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Histological changes in lung tissues related with sub-chronic exposure to ambient urban levels of $PM_{2.5}$ in Córdoba, Argentina

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HIGHLIGHTS

- A good correlation was observed between $PM_{2.5}$ and lungs chemical composition.
- Mild infiltration, increment of macrophages and PAS (+) cells was observed in animal exposed to $PM_{2.5}$.
- Treated animals also showed an increase in the number of cell with comets, with a high DNA fragmentation rate.
- Sub-chronic exposure to urban ambient levels of $PM_{2.5}$ caused pathological responses, even at low concentrations.

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ABSTRACT

Concentration of fine particulate matter ($PM_{2.5}$) is one of the most important environmental parameters to estimate health impacts attributable to air pollution. Despite the fact there are many studies regarding $PM_{2.5}$ effects on human health, most of them were performed under conditions that do not simulate the natural particles interaction with the organism. In the present paper, we studied the effects of mammals' sub-chronic exposure to $PM_{2.5}$ on the lower respiratory tract, addressing realistic exposure conditions to normal urban air. Thus, we exposed Wistar rats under controlled settings to the same normal urban air, with and without particles. Next, we analyzed chemical composition of $PM_{2.5}$ and lungs samples, performed a histologic examination and run the comet assay to assess genotoxic effects. We found a strong agreement between lung tissues and $PM_{2.5}$ elemental composition suggesting that metals found in lungs came from the particles inhaled. Histological analysis showed a mild to moderate infiltration, with a reduction of alveoli lumen and increment of alveolar macrophages and periodic acid-Schiff (PAS) (+) cells in treated animals. We also observed an increase in the number of nuclei with comets, mostly comets type 3, with a high DNA fragmentation as well. These results provide strong evidence that sub-chronic exposure to low particle levels, even below the 24 h WHO standard, can cause injuries in lungs tissues and DNA damage, as well.

1. Introduction

According to the WHO, about 90% of people worldwide breathe air that does not comply with its Air Quality Guidelines (World Health Organization, 2006) and about 3 million people die each year due to ambient air pollution, which represents 11% of the total annual deaths (World Health Organization, 2016). Annual mean concentration of $PM_{2.5}$ is highly relevant for estimating health impacts and is used as an exposure indicator for calculating the burden of disease attributable to ambient air pollution. Several studies report strong association between chronic and acute exposure to $PM_{2.5}$ and increased mortality and morbidity (Anderson et al., 2012; Adam et al., 2015). For example, chronic exposures to particles are related to pathologies such as asthma (Kim et al., 2015), COPD (Ko and Hui, 2012), atherosclerosis (Prueitt et al., 2015), arteriosclerosis, hypertension (Franklin et al., 2015) and many others (Laing et al., 2010; Brook et al., 2013a, 2013b; Meo et al., 2015; Costa et al., 2017). However, little is known about the

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biological mechanisms that might explain the chronic epidemiological results and many toxicological studies are urgently needed to fill de gap in knowledge related to the exposure-response relationships.

$PM_{2.5}$ penetrates deep into the lungs and in the cardiovascular system, thus they are more hazardous for human health than larger particles (World Health Organization, 2016). Fine particles can easily reach pulmonary alveoli where they interfere with debris clearance and toxics transport initiating an inflammatory response (Zereini and Wiseman, 2010). Their toxicity may not only be due to this mechanical effect related with particles concentration, but also with particles composition. Thus, their toxicological effects could be partially explained by their adsorbed compounds (Cakmak et al., 2014). Indeed, it has been observed that the chemical composition as well as the elemental concentration are associated with changes in respiratory indexes (Lagorio et al., 2006; Cakmak et al., 2014).

Besides differences in chemical composition, particles can bioaccumulate and cause injuries through oxidative stress and molecules alteration (Zereini and Wiseman, 2010; López et al., 2011). In our previous studies employing ambient urban particles we observed significant associations between their concentration and composition, and metabolic changes, oxidative stress and DNA alterations in kidney, liver and heart animal tissues (Busso et al., 2016) and human cells (Carreras et al., 2013). Therefore, it could be expected that these molecular alterations may derive in tissues macroscopic changes, that can be easily measured.

Other studies have already assessed the influence of ambient urban particles on mammals assessing exposures to concentrated particles (Tesfaigzi et al., 2002; Barrett et al., 2011), their individual adsorbed compounds (Fortoul et al., 2005; Silva et al., 2010), under simulated conditions (Reed et al., 2008), intermittent periods of time (Godleski et al., 2011) or employing non-real entry pathways (instillation, tracheostomy, etc.) (Martin et al., 2007; Riva et al., 2011). However, none of them address the human exposure to atmospheric pollutants, nor the particles natural route of entry into the body, therefore the results could not be completely extrapolated to human health effects. In the present work, we study the effects of real, urban ambient $PM_{2.5}$ sub-chronic exposures on the lower respiratory tract of mammals, addressing realistic exposure conditions to atmospheric pollutants, both particles and gases.

2. Materials and methods

2.1. Sampling site

Cordoba city is located in the center of Argentina (31°25′00″S; 64°11′00″W) and is the second most populated city in Argentina (1.4 million inhabitants). It has a funnel-shaped topography with an increasing positive slope from the center towards the surrounding areas. This somewhat concave formation reduces the air circulation and causes frequent thermal inversions during the autumn and winter seasons. The climate is sub-humid, with an average annual rainfall of 790 mm, concentrated mainly in summer. The mean annual temperature is 17.4 °C and the prevailing winds come from the NE, S, and SE.

The main sources of atmospheric pollutants are related with traffic and road dust resuspension (López et al., 2011; Carreras et al., 2013). The city also has important stationary sources, including metallurgic and mechanical industries that are located in surrounding areas (Olcese and Toselli, 2002).

2.2. Study design

Healthy rats were exposed in two whole-body exposure chambers to normal urban air. In one chamber, treatment animals were exposed to $PM_{2.5}$ or smaller particles while control animals were exposed in the other chamber only to particles smaller than $PM_{2.5}$ (Supplemental material). Concentration of atmospheric gases was not controlled, so their proportion were expected to be similar in both chambers. After a three months period, lung tissues of exposed animals were analyzed for inorganic composition, histological changes, and DNA damage.

2.3. Exposure protocol

The study was conducted under the NIH guidelines of Institutional Animal Care and Use Committee (IACUC). Twelve male Wistar rats were bred and kept until their use in the Specific Pathogen Free bioresources of the INIMEC (UNC), in an area with ultra-filtered air. At the age of 5 weeks, they were randomly divided into two groups with equal numbers of individuals (control and treatment) and exposed in a mobile animal facility with vacuum closure for a period of three months (2160 h), which is considered sub-chronic exposure (Barile, 2013), at the same time samples of $PM_{2.5}$ were taken. Water and food was always supplied *ad libitum* and beds were renewed weekly with sieved sawdust.

The facility consisted of two chambers of 60 cm (width) 60 cm (length) and 60 cm (high), each one provided with a source of warm light (12 h photoperiod), a heating plate connected to an adjustable internal thermostat (20–27 °C) and a thermometer to record flagrant, maximum and minimum temperatures (Supplemental material). Each whole-body exposure chamber had an air intake connected to a pipe system coupled to an impactor, a door with vacuum closure and an air output attached to a vacuum pump. The pipe system had a breaker section that allows to deflect air stream to a flowmeter. The pump generates a stream of air from the outside that passes through the impactors, the flowmeter (when needed) and the chambers, to be then expelled by the pump outlet, about 15 m away. Thus, in the control chamber all particles were removed with an impaction plate and a $PM_{2.5}$ PTFE filter; while in the treatment chamber only large particles (>$PM_{2.5}$) were removed by an impaction plate.

A constant airflow of 12.5 ± 2 L min^{-1} was employed, which arises from a compromise between efficient particle filtration and an air level that may not cause hypoxia. In both cases, flow allowed a full replacement of the internal atmosphere at least 15 times per hour, ensuring no toxic levels of ammonia and other harmful gases.

2.4. $PM_{2.5}$ sampling and mass determination

Two daily samples of $PM_{2.5}$ were collected using 16 L min^{-1} Harvard Impactors (HI) at 12.5 L min^{-1} with an expected cut point a little bit over 2.5 μm. 47-mm polytetrafluoroethylene filters with a 2.0 μm pore (*Zefluor, Millipore*) were employed. One impactor, was connected to the control exposure chamber and was employed for the determination of mass and inorganic composition, while the other impactor, was employed for $PM_{2.5}$-PAHs quantification. Samplers were placed 7 m high on the roof of the Chemistry Department at the School of Exact, Physical and Natural Sciences of the National University of Córdoba (31°26′10.9″S; 64°11′38.8″W), from August to October, 2014 in Córdoba city, meaning that we collected 90 daily samples for each determination.

$PM_{2.5}$ mass was determined by gravimetric difference using a microbalance (0.01 mg mass resolution, *Sartorius*) (Carreras et al., 2013; Busso et al., 2016). $PM_{2.5}$ concentration was expressed as mean ± standard deviation (SD).

2.5. PAHs composition

Particle-associated PAHs were determined as previously

reported (Busso et al., 2016). Concentration of Naphtalene, Acenaphtene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo[*a*]anthracene, Chrysene, Benzo[*b*]fluoranthene, Benzo[*k*]fluoranthene, Benzo[*a*]pyrene, Dibenzo[*a,h*]anthracene, Benzo[*g,h,i*]perylene and Indeno[*1,2,3-c,d*]pyrene were obtained for 90 filters employing High Performance Liquid Chromatograph (*Dionex Ultimate 3000, Thermo Scientific*), with a C-18 reverse-phase column (*Vydac* 201TP 250 mm 2.1 i.d. 5 μm) and fluorescence detector. UV detection was employed for Indeno[1,2,3-*cd*]pyrene. Five-point calibration curve was obtained for all PAHs employing standards (Sigma), ranging from 0.1 to 25 μg L^{-1} (R^2 for all compounds were over 0.9991; $p < 0.005$).

2.6. Gases

In both chambers, atmospheric concentrations of SO_2, NO_2, O_3 and CO were monitored employing automatic electrode sensors (CairClips SO_2, NO_2, O_3-NO_2, CO coupled to a CairTub, *Cairpol*) with a sensibility of parts per billion by volume (ppbv). Moving averages of 1 h (SO_2 and NO_2) and 8 h (O_3 and CO) were calculated.

2.7. Tissue collection

In order to obtain tissue samples, animals were anesthetized with ketamine (100 mg kg^{-1} body weight) and xylazine (20 mg kg^{-1} body weight) and weighed (Busso et al., 2016). The thoracic cavity was opened, lungs and bronchia were removed, washed with cold PBS (4 °C) and weighed (total fresh weigh, TFW). Next, one lung of each animal was submerged in 10% m/v formalin buffered solution, for histological analysis (Bancroft et al., 2013) and the other was divided in two fragments. One fragment was weighed (wet weight, WW), washed with Milli Q water and preserved at −80 °C for elemental composition, while the other was chopped, washed with cold PBS, submerged in 1 mL of Hanks' balanced salt solution (HBSS) and conserved at 4 °C in the dark for comet assay.

2.8. Elemental composition

Elemental composition of filters with $PM_{2.5}$ (Wang et al., 2006) and lung tissues (Chen et al., 2007) was determined by Mass Spectroscopy Inductively Coupled Plasma (*Agilent 7500cx*). Three calibration curves (high, middle and low concentration) were made employing pure standard (*Sigma*) (R^2 for all elements were over 0.9983; $p < 0.005$). Frozen tissues were dried at 60 °C for dry weight determination (DW). Next, all samples were digested with 2 mL of 65% HNO_3 (*Merck, Millipore*) at 130 °C in closed reflux tubes, filtered with 0.22 μm pore filter (*Millipore*) and carried to a final volume of 3 mL. When necessary, dilutions were prepared employing Milli Q water. Levels of K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn As, Cd, Hg, Tl and Pb were determined in all filters (90) and tissues (12).

2.9. Histological analysis

Lungs collected in formalin were dehydrated (50%, 70%, 90%, 95% and 100% v/v ethanol solution), clarified in xylol and embed in paraffin by an automated tissue processor (*Leica TP1020*). Then, fragments were cut with a rotary microtome (2 μm), mounted on slides and fixed. Tissue slides were next deparaffinized and stained with hematoxylin/eosin (HE) and periodic acid-Schiff (PAS) (Bancroft et al., 2013). Preparations were observed at 400 and 1000x (*Olympus CX31*). 20 fields of each slide were explored looking for leucocyte infiltration, fibrosis, anisokaryosis, and anisocitosis (Kumar et al., 2014). Number of macrophages in alveoli (NM), number of PAS (+) cells in bronchia and percentage of alveoli lumen (%AL) was also determined (Martin et al., 2007). Each field was analyzed and scored with a scale from 0 to 3 to classify the damage (0: no observed, 1: mild, 2: moderate and 3: severe) and a slide mean was calculated. %AL of lung's center and periphery was determined in 20 photographs per animal (400x) by a computer image analysis software (*Bio7*, Freeware). Results of each group were expressed as mean ± standard error (SE).

2.10. Comet assay

An alkaline unicellular assay was performed in order to assess DNA damage. Each chopped fragment of lung was mixed creating a flow with an automatic pipette to disperse the cells. Tissue fragments were removed and cellular suspension was centrifuged (670g, 2 min, 4 °C). Supernatant was discarded and cells were resuspended in 1 mL of fresh HBSS. Next, comet assay was performed as described by Busso et al. (2016). 2 slides of each animal lung were observed and photographed employing an epifluorescence microscope (*Nikon*). 200 nuclei per animal were counted

Table 1
Concentration (μg m^{-3}) and elemental composition (ng m^{-3}) of $PM_{2.5}$ collected in Cordoba city and comparison with other studies.

City (Ref.)	Córdoba, Argentina (Present Study)		Córdoba, Argentina (López et al., 2011)		Buenos Aires, Argentina (Murruni et al., 2004)		Porto Alegre, Brazil (de Miranda et al., 2012)		Medellin, Colombia (Gomez et al., 2011)		Santiago de Chile, Chile (Sax et al., 2007)	
Determ.	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
$PM_{2.5}$	10.3	3.8	67.0	17.8	14.0	8.0	19.3	14.3	36.9	21.7	52.0	–
K	147.5	112.3	170.0	145.0	–	–	322.3	320.2	275.0	318.0	238.4	–
Ca	87.0	142.5	289.0	218.0	–	–	38.3	40.6	68.0	97.0	91.3	–
Ti	16.3	4.8	22.0	23.0	6.0	2.0	5.2	6.0	9.0	19.0	–	–
V	0.9	0.4	7.0	2.0	4.0	2.0	1.5	1.0	0.0	2.0	–	–
Cr	43.6	9.2	3.5	0.8	2.0	1.0	12.1	69.1	0.0	3.0	5.2	–
Mn	7.3	2.8	13.0	9.0	2.0	1.0	6.2	19.7	4.0	11.0	16.0	–
Fe	106.8	115.0	345.0	211.0	89.0	45.0	90.7	134.3	157.0	131.0	271.4	–
Co	*	*	0.6	0.2	–	–	–	–	14.0	25.0	–	–
Ni	12.9	2.9	2.8	0.7	2.0	1.0	4.7	13.9	*	*	1.2	–
Cu	19.4	30.0	9.0	5.0	7.0	5.0	5.2	7.5	*	*	24.2	–
Zn	111.0	36.3	28.0	22.0	26.0	20.0	18.7	20.2	78.0	97.0	76.3	–
As	2.2	0.6	–	–	–	–	–	–	1.0	8.0	–	–
Cd	0.2	0.1	–	–	–	–	–	–	*	*	–	–
Hg	0.0	0.0	–	–	–	–	–	–	–	–	–	–
Tl	*	*	–	–	–	–	–	–	35.0	383.0	–	–
Pb	7.2	2.9	0.0	0.0	24.0	9.0	5.4	5.4	208.0	447.0	33.2	–

Asterisks (*) represents values below detection limit and scripts (–) represents not reported values.

Table 2
Concentration of PAHs associated to fine particles collected in Cordoba city (pg m^{-3}) and comparison with other studies.

PAHs	Córdoba, Argentina (Present Study)		Córdoba, Argentina (Amarillo et al., 2014)		La Plata, Argentina (Rehwagen et al., 2005)	
	Mean	SD	Mean	SE	Mean	SD
Nap	16	10	4985	586	137	–
Ac	67	28	308	70	5	–
Fl	45	38	773	143	15	–
Phe	68	45	878	95	112	–
Ant	3	2	27	4	15	–
Flu	12	8	681	135	169	–
Pyr	10	4	394	81	231	–
B[*a*]A	6	2	513	77	209	–
Chr	12	10	516	93	344	–
B[*b*]F	6	4	778	116	756	–
B[*k*]F	9	5	182	27	263	–
B[*a*]P	8	17	357	61	381	–
DB[*ah*]A	32	34	106	15	60	–
B[*ghi*]P	24	20	827	120	1307	–
Ind	49	30	–	–	738	–
Total PAHs	354	172	11330	419	4605	–
2-rings PAHs	43	25	2022	266	52	–
3-rings PAHs	28	18	529	78	99	–
4-rings PAHs	9	5	477	79	361	–
5-rings PAHs	20	26	232	38	221	–
6-rings PAHs	37	25	827	120	1023	–

Naphtalene (Nap), Acenaphtene (Ac), Fluorene (Fl), Phenanthrene (Phe), Anthracene (Ant), Fluoranthene (Flu), Pyrene (Pyr), Benzo[*a*]anthracene (B[*a*]A), Chrysene (Chr), Benzo[*b*]fluoranthene (B[*b*]F), Benzo[*k*]fluoranthene (B[*k*]F), Benzo[*a*]pyrene (B[*a*]P), Dibenzo[*a,h*]anthracene (DB[*ah*]A), Benzo[*g,h,i*]perylene (B[*ghi*]P), Indeno[*1,2,3-c,d*]pyrene (Ind).

and analyzed employing an image analysis software (Comet Score, *TriTek Corp.; Sumerduck, USA*). For each animal tissue, percentage of nuclei with comets, percentage of DNA in tail (%T-DNA) and tail moment value (TMV, %) was measured. Results were expressed as mean ± standard error of each group.

2.11. Statistical analysis

To assess statistical differences between treatments non-parametric analysis Mann-Whitney *U*-test was employed. Statistical analyses were performed employing IBM SPSS 19.0 (*IBM Corp.*, Armonk, NY, USA). Differences with a p value < 0.05 were considered statistically significant.

Regarding elemental composition, we calculated a theoretical mass accumulation for each element, considering the elemental concentration in $PM_{2.5}$ (ng m^{-3}), the maximum ventilation rate (m^3 min^{-1}) and exposure minutes per day (min) (Sharp and Villano, 2012). Next, we calculated the mass accumulated over the 90 days exposure period (total mass) summing up the daily mass. In addition, we calculated the actual mass accumulated in lung tissues multiplying the elemental tissue concentration (ng mg DW^{-1}) by the dryness rate (mg DW mg WW^{-1}) and total organ mass (mg WW). A linear regression was performed between theoretical and actual accumulated mass.

Table 3
Diagnostic ratios of PAHs sources.

Ratios	Value	Reference	Source
Flu/(Flu + Pyr)	0.54	>0.5	Diesel
B[*a*]P/(B[*a*]P + Chr)	0.41	0.5	Diesel
B[*a*]A/(B[*a*]A + Chr)	0.35	0.38–0.64	Diesel
B[*b*]F/B[*k*]F	0.65	>0.5	Diesel
Fl/Pyr	4.38	>0.6	Vehicular
(Flu + B[*b*]F + B[*k*]F)/DB[ghi]P	1.12	1.6	Diesel

3. Results

We collected 90 samples of $PM_{2.5}$ from August to October, 2014. The average $PM_{2.5}$ and elemental composition concentration for the mentioned period is shown in Table 1. Maximum and minimum levels registered were 18.9 and 4.9 μg m^{-3}, respectively. Overall mean was slightly above annual WHO standards (10 μg m^{-3}) and no daily sample was above the 24 h WHO standard (25 μg m^{-3}) (World Health Organization, 2016).

$PM_{2.5}$ showed an elemental composition rich in K, Ca, Fe and Zn. V, Cr, Ni and Zn concentration were an order of magnitude higher than those reported by López et al. (2011), while the other elements concentrations were similar or lower. These differences could be attributed to the high variability of daily data, meteorological conditions and sampling site characteristics. The presence of Ti, Ni, Cu and Zn suggests that the origin of the PM is anthropic, mainly from vehicular traffic (Kabata-Pendias and Mukherjee, 2007), which agrees with the main PAHs emission sources estimated by diagnostic ratios (Table 3). However, the abundance of K, Ca, Fe, As and Pb suggest also a contribution of dust resuspension (Zereini and Wiseman, 2010). There are only few studies dealing with inorganic composition in Latin America. Overall, we found that elements concentrations were in the same order of magnitude than those reported for Porto Alegre (Brazil), Medellin (Colombia) and Santiago de Chile (Chile) (Table 1).

Regarding PAHs, individual concentrations are shown in Table 2. PAHs determined were selected because they are signaled as the most abundant urban carcinogens, mutagens and teratogens (Agency for Toxic Substances and Disease Registry, 2016) and many of them have been classified as priority pollutants by both the United States Environmental Protection Agency (2017) and the European Environment Agency (Lerda, 2010). PAHs levels were one order of magnitude lower than the informed for La Plata, Argentina (Rehwagen et al., 2005) for almost all compounds and 12 times lower than the ones reported for the same sampling site during 2012 for total suspended particles (TSP) (Amarillo et al., 2014). The lower values observed were in accordance with the fact that $PM_{2.5}$ represented a 6.85% of TSP above mentioned (14 times lower). The 2-rings and 6-rings PAHs were predominant, comprising compounds that can also produce oxidative stress (Busso et al., 2016). To identify probable PAHs emission sources several diagnostic ratios were calculated (Slezakova et al., 2013). Even though diagnostic ratios have limitations and their results cannot be considered conclusive, they can be useful for an exploratory analysis (Park et al., 2011). All the ratios calculated suggested that diesel powered vehicles were the main source of PAHs in the atmosphere of Cordoba city during the study (Table 3).

Temperature during the exposure period, outside and inside the chambers was 23.2 ± 3.9 °C and 25.5 ± 2.6 °C, respectively. Average concentration of atmospheric pollutants NO_2, SO_2 and CO (mean ± SD) were 46.41 ± 9.43, 13.72 ± 4.06 and 101.0 ± 40.5 ppbv, respectively. O_3 concentration was always under the instrument sensitivity limit (20 ppb). Throughout the experiment, gases concentration did not exceed international standards (United States Environmental Protection Agency, 2016).

We also analyzed the inorganic chemical composition of lung tissues and calculated their concentration (Table 4). K, Ca and Fe were excluded from this analysis because there are major components in mammal tissues and V, Mn, Co and Tl were not informed because they were below the detection limits. Despite we did not found any significant difference between control and treated animals, concentration in control animals tended to be

Table 4
Concentration of inorganic elements in lung tissues (ng element mg DW^{-1}).

Element	Treatment (n = 6)		Control (n = 6)	
	Mean	SE	Mean	SE
Ti	3.58	0.56	2.74	0.18
Cr	8.23	4.98	2.67	0.42
Ni	19.00	7.90	10.82	5.47
Cu	19.91	11.17	7.82	0.60
Zn	221.70	67.40	133.76	19.48
As	6.59	1.37	3.87	0.40
Cd	0.11	0.06	0.08	0.02
Hg	3.16	0.51	2.21	0.57
Pb	4.63	2.87	1.59	0.55

slightly lower than animals under treatment. In order to analyze the relationship between metals measured in lungs and those measured in $PM_{2.5}$ samples, a linear regression was performed between the theoretical and actual accumulated mass in lung tissues for each element ($R^2 = 0.874$; Fig. 1). The fact we found a fairly good regression coefficient suggests that most elements have an aerial origin.

To determine the damage produced by exposure to $PM_{2.5}$, we analyzed all lung tissues from treated (6) and control (6) animals. Results are presented in Table 5. Somatic index and tissue hydration percentage showed no statistical difference. Mild infiltration (Fig. 2a and b) was observed in treated animals, which is mainly attributable to animals with mild (score 2) and moderate (score 3) observable damage. Infiltration was diffuse in treatment and control animals, losing intensity from the center to the periphery. Treated animals also presented a major NM in comparison with the control rats (Fig. 3a and b). The same trend was observed with the number of PAS (+) cells (Fig. 4a, b). Regarding the %AL in the periphery (Fig. 2a and b), control animals showed difference from treated animals. This difference was enhanced when comparing lung centers.

To evaluate the DNA damage produced after $PM_{2.5}$ exposure, an alkaline unicellular electrophoresis was performed employing lung tissues. Results are shown in Fig. 5. The percentage of nuclei with comets was twice higher in treatment animals than in control ones. Considering the classification by DNA percentage in the comet tail (Busso et al., 2016), a significant difference between control and treatment animals was observed only in type 3 comets (50–74% DNA in tail) ($p = 0.0411$). Same trend was observed with the TMV: nuclei of treatment cells showed a 52.9 ± 3.9% TMV, while nuclei of control ones presented a 37.8 ± 6.5% TMV ($p = 0.0411$).

4. Discussion

In the present paper, we demonstrated that $PM_{2.5}$ associated compounds induce a proinflammatory response in rats' lungs, even when PM concentrations were below daily mean standards and near the annual WHO air quality guideline for $PM_{2.5}$, in agreement with epidemiological studies showing there is no safe threshold for particles in the atmosphere (Adam et al., 2015). Indeed, we verified that particles may not only induce histological changes, but also contribute to DNA degradation process. We present strong evidence that urban environments with mild to low pollution can led to physiological and genetic damage in lung tissues due to the presence of particles that could be extrapolated to human health, since animals' exposure conditions simulate the exposure of a human being living in urban environments.

Previous studies have only characterized inorganic composition of fine particles of the city of Córdoba (López et al., 2011) or PAHs composition of TSP (Amarillo et al., 2014). In the present study, we characterized not only $PM_{2.5}$ composition, but also determine its inorganic and PAHs composition. We found that $PM_{2.5}$ concentration was lower than the reported by López et al. (2011). This could be attributed to meteorological conditions and sampling site characteristics. However, we found similar levels to those reported in urban areas such as Buenos Aires (Murruni et al., 2004) or Porto Alegre, during the winter period (de Miranda et al., 2012) and lower than the reported in cities with similar geographic conditions, like Medellin (Gomez et al., 2011) and Santiago de Chile (Sax et al., 2007).

Histological analysis of treated animals showed mild infiltration, decreasing from the center to the periphery, and increased NM in the respiratory lumen. This in accordance with the fact that alveoli lumen in these animals tend to diminish in the center, which may be due to leucocyte recruitment, associated to the infiltration observed. In contrast, the percentage of lumen in the periphery was higher than in control animals, which may be related to rupture of the alveolar septa. Even though this cannot be only attributable to a physical damage of $PM_{2.5}$, the increased number of leukocytes may be related not only with an activation of tissue repair and regeneration systems, but also with compounds, biological molecules and microbiological agents associated. PAS (+) cells in bronchia

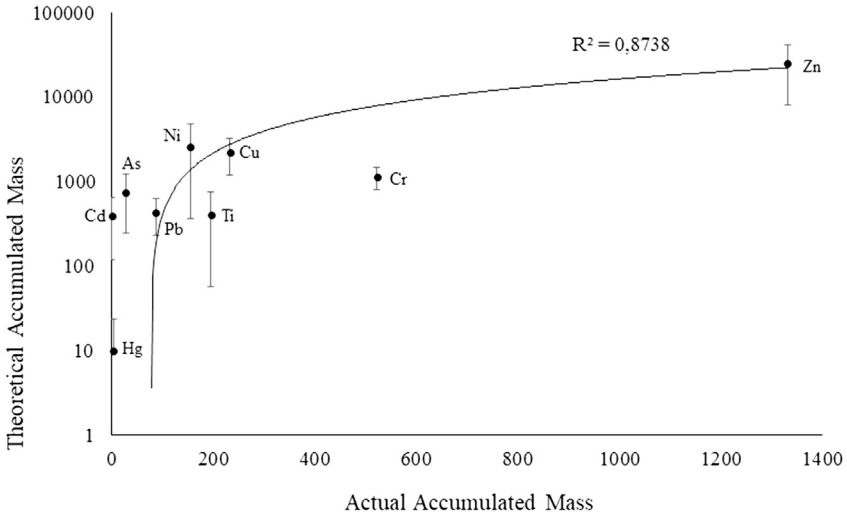

Fig. 1. Correlation between theoretical and actual accumulated mass.

Table 5
Somatic and histological determinations (mean ± SE).

Parameter	Control	Treatment	p value
Somatic index (g TFW g animal^{-1})	0.0048 ± 0.0003	0.0057 ± 0.0008	>0.05
Tissue hydration (%)	87.07 ± 0.96	85.90 ± 1.86	>0.05
Infiltration (score)	0.40 ± 0.06	1.12 ± 0.14	0.0022
Fibrosis (score)	No observed	No observed	–
Anisokaryosis (score)	No observed	No observed	–
Anisocitosis (score)	No observed	No observed	–
Number of macrophages in alveoli (cells per field)	4 ± 0.4	26 ± 0.8	0.0032
PAS (+) cells (cells per field)	9.5 ± 0.8	59.8 ± 10.6	0.0022
Alveoli lumen - Center (%)	75.65 ± 2.55	46.15 ± 2.54	0.0022
Alveoli lumen - Periphery (%)	85.18 ± 1.08	88.38 ± 0.67	0.0087

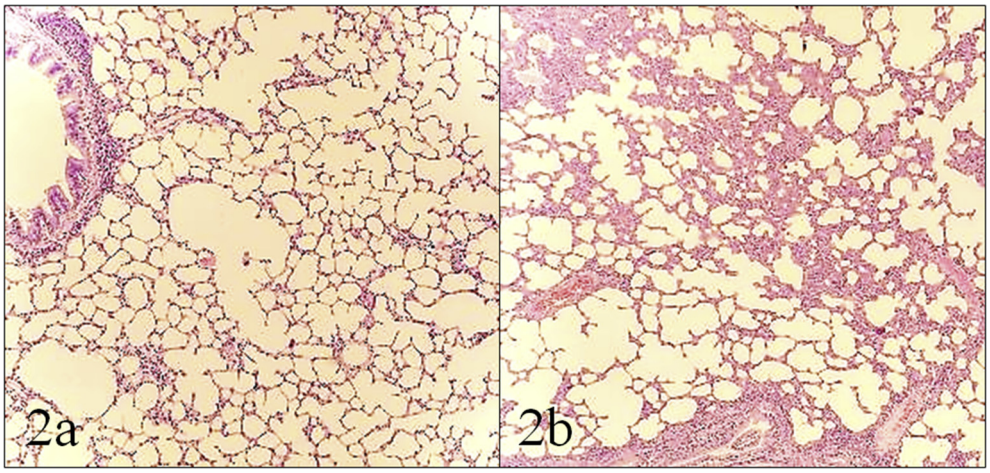

Fig. 2. a, b – Lungs slides, Hematoxylin-Eosin stain. Treated animals (2b) showed mild to moderate infiltration in comparison with controls (2a). See also the decrease in alveolar space.

were also higher in treated animals. Since PAS stains mucus components like carbohydrates, glycoproteins and proteoglycans (Bancroft et al., 2013), an increment of PAS (+) cells might indicate an increase in mucus production. This process has already been observed when the lower respiratory tract was exposed to air pollutants (Kilburn, 1967). Therefore, under a chronic exposure it is expected that a greater number of cells will activate the mucus production, contributing to exacerbate pathologies, such as COPD or asthma. Despite the fact that animals were exposed to a complex mixture of air pollutants, infiltration observed, reduction of alveoli lumen, and increment of alveolar macrophages and (PAS) (+) cells are consistent with the results collected by Zereini and Wiseman (2010) and Anderson et al. (2012). Although the concentration of measured gases did not exceed international standards, we cannot ignore that gas-particle interaction might exacerbate the inflammatory processes, through the facilitation of either oxidative or

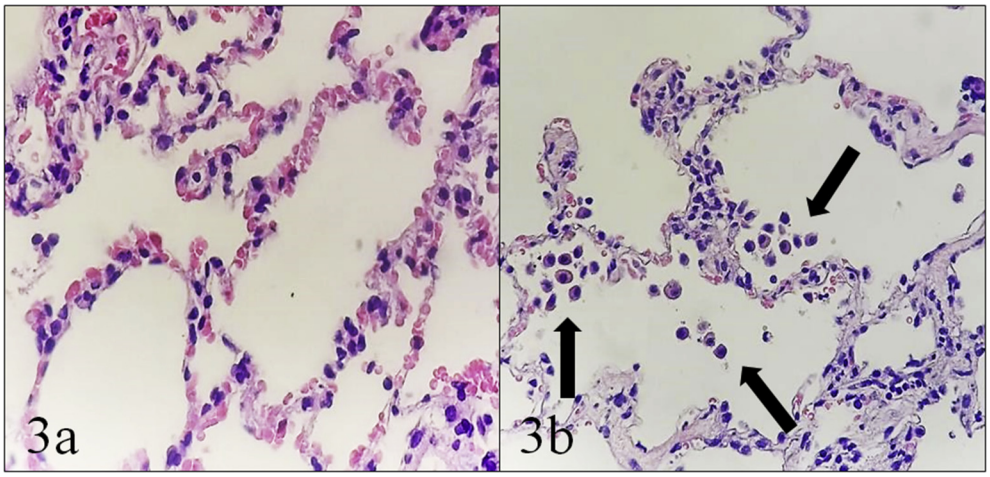

Fig. 3. a, b – Lungs slides, Hematoxylin-Eosin stain. Control (3a) vs. treatment (3b) animals. Arrows indicates the incremented number of mononuclear cells in alveoli of treated animals.

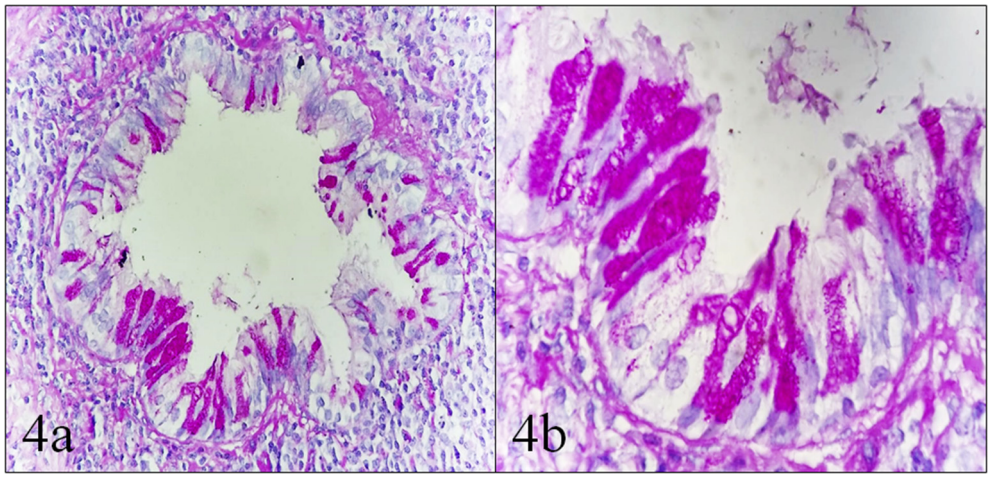

Fig. 4. a, b – Lungs slides, PAS stain. Bronchia PAS (+) cells at 400x (4a) and 1000x (4b).

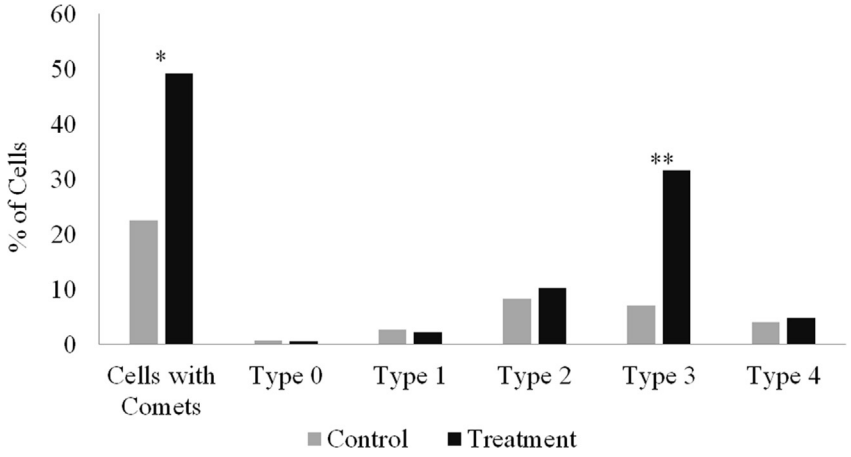

Fig. 5. Percentages of cells with comets in lungs. Bars represent mean values (* = $p < 0.05$; ** = $p < 0.01$).

irritant processes, even with low $PM_{2.5}$ concentrations. However, the fact that both control and treated animals were exposed to the same levels of gaseous pollutants suggests the idea that the observed damage is mainly due to particles.

As stated before, many studies have been conducted exposing animals to suspended particles. However, they do not simulate realistic conditions, as they test individual particles-bounded compounds (Fortoul et al., 2005; Silva et al., 2010), concentrated $PM_{2.5}$ (Barrett et al., 2011), primary particles emissions (Reed et al., 2008), one source emissions (Tesfaigzi et al., 2002) or urban ambient concentrations, but for intermittent time periods (Godleski et al., 2011) or under conditions that do not simulate the natural particle entrance to the organism (Seagrave et al., 2006; Martin et al., 2007; Riva et al., 2011). Our exposure protocol imitates a truthful exposure to a realistic mix of inhalable gases and particles and our findings were consistent with those reported in the mentioned studies (Table 6). The differences in histological changes we have observed may be attributable mainly to the exposure timing (duration and frequency) and particles source. Regarding timing, intermittent exposures can induce acute responses without habituation or pathological responses, such as increment of PAS (+) cells. On the other hand, the mild infiltration observed can be explained by the particles source, since the particles tested may be oxidized or contain biological agents, differing from those employed in some of the mentioned studies. Nevertheless, the overall similarities suggest that the concentration of particles is not the only characteristic responsible for $PM_{2.5}$ toxicity. Indeed, their toxicity could be related to both particles composition and exposure timing; even more if we consider that many of its components can actually bioaccumulate.

The results of the comet assay demonstrated a significant DNA damage in exposed animals. DNA fragments were not only higher, but also more scattered than control's (TMV), which indicates these fragments were smaller. Therefore, the cells would be exposed either to low concentrations of high toxicity compounds, such as PAHs, or to substances with lower toxicity acting synergistically, such as some metals. DNA damage may be the consequence of metals or PAHs-mediated oxidative stress (Busso et al., 2016), indirect effects produced by the metabolism of toxic substances (Laing et al., 2010) and direct modifications to DNA molecule (Azqueta et al., 2009). Moreover, many of the $PM_{2.5}$-bounded compounds, can cause cytotoxic effects with disturbances in cell's mediators that can lead to genetic alterations, too (Øya, 2009). Our results are consistent with many other studies, that reports an increment of lung cell with comets when exposed to $PM_{2.5}$. However, many of these experiments were performed employing $PM_{2.5}$ extracts (Oh et al., 2011), with instillation (Zhang et al., 2011) or in vitro studies (Mohseni Bandpi el at., 2016). Despite the fact that $PM_{2.5}$ concentration was much lower in this study than in other studies whose values ranged from 10 to 100 $\mu g\ m^{-3}$, our results

Table 6
Comparison of exposure conditions and results between studies.

Exposure	Present Study	Seagrave et al. (2006)	Riva et al. (2011)	Martin et al. (2007)	Barrett et al. (2011)	Tesfaigzi et al. (2002)	Reed et al. (2008)	Godleski et al. (2011)
Organisms	Rat	Rat	Mouse	Mouse	Mouse	Rat	Rat	Rat
Duration	90 days	1 days	1 days	7 days	3 days	90 days	180 days	78 days
Frequency	24 h/day	1 dose	1 - 3 doses	3 doses/day	6 h/day	3 h/day	6 h/day	6 h/day (one-day intermittent)
						5 days/week	7 days/week	
Particulate Matter	$PM_{2.5}$	$PM_{2.5}$	$PM_{2.5}$	TSP	<50 μm	63 - 74% <1 μm	0.10 ± 1.4 μm	–
						26 - 37% >1 μm		
Particles Concentration	10.3 μg/m^3	0.75–3 mg/animal	5 μg/animal	0.17 mg/kg	117 μg/m^3	0.021–9.8 μg/m^3	9 - 43 μg/m^3	123 - 212 μg/m^3
Source	Traffic	Wood smoke (primarily)	Traffic	Traffic	Coal combustion	Wood smoke	Primary diesel exhaust	Coal power plant emissions
Entrance Way	Respiratory (normal breathing)	Intratracheal instillation	Intranasal instillation	Intranasal instillation	Respiratory (normal breathing)	Respiratory (normal breathing)	Respiratory (normal breathing)	Respiratory (normal breathing)
Observed Effects	Mild leucocyte infiltration. Increment of macrophages in alveoli. Reduction of alveoli space. Increment of PAS (+) cells.	Leucocyte infiltration. Histological changes. Cytotoxicity.	Increment of macrophages in alveoli. Reduction of alveoli space.	Increment of phagocytes in alveoli. Reduction of alveoli space. Increment of PAS (+) cells.	Increment of macrophages and neutrophils in alveoli.	Mild chronic inflammation and squamous metaplasia in larynx. Alveolar macrophage hyperplasia and pigmentation. Increment of PAS (+) cells.	Increment of macrophages in alveoli. Thickening of alveolar septa. Increment of PAS (+) cells.	Increment of macrophages in bronchoalveolar lavage. No histological changes.

showed a higher %T-DNA. This may be related with the exposure setup, namely a sub-chronic exposure to a mix of aerial toxics with a high penetration ability that may lead to an increment in the number of comets in animals' lungs, with a higher percentage of DNA in tails as well.

The present study demonstrates a straightforward relationship between fine particles, and anatomic and physiological alterations in lung tissues. Even though, we acknowledge several limitations, such as the small number of animals studied and the fact that we did not measure molecular markers, we were able to demonstrate that exposure to low levels of urban $PM_{2.5}$ causes infiltration, increases the NM and PAS (+) cells and alters %AL, even under sub-chronic exposure to medium-low levels of ambient particles. This suggests that the observed effects are mainly related with $PM_{2.5}$ exposure timing and particles source, implying a stronger importance of particles' composition than concentration. Our results will certainly contribute to understand biological mechanisms supporting the chronic effects observed in epidemiological studies at medium-low exposures.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atmosenv.2017.08.061.

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