	**SS**
Age (yr)	61.00(8.8)	69.95(10)	68.02(7.5)	NS
Sex (F/M)	10/1	9/17	4/13	NS
TS (Pack/yr)	-------	40.5(18)	46(15.5)	NS
BMI	27.6(3)	26.70(4)	26.18(2.5)	NS
FEV_1 p(%)	106 (21.88)	78.55(12)	58.15(6)	<0.0001
FVC p(%)	104.7 (21.07)	103.86(8)	61.33(17)	<0.0001
FEV_1/FVC	79.0(4)	57.99(8)	36.27(9.5)	<0.0001

The data are the MEAN (SD); TS= Tobacco smoking; BMI=body mass index; FEV_1=first forced expiratory volume; FVC=forced vital capacity. *Chi square test. SS: statistical significance

absorption spectroscopy (Varian, Spectra A-2). A sub-sample of the PM_{10} filters were analyzed by electron microscopy (JEOL, JSM-5900 LV) coupled with energy dispersive X-ray spectroscopy (Oxford) to determine the size distribution and individual composition of the particles. The complete $PM_{2.5}$ filter was swept with a powder puff, with particles collected in a polyethylene vial. The amount of particles recovered using this technique ranged from 18 to 80 mg.

3.2. Patients

A total of 43 patients with a diagnosis of COPD and 11 healthy volunteers were enrolled in this study. COPD patients were ex-smokers and belonged to a cohort of subjects who were evaluated during a clinically stable period that was free of exacerbations during the previous six weeks. Subjects had a history of smoking at least 10 packs of tobacco products per year. COPD diagnosis was established according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines with the severity of disease divided into moderate and severe (Table 1) (9). Subjects who met the criteria for COPD but had an alternative respiratory disorder (i.e., bronchiectasis or asthma) were excluded. Spouses of COPD patients who had no history of chronic illness, such as diabetes, rheumatoid arthritis, or lung disease, were recruited as controls. Individuals with a history of tobacco smoking were excluded. The protocol of this study (B10-08) was approved by the Ethics Committee at the National Institute of Respiratory Diseases. All subjects were informed, and their written consents were obtained.

3.3. Treatment strategy

Subjects underwent treatment for post-bronchodilator spirometry following the procedures recommended by the ATS and ERS (13). We used Mexican standard reference equations for predicted values (14). For spirometry, we used a dry rolling-seal volume spirometer (Sensormedics, Yorbalinda, CA). Subjects also underwent testing for their blood count and lipid profile.

Blood samples (5 mL) from both healthy volunteers and COPD patients were obtained by venipuncture and centrifuged. The plasma and erythrocyte ghosts were obtained and used for stimulation with particles and the Fenton reaction.

3.4. Stimulation with $PM_{2.5}$

The particles were resuspended at a concentration of 1 mg per test in Krebs-Ringer phosphate buffer, pH 7.4.

3.5. Hydroxyl (HO) radical generation

Hydroxyl radical generation was obtained via the Fenton reaction (15) using 1 mM H_2O_2 and 5 mM $CuSO_4$. After incubating 5 min at 37°C, the reaction was stopped by the addition of 5 mM of citrate.

Following stimulation, open erythrocyte membranes were prepared by hypotonic lysis of RBC cells obtained from whole blood, as originally described by Steck, and subsequently used for biomarker analysis (16).

3.6. Biochemical analysis

To evaluate lipoperoxidation, 1-methyl-2-phenylindole (Sigma-Aldrich, MO) was used as standard. Aliquots of plasma were used to measure malondialdehyde (MDA) at 586 nm, and the values obtained were expressed as nmol of MDA per mg of dry weight (dw) (17). To evaluate lipid peroxides, we used the assay conditions described by Yagi *et al.* (18). The test solution was mixed with a color reagent of a commercially available kit for the enzymatic determination of cholesterol (CHO-iodide; Roche). This assay quantifies lipid peroxides by testing their ability to convert iodide to iodine, which can be measured photometrically at 365 nm. Calibration curves were obtained using peroxides, such as t-butylhydroperoxide. The concentration of lipid peroxides was calculated using the molar absorptivity of I_3 measured at 365 nm ($\epsilon = 2.46 \pm 0.25 \times 10^4\ M^{-1}\ cm^{-1}$). The conjugated dienes were obtained after a single extraction with methanol-chloroform and quantified using a molar extinction coefficient of $28 \times 10^3\ M^{-1}\ cm^{-1}$ and photometric analysis at 284 nm (19).

Protein damage was evaluated by determining the carbonyl group content of the erythrocyte membranes using 2,4-dinitrophenylhydrazine (DNPH), which reacts with the protein carbonyl derivates to form stable hydrazones that absorb at 370 nm (20). A molar extinction coefficient of $21 \times 10^3\ M^{-1}\ cm^{-1}$ was used to quantify carbonyl content. PTPase activity was determined using p-nitrophenyl phosphate (p-NPP) as a substrate according to previously published procedures (21). The release of p-nitrophenol from p-NPP was measured spectroscopically at 410 nm. Protein sulfhydryl groups were evaluated using 5,5'-dithiobis (2-niotrobenzoic acid) (DTNB), with the absorbance measured at 412 nm (22). Glucose 6-phosphate dehydrogenase (G6PD) activity was measured as the absorbance at 340 nm, as previously described (23).

Table 2. Scanning electron microscopy classification of morphology and elemental composition in individual $PM_{2.5}$ particles

Element	Spherical n=20			Cluster n=27			Irregular n=70			Soot Aggregate n=38		
	Min	Max	n	Min	Max	n	Min	Max	n	Min	Max	n
C	12.97	60.23	20	20.63	58.73	27	18.81	60.8	70	23.08	53.98	38
O	27.19	45.38	20	28.91	44.33	27	25.17	53.52	70	21.51	41.89	38
Na	0.35	0.94	7	0.31	11.27	15	0.31	3.32	38	0.32	1.61	15
Mg	0.23	8.91	9	0.31	6.69	10	0.34	13.02	33	0.25	1.22	7
Al	0.43	9.6	10	0.47	7.31	22	0.65	17.91	52	0.37	3.77	11
Si	6.86	28.27	20	5.07	29.54	27	3.65	51	69	8.11	44.93	38
P	nd	nd		nd	nd		nd	nd		nd	nd	
S	nd	nd		nd	nd		0.51	1.87	3	nd	nd	
Cl	0.09	2.56	3	0.35	3.15	4	0.25	7.72	9	nd	nd	
K	0.31	4.26	11	0.31	4.91	15	0.16	7.21	50	0.24	4.55	18
Ca	0.31	3.28	17	0.28	10.23	22	0.23	21.87	63	0.22	2.88	22
Ti	nd	nd		nd	nd		nd	nd		nd	nd	
V	0.29	1.08	2	nd	nd		nd	nd		nd	nd	
Cr	nd	nd		nd	nd		nd	nd		nd	nd	
Mn	nd	nd		nd	nd		nd	nd		nd	nd	
Fe	0.56	50.57	8	0.37	6.19	14	0.38	32.76	43	0.38	0.92	5
Cu	0.47	1	4	nd	nd		0.66	1.63	7	0.52	1.54	4
Zn	nd	nd		nd	nd		nd	nd		nd	nd	

nd=not determined

3.7. Statistics

Data are expressed as the mean ± standard deviation. One-way ANOVA and Bonferroni's Multiple Comparison Test, linear regression, and Pearson's correlation were used for statistical analysis. Differences were considered significant when the p-value was <0.05. Data analysis was performed using the Statistical Package for Social Sciences (version 10.0 for Windows; SPSS Inc., Chicago, IL).

4. RESULTS

4.1. Respiratory probes

This study included a total of 26 patients as follows: Moderate group: [7 patients (16.2%) in GOLD stage I , 19 patients (44.1%) in GOLD stage II] and 17 patients in the "severe" group [10 patients (23.2%) in GOLD stage III and 7 (6.2%) in GOLD stage IV]. The only difference between GOLD stage groups was in the spirometry parameters, as calculated using ANOVA analysis (Table 1). There were significant differences between the control and patient groups with respect to post-bronchodilator FEV_1 percent predicted, forced vital capacity (FVC) percent predicted, and the FEV_1/FVC ratio (Table 1). There were no significant differences between the two groups with respect to age and body mass index (BMI). The mean cumulative tobacco consumption for COPD patients was 46 ± 26 pack-years. COPD patients had statistically higher hematocrit values in the blood count.

4.2 Particle Characteristics

We utilized a mixture of particles from industrial sources. From the nearly 100 filters that were evaluated, two PM morphologies were most frequent. Representative scanning electron microscopy images are shown in Figures 1 and 2. The morphological form classification and elemental composition in the fine fraction (< 2.5 μm) particles measured at the CENICA site are shown in Table 2. Figures 1 and 2 illustrate the diverse forms observed in the ultra-microscopic analysis. Different morphological forms have a characteristic composition that can be correlated with the emission source. The predominant forms were spherical (n = 20), clusters (n = 27), irregular (n = 70) and aggregate or reticular forms of soot aggregates (n = 38). Table 2 displays the individual X-ray fluorescence analysis that correspond to Figures 1 and 2. The quantitative analyses indicate that carbon and oxygen, likely derived from incomplete combustion of fossil fuels, were the principal components. These elements accounted for 68% to 94% of the coarse fraction and 74% to 76% of the fine fraction. Metallic elements contributed to 2.5% of the coarse and 2% of the fine fractions. Iron was most abundant in spherical particles (0.56% to 50.57%) and copper in the soot aggregates and irregular particles (> 1.5%). Aluminum, calcium and potassium were also identified and detected principally in irregular particles, while zinc was not found in the samples. The particle size and shape are related to the original source of generation. For example, the soot aggregate which principally accounted for the nanoparticles (< 0.25) is generated by vehicular emissions.

4.3. Erythrocyte oxidative injury by $PM_{2.5}$ free fraction.

Figure 3 shows the lipoperoxidative changes in RBC membranes obtained from COPD patients. When compared with HV values, it is apparent that the degree of oxidative injury correlates with progression of the disease. Following incubation with $PM_{2.5}$, diene conjugate formation increased 17.5% (moderate COPD patients) and 16.6% (severe COPD patients) (Figure 3-A) compared with untreated membranes. The LHP values were also increased after $PM_{2.5}$ incubation, indicating that pre-existing oxidative injury in COPD patients was exacerbated (Figure 3B). LHP formation was also increased in the treated control group

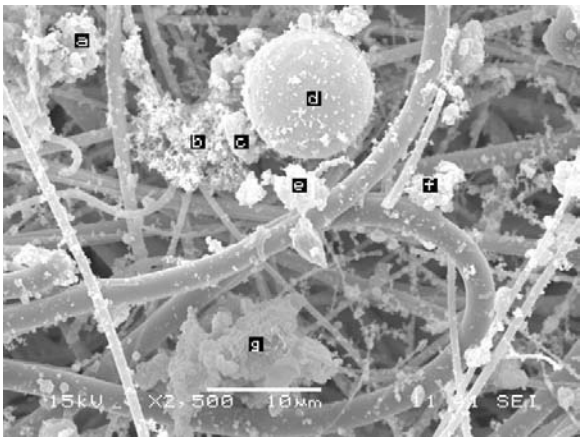


Figure 1. Respirable particles sampled from southeast of Mexico City. Letters a, c and g correspond to PM_{10} aggregates; e and f are $PM_{2.5}$ fragments; d is a PM_{10} sphere and b corresponds to carbon reticular non particles. The most abundant elemental components in these particles are carbon, oxygen, silicon, potassium, calcium and iron.

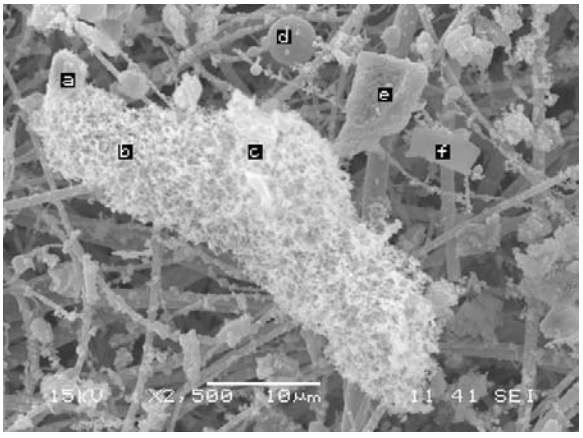


Figure 2. Respirable particles sampled from southeast of Mexico City. Letters a, c and e correspond to $PM_{2.5}$ fragments; f is PM_{10} fragment; d is a combustion PM_{10} sphere and b is a reticular carbon particle that is part of a nanoparticle network.

(0.03 ± 0.01 to 0.05 ± 0.007 mmol $I_3/10^6$ RBC) but never reached the levels obtained from patients in moderate or severe stage of COPD (0.06 ± 0.007 to 0.067 ± 0.017). The MDA concentration significantly decreased in both COPD stages ($P < 0.001$) (Figure 3C) when compared with the control group, which did not show a significant decrease in MDA after $PM_{2.5}$ incubation (0.28 ± 0.05 to 0.31 ± 0.04, nmol of carbocyanine/mg dw). Figure 3C (lower panel) shows the Fenton reaction values, which are similar to those obtained after PM incubation. Figure 4A shows the amount of free protein carbonyls. Significant increases ($p < 0.001$) were observed in both the moderate (14.3 ±2.9 to 21.29 ± 5.1 nmol) and severe (18.19 ± 1.08 to 23.81 ± 23.81 nmol) COPD groups after $PM_{2.5}$ incubation. The carbonylation values in both cases were higher when compared with those produced by the Fenton reaction (Figure 4A). Figure 4B shows the significant inverse regression of the carbonyl concentration versus the glucose-6 phosphate dehydrogenase activity ($p < 0.0001$, $r = -0.6966$). Figure 5 shows the non-protein SH group concentration (Figure 5A) and the glucose-6 phosphate activity (Figure 5B) measured in the RBC supernatant obtained from HV and COPD patients before and after the RBC-$PM_{2.5}$ incubation. The SH group concentration was significantly decreased ($p < 0.0001$) in COPD patients (6.5 ± 1.9 and 4.38 ± 0.9 nmol/mg protein for moderate and severe COPD patients) when compared with healthy volunteers (8.06 ± 1.31 nmol/mg protein). As shown in Figure 5B, the RBC glucose-6 phosphate dehydrogenase activity of COPD patients was also significantly ($p < 0.0001$) less than that observed in healthy volunteer group (4.57 ± 0.79 nmol NADPH/mg protein) in both the moderate (3.04 ± 0.63 nmol NADPH/mg protein) and severe COPD groups (0.59 ± 0.22 nmol NADPH/mg protein). $PM_{2.5}$ incubation induced further significant decreases in the patients with moderately severe COPD. There was a significant ($p < 0.01$) direct correlation ($r = 0.4825$) between SH group concentration and glucose-6 phosphate activity (Figure 5C). The values obtained with the *in vitro* generation of ROS are described in the footnotes of Figures 5B and 5C. The activity of phospho-tyrosine phosphatase showed a significant decrease in the three groups when the supernatant was incubated in presence of the $PM_{2.5}$ suspension (Figure 6) The inhibition was greater in the control group (HV) ($p< 0.0001$), reaching a value of 53.52% less than basal control activity (from 0.71 ± 0.2 to 0.33 ± 0.06 nmol p-nitrophenol/10^6 RBC/min). The phospho-tyrosine activity also decreased in RBCs obtained from COPD patients, but the change was significant only in the moderate group. The Fenton reaction significantly decreased the PTPase activity in the control group when compared with the $PM_{2.5}$ incubation.

5. DISCUSSION

Protecting our health from the surrounding environment is a challenge, particularly for vulnerable populations, such as the elderly, children, pregnant women and their fetuses, residents in areas with poor to marginal outdoor air quality, and people suffering from chronic diseases. Exposure to particulate matter is associated with both short- and long-term cardiovascular and respiratory diseases and may increase the risk of cancer. Short-term exposure to particulate matter ($PM_{2.5}$) is associated with cardiovascular mortality in adults (24). Short-term exposure is also significantly associated, although less strongly, with excess emergency room visits and hospital admissions due to cardiovascular and respiratory symptoms (25). This clinical phenomenon is not observed in populations that have adapted to air pollution because their capacity for efficient antioxidant enzyme induction has been increased following years of exposure to contaminants (26, 27). Long-term exposure appears to increase the risk of developing chronic bronchitis, chronic obstructive pulmonary disease (COPD), and lung cancer. The cumulative effects of chronic exposure to $PM_{2.5}$ have been estimated to reduce the life expectancy for the US population by 1.3 years (28).

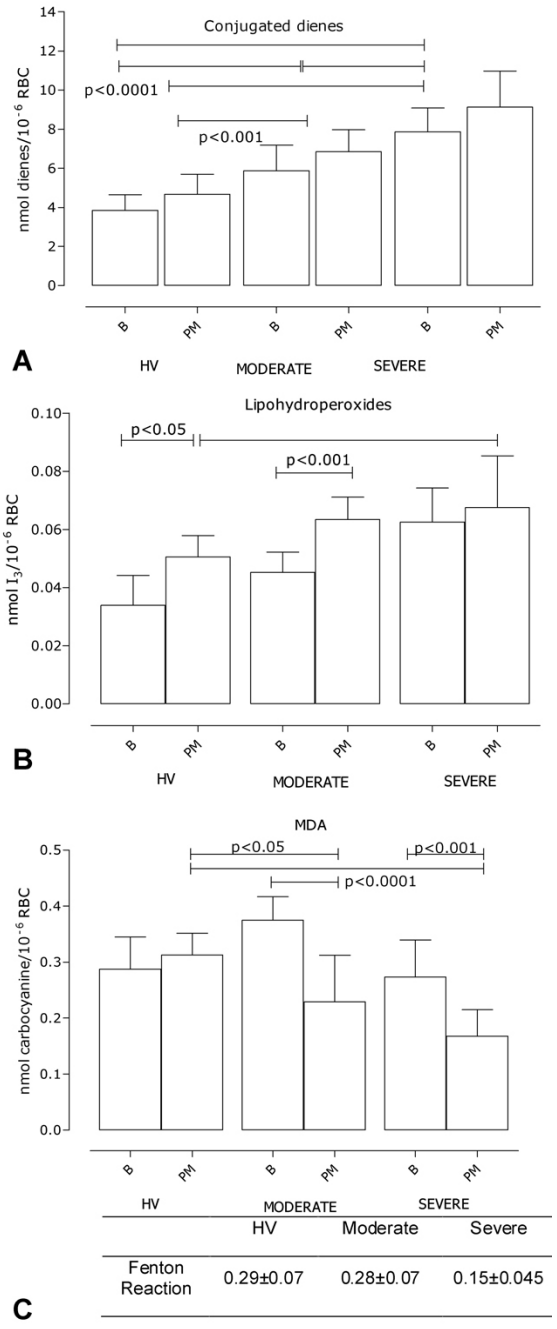


Figure 3. The (A) Conjugated dienes, (B) Lipohydroperoxide (LHP), and (C) Malondialdehyde (MDA) concentration measured in erythrocyte ghosts from moderate and severe COPD patients and healthy volunteers (HV) before and after $PM_{2.5}$ incubation.

Chronic obstructive pulmonary disease affects various lung structure and function, leading to air flow limitation (8). In addition to these pulmonary abnormalities, COPD is associated with significant detrimental effects in organs distant from the lungs. These systemic effects of COPD include weight loss, nutritional abnormalities, skeletal muscle dysfunction, an increased risk of cardiovascular disease, and several neurological and skeletal effects (7). RBC oxidative injury in these patients affects enzymatic activities including the band-3 associated phospho-tyrosine phosphatase (29). The mechanisms underlying these systemic effects are unclear, although they are probably interrelated and multifactorial and may include inactivity, systemic inflammation, tissue hypoxia, and oxidative stress. However, the mechanisms that induce the increased susceptibility of COPD patients to air pollution and particulate matter have not yet been identified. In this study, we have shown *in vitro* the differences in the response of RBC from healthy volunteers compared with RBC from COPD patients incubated with $PM_{2.5}$. An increase in oxidative stress in RBC has been reported in many pathological conditions, including COPD (7, 15, 31). Therefore, the decrease in PTPase activity is considered an excellent indicator of oxidative injury (29).

Our results indicate that depending on composition of PM, the oxidative injury of RBCs in COPD patients is increased in presence of $PM_{2.5}$, which can generate ROS by different mechanisms (Table 2). ROS generation can partially result from redox cycling of hydrophobic substances, including polycyclic aromatic hydrocarbons (PAHs) and persistent quinoid radicals, which are usually bound to fine particles of the soot aggregates (Figure 1 and Figure 2) (28). Compounds found in the water-soluble fraction of PM elicit cell damage via reactive transition metal-dependent formation of hydroxyl radicals, implicating an important role of hydrogen peroxide. Together, these data indicate the importance of mechanisms involving redox cycling of quinones and Fenton-type reactions by transition metals in the generation of ROS (15). Table 2 shows the concentration of Fe^{++} and Cu^{+} transition metals distributed principally in spherical and irregular particles. In our assays, these particles induced injury in RBC membranes *in vitro*, increasing conjugate dienes and LHP formation (Figure 3A, Figure 3B). The generation of ROS from $PM_{2.5}$ can be due to iron and copper producing a Fenton reaction in biological fluids (15). Aromatic compounds present in the soot aggregate may also contribute to the generation of free radicals in our experiments (Table 2). However, to consider such a possibility, a hydrophobic medium is necessary to generate appropriate conditions for the ROS generation. The Fenton reaction used as a control for RBC membrane injury consistently showed similar degrees of oxidative damage compared with $PM_{2.5}$ (Figure 3C, 4A , 5B, Figure 6).

The observed decreases in both non-protein sulfhydryl concentration and G6PD activity were magnified by PM addition, showing a significantly inverse correlation (Figure 5 Figure 4A, r = 0.-6966, Figure 5) when compared with the G6PD activity that decreased as consequence of COPD progression. G6PD is an intracellular enzyme that plays a key role in the protection of erythrocytes against oxidative stress. Reduced activity of G6PD renders RBCs susceptible to hemolysis under oxidative conditions by oxidant drugs and infection (33). The functional consequence of decreases in NADPH and non-protein SH availability in the RBCs is a reduction in antioxidant

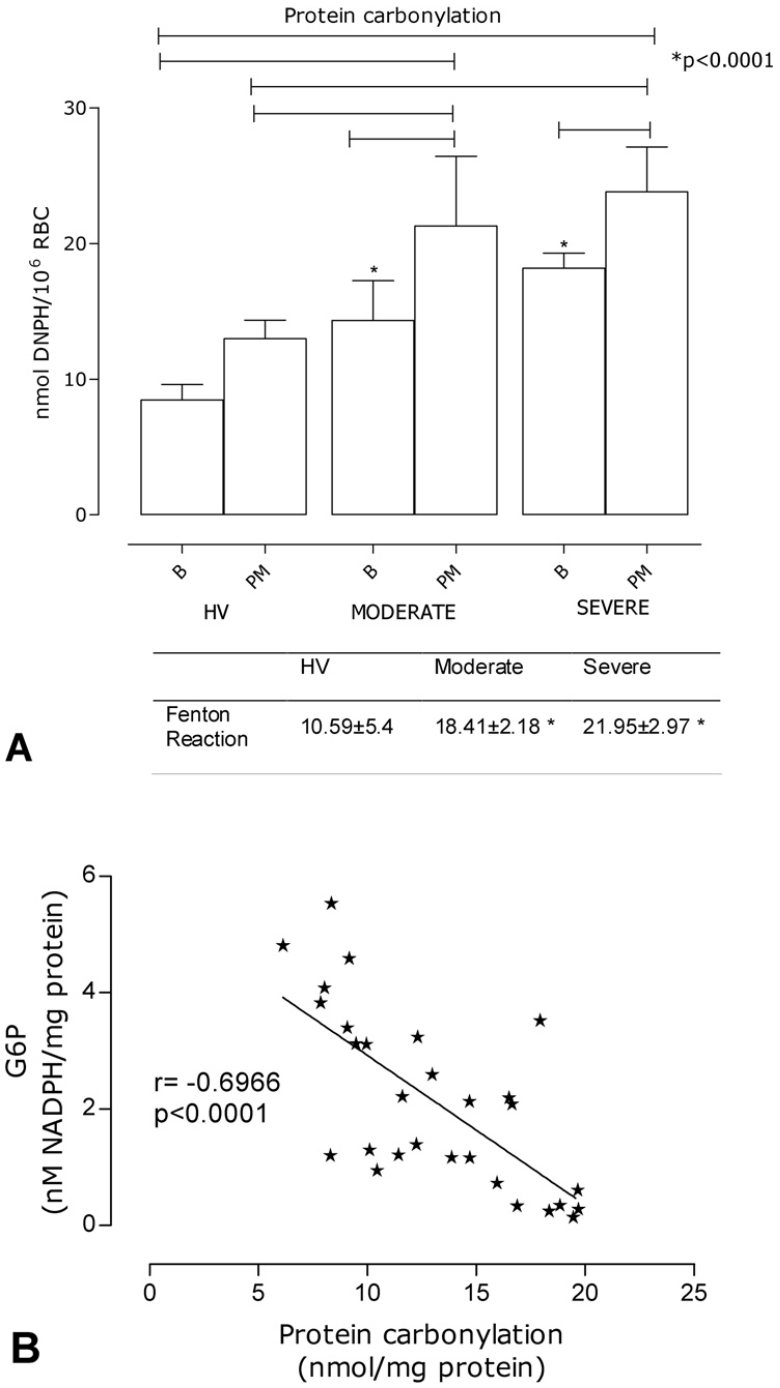
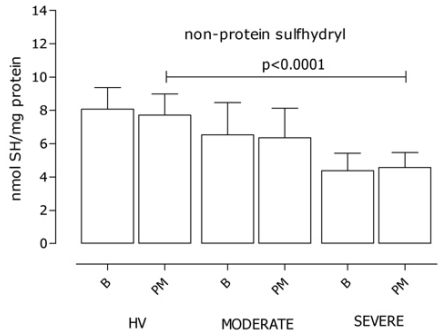


Figure 4. (A) The protein carbonylation of RBC ghosts and its correlation with (B) G6PD activity of RBC in two COPD stages before and after $PM_{2.5}$ addition. Effect of the Fenton reaction.

capacity and the loss of membrane molecular repair (Figure 5B)(34).

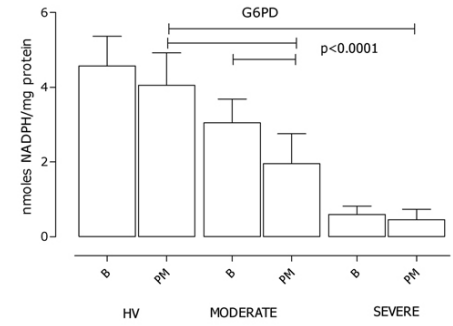
6. CONCLUSION

The interpretation of previous *in vitro* studies has proven difficult because particles of different chemical compositions were used, target cells were different and experimental parameters, such as duration and end points, also differed. Particularly, results from "high doses" should be viewed with caution if they are orders of magnitude higher than predicted from relevant ambient exposures. The adverse health effects of PM are mediated by the carbonous particles of their reactive chemical compounds.



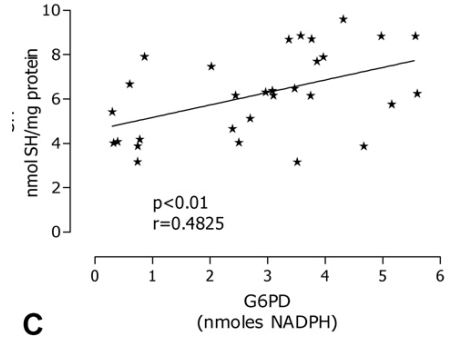
	HV	Moderate	Severe
PM	7.7±1.2	6.34±1.77	4.56±0.9
Fenton Reaction	5.6±1.4 *	5.89±1.44	4.0±1.2

A



	HV	Moderate	Severe
PM	4.05±0.86	1.95±0.8	0.35±0.19
Fenton Reaction	3.87±0.45	1.79±0.55	0.45±0.27

B

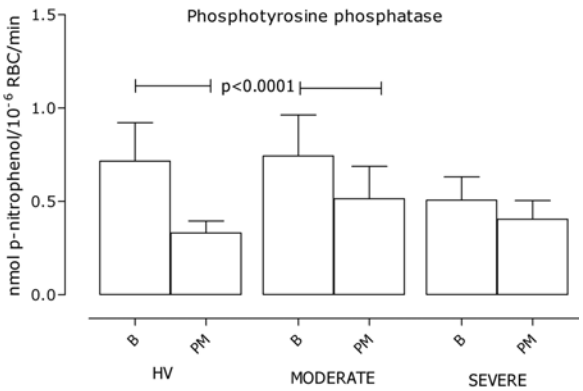


C

Figure 5. (A) The non-protein sulfhydryl concentration of RBC ghosts and its correlation with (B) G6PD activity in RBC. (C) Glucose-6 phosphate dehydrogenase activity in RBC in two COPD stages before and after $PM_{2.5}$ addition. Effect of the Fenton reaction.

Experimental evidence has shown that PM contains redox-active transition metals.

In summary, we demonstrated that, due to a lack of sufficient NADPH and non-protein SH groups for antioxidant activity, RBCs from a population susceptible to air pollution are more vulnerable to damage than RBCs from healthy volunteers. Oxidative injury to membrane lipids and proteins were also demonstrated concomitant with a decrease of PTPase activity and altered function of anionic exchanger (AE_1). Finally, it was possible to determine that cells with preexisting injury resulting from chronic disease (COPD) showed a greater chance of damage in the presence of particulate material from air



	HV	Moderate	Severe
PM	0.33±0.06	0.51±0.17	0.4±0.09
Fenton Reaction	0.39±1.37	0.58±0.045	0.45±0.12

Figure 6. Phospho-tyrosine phosphatase activity of erythrocyte membranes in two COPD stages before and after $PM_{2.5}$ addition. Effect of the Fenton reaction.

pollution compared with the susceptibility of cells from healthy volunteers. This is one of the first reports to demonstrate differences in the responses of cells from healthy and susceptible populations to pollution.

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Abbreviations: ROS: reactive oxygen species, COPD: Chronic Obstructive Lung Disease, GOLD: Global Initiative for Chronic Obstructive Lung Disease, PTPase: phospho-tyrosine phosphatase, Air pollution, RBC damage. Tissue Hypoxia, Oxidative Stress, Erythrocyte, Anionic Exchanger, Glucose 6 Phosphate Dehydrogenase, Free Radicals, Phosphotyrosine Phosphatase, PM: Particulate matters.

Key Words: Tissue, Hypoxia, COPD, Oxidative Stress, Erythrocyte, Anionic Exchanger, Glucose 6 Phosphate Dehydrogenase, Free Radicals, Phosphotyrosine Phosphatase, PM, Particulate matter

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