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RESEARCH ARTICLE

# Organic compounds present in airborne particles stimulate superoxide production and DNA fragmentation: role of NOX and xanthine oxidase in animal tissues

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**Abstract** Suspended particulate matter trigger the production of reactive oxygen species. However, most of the studies dealing with oxidative damage of airborne particles focus on the effects of individual compounds and not real mixtures. In order to study the enzymatic superoxide production resulting from the exposition to a complex mixture, we derived organic extracts from airborne particles collected daily in an urban area and exposed kidney, liver, and heart mammal tissues. After that, we measured DNA damage employing the comet assay. We observed that in every tissue, NADPH oxidase and xanthine oxidase were involved in $O_2^-$ production when they were exposed to the organic extracts, as the lucigenin's chemiluminescence decays when enzymes were inhibited. The same trend was observed with the percentage of cells with comets, since DNA damage was higher when they were exposed to same experimental conditions. Our data allow us to hypothesize that these enzymes play an important role in the oxidative stress produced by PAHs and that there is a mechanism involving them in the $O_2^-$generation.

**Keywords** NOX · PAHs · Superoxide · Suspended particulate matter · Xanthine oxidase · Comet assay

Guillermo Benjamín Silva died last november.

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## Introduction

One of the major environmental problems today is air pollution caused by the presence of suspended particles. Urban populations are actually exposed to levels of particulate matter (PM) that excess natural levels due to anthropogenic processes (Ignotti et al. 2010). Particles that are 10 μm in diameter or smaller can pass through the upper respiratory system, and reach the alveoli affecting several organs, even the heart function (United States Environmental Protection Agency. Office of Air Quality Planning and Standards 2009). It is already accepted that the exposure to airborne particles is related with an increased mortality and morbidity in humans (Peng et al. 2008; Zanobetti et al. 2000). In addition, studies under controlled conditions have shown that chronic and sub-chronic exposure to suspended particulate matter induces genomic degradation processes (André et al. 2011) and cellular stress (Laing et al. 2010). They also suppress the activity of protein-synthesis associated enzymes (Laing et al. 2010; Mendez et al. 2013) and trigger the production of reactive oxygen species (Behndig et al. 2006), which leads to DNA and cellular damage (Goetz and Luch 2008; Topinka et al. 2008). However, the assessment of particles' harmful effects is complicated because they are a complex mixture of components with variable chemical compositions, toxicity, and carcinogenicity. Although toxicity may be due to a direct action of particles on the respiratory tissue, the particle composition varies extensively, and toxicological effects may also be mediated by compounds present in or associated with ambient particles (Lei et al. 2004).

Recent findings have shown that organic compounds adsorbed on the surface of airborne particles, such as polycyclic aromatic hydrocarbons (PAHs), can induce oxidative stress and cancer (Carreras et al. 2013; Risom et al. 2005; Xue and Warshawsky 2005). In addition, PAHs have been

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the most widely investigated in studies exploring the mutagenic and potentially carcinogenic activity of ambient particulate matter (Carreras et al. 2013). Once inside the body, PAHs are distributed by blood and metabolized in different organs causing oxidative damage all over the process (Muñoz and Albores 2011). Indeed, they are considered as one the most ROS-producing compounds (Goetz and Luch 2008) since they can undergo enzymatic oxidation by different well-known ways (Xue and Warshawsky 2005). They also induce several biochemical responses that contribute to the long-term production of ROS by alternative ways (Hordijk 2006; Goulaouic et al. 2008; Pinel-Marie et al. 2009; Laing et al. 2010). Under normal conditions, superoxide ions ($O_2^-$) are endogenously produced to perform a number of physiologic function (Dröge 2002), but under pathological conditions, excessive $O_2^-$ production induce negative responses including obesity, hypertension, atherosclerosis, and multiple forms of cancers (Bedard and Krause 2007; Silva and Garvin 2010). There are several $O_2^-$ sources in cells such as NADPH oxidase (NOX), xanthine oxidase (XO), and mitochondria (Lushchak 2011), although the main enzymatic $O_2^-$-synthetizing mechanism are the first ones.

The findings of previous studies provide important information on human exposition to oxidants, mostly focused on the effects of specific compounds on $O_2^-$ production or specific pathways. However, humans are constantly exposed to a complex mixture of pollutants with constantly changing conditions and it is still unresolved how much this genuine ROS-producing capacity of PM contributes to the overall generation of ROS in human tissues (Risom et al. 2005).

To study the superoxide production resulting from exposition to a complex organic mixture, we derived organic extracts from airborne particles collected daily in an urban area and exposed kidney, liver, and heart mammal tissues. In addition, since excessive $O_2^-$ may produce DNA fragmentation (Goetz and Luch 2008), we quantified DNA damage employing the comet assay which is a robust method widely used in genotoxicology (Carreras et al. 2013; Azqueta et al. 2015).

## Materials and methods

### Particulate matter sampling

Particles of 10 μm or less ($PM_{10}$) were chosen as representative of all particulate mass fractions. Air samples containing $PM_{10}$ matter were collected every 24 h at a constant flow of $16 \pm 4$ L $min^{-1}$ using a $PM_{10}$ collector (Harvard Impactor) with a 46.2-mm glass fiber filters (AP20, Millipore). The sampler was 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 from June to October, 2013 in Cordoba city. $PM_{10}$ mass was determined by gravimetric difference using a microbalance (0.01 mg mass resolution, Sartorius) (Carreras et al. 2013).

### Organic compounds extraction

The extraction of organic compounds (organic extract, OE) was performed according to Carreras et al. (2013). Each filter was immersed in 40 mL of dichloromethane (ACS grade, Sintorgan) and sonicated for 30 min in sealed containers, at room temperature. The supernatant was collected and the extraction process was repeated to ensure that all compounds of interest had been extracted. Both extract were next mixed and concentrated with rotary vacuum evaporator at 37 °C, and then evaporated under a soft nitrogen flow up to 3 mL. The concentrates were filtered with a glass syringe (0.22 μm) and brought to 5 mL with dichloromethane. A pool of the same fortnight filters was made with 2 mL of each extract and divided in two equal parts. Both were taken to dryness with $N_2$ separately and finally stored at −20 °C until analysis. For the $O_2^-$-generation experiment, one fraction was re-suspended in 2 mL of Triton X-100 0.1 μg $L^{-1}$ (Sigma), while the other fraction, for HPLC determination, was dissolved in 0.1 mL of acetonitrile (mobile phase, HPLC grade, Sintorgan). Analysis of acenaphthene, anthracene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[ghi]perylene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorene, naphthalene, phenanthrene, and pyrene was made using high-performance liquid chromatography (PerkinElmer Series 200), with a C-18 reverse-phase column (Vydac 201TP 250 mm 2.1 i.d. 5 μm) and fluorescence detector (Delgado et al. 2004). Pure standards (PAH Calibration Mix, Sigma) were used for quality control and calibration. Seven-point calibration curves were obtained for all PAHs, ranging from 1 to 200 μg $L^{-1}$ ($R^2 > 0.98$; $p < 0.005$).

### Animal tissues

This study was conducted by the NIH guidelines of Institutional Animal Care and Use Committee (IACUC). According to Herrera et al. (2010), five male Wistar rats from 4 to 5 weeks of age were bred and kept until their use in the Specific Pathogen Free bioresurces of the INIMEC (UNC), in an area with ultra-filtered air. Water and food was supplied ad libitum. In order to obtain tissue samples of the heart, kidneys, and liver, animals were anesthetized with ketamine (100 mg $kg^{-1}$ body weight) and xylazine (20 mg $kg^{-1}$ body weight). The abdominal and thoracic cavity was opened and the liver, heart, and kidneys were removed, washed, and preserved with cold PBS (4 °C). Organs were selected in order to study health effects in the transformation, distribution, and elimination of the toxics mentioned, respectively.

### Cellular suspensions

To avoid further DNA damage and reduce false positives, all the process since this point was made under attenuated light and low temperature (4 °C). The cellular suspensions were obtained following the protocol made by Neogy et al. (2008). Representative slices of ~700 mg of tissue were weight to normalize $O_2^-$ measurements, fragmented into small portions, and put in a 10-mL tube containing 0.5 mL of cold PBS. Each tissue was mechanically homogenized and centrifuged at 110 g for 2 min at 4 °C, after which the supernatant was removed and the cell pellet washed with 3 mL of cold PBS, creating a flow with automatic pipette to disperse the cells. To obtain a clear suspension, the process was repeated four times. After this, the supernatant was discarded and the pellet re-suspended with cold PBS to a final concentration of ~200 mg $mL^{-1}$. Finally, a soft flow was generated to disperse the cells.

### Exposure and $O_2^-$ measurement

Tissues were exposed to organic extracts of $PM_{10}$ and conventional enzymatic inhibitors (Carro et al. 2010; Silva and Garvin 2010) according to Herrera et al. (2010). The reagents used and their final concentrations were 10-methyl-9(10-methylacridin-10-ium-9-yl)acridin-10-ium dinitrate 5 μM (Lucigenin, Sigma), apocynin 10 μM (Acetovanillone, Sigma) as a NOX inhibitor, allopurinol 100 μM (Sigma) as a XO inhibitor, and Standard Mix of 14 PAHs 100 μg $L^{-1}$ (PAH Calibration Mix, Sigma). Apocynin and allopurinol concentrations were determined to guarantee enzyme inhibition (Carro et al. 2010; Herrera et al. 2010; Silva and Garvin 2010). PBS was used to maintain equal volumes in all the cases, and oxygenation was present as a stirrer without causing bubbles on the surface. Tissues exposition protocol was (a) $PM_{10}$ sample: OE and PBS; (b) NOX $O_2^-$ production: OE, apocynin, and PBS; (c) XO $O_2^-$ production: OE, allopurinol, and PBS; (d) total inhibition (+) control (baseline): OE, apocynin, allopurinol, and PBS; (e) OE solvent (−) control: Triton X-100 0.1 μg $L^{-1}$, and PBS; (f) OE (−) control: PBS; (g) PAHs Mix (+) control: 100 μg $L^{-1}$ PAHs Mix and PBS; (h) NOX-PAHs $O_2^-$ production: apocynin, 100 μg $L^{-1}$ PAHs Mix, and PBS; and (i) XO-PAHs $O_2^-$ production: allopurinol, 100 μg $L^{-1}$ PAHs Mix, and PBS. Each treatment was made by triplicate.

On each case, cellular suspensions and inhibitors were first added and incubated for 15 min at 37 °C. Next, lucigenin, OE (25 μL), standard (25 μL), solvents (25 μL), and PBS were added and incubated for another 15 min, after which three measures were taken using a luminometer (Thermo Scientific) with 30-s delay between lectures. The dose of OE was calculated to ensure abundance and availability of PAHs.

### Alkaline unicellular electrophoresis (comet assay)

In order to check the degree of DNA damage after PAH-induced $O_2^-$ production, an alkaline unicellular electrophoresis (comet assay) was performed, following Speit and Hartmann (2005) and Arrebola and Fernandez (2010). The exposition protocol for the (a), (b), (c), (d), (f), and (g) cases was repeated, without lucigenin at equal volumes. Incubation time was set to 30 min for the liver and kidney tissues, and 90 min for the heart tissue. On each case, 2 samples were scanned and analyzed, counting at least 100 cells. Comet cells were analyzed with Comet Score software (TriTek Corp.; Sumerduck, USA) and categorized according to the percentage of tail DNA (T-DNA): 0 (minimum damage, up to 5 % T-DNA), 1 (low damage, up to 25 % T-DNA), 2 (average damage, up to 50 % T-DNA), 3 (high damage, up to 75 % T-DNA), and 4 (massive damage, up to 100 % T-DNA) (Collins et al. 2014). Tail moment value (TMV) was also calculated, as this parameter reflects better DNA dissemination.

### Statistical analysis

Significant statistical differences between treatments were assessed with one-way ANOVA or Student's *t* test, as applicable. Following the rejection of the null hypothesis, LSD-Fisher's test was performed to determine the statistical significance of the results ($\alpha = 0.05$). In addition, Pearson coefficients were calculated to determine correlation between $O_2^-$ production data and the percentage of cells with comets. All statistical analyses were carried out using IBM SPSS 19.0 (IBM Corp., Armonk, NY, USA).

## Results

To investigate the role of PAHs on $O_2^-$ production, we first quantified PAH concentration and composition on collected air samples. The average concentration of total PAHs in $PM_{10}$ air samples was 9.72 ng $m^{-3}$, and their average concentration in the extracts was 718 μg $L^{-1}$. They contained acenaphthene (38.7 ng), anthracene (2.4 ng), benz[a]anthracene (130.1 ng), benzo[b]fluoranthene (189.7 ng), benzo[k]fluoranthene (65.2 ng), benzo[g,h,i]perylene (309.7 ng), benzo[a]pyrene (105.4 ng), chrysene (100.9 ng), dibenz[a,h]anthracene (3.6 ng), fluoranthene (139.8 ng), fluorene (185.8 ng), naphthalene (43.5 ng), phenanthrene (46.2 ng), and pyrene (98.8 ng). These values are similar to PAHs values measured in other urban areas such as Baltimore (0.33 to 6.52 ng $m^{-3}$) and New Brunswick (0.38 to 11.6 ng $m^{-3}$) in USA (Dachs et al. 2002; Gigliotti et al. 2000). Also, on comparing the values measured in the present study with measurements from other regions of Argentina, it could be seen that total PAHs

values were similar to the ones informed for an industrial site in Buenos Aires province (Rehwagen et al. 2005). As previously described, this implies that the particulate matter collected may have toxicological relevance for public health.

To investigate the enzymatic $O_2^-$ production, we exposed the animal liver, heart, and kidney tissues to organic extracts derived from PM. Results demonstrate that both enzymes, NOX and XO, are involved in $O_2^-$ production, since chemiluminescence decays when they are inhibited in the three tissues studied. In addition, both enzymes show significant differences from a condition in which there is no stimulus or the response is blocked (Fig. 1). There was no statistical difference between tissues exposed to $PM_{10}$ OE and PAHs mix, although the $O_2^-$ production was slightly higher in samples exposed to real mixtures of organic compounds, suggesting the presence of other substances that are related also with $O_2^-$ production. In the liver tissue (Fig. 1(i)), we observed a significant difference between enzymes, showing that NOX is more important than XO in $O_2^-$ production, either if the stimulus was produced by a $PM_{10}$ OE or a PAHs standard. In the heart tissue (Fig. 1(ii)), both enzymes are equally involved in $O_2^-$ production at any case. In contrast, in samples exposed to PAHs mix, the $O_2^-$ production was significantly higher in XO treatments. In the kidney tissue

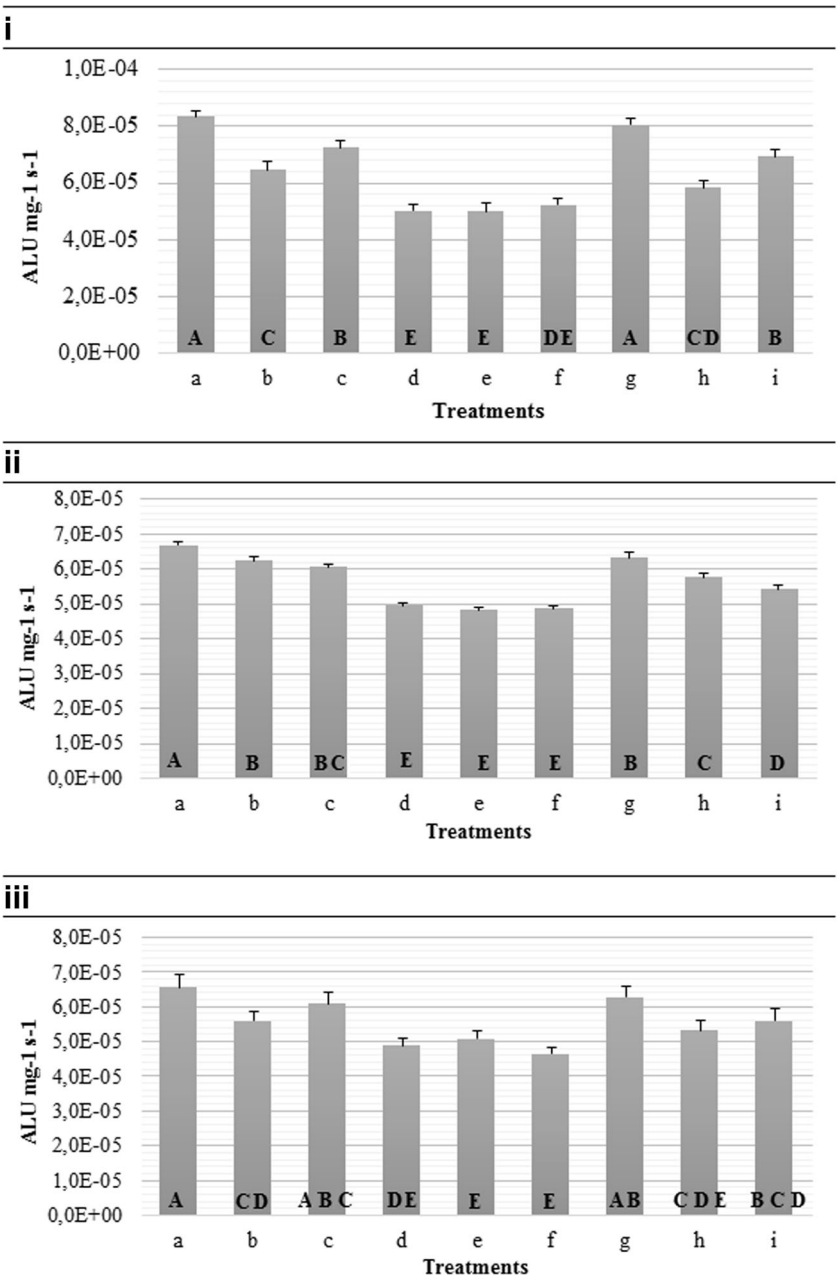


**Fig. 1** Chemiluminescence measured as arbitrary luminescence units (ALU) in the liver (*i*), hearth (*ii*), and kidney (*iii*) tissues: (*a*) $PM_{10}$ OE; (*b*) NOX and (*c*) XO $O_2^-$ production when inhibited and exposed to $PM_{10}$ OE sample; (*d*) $PM_{10}$ OE with total inhibition (+) control (baseline); (*e*) $PM_{10}$ OE solvent (−) control; (*f*) $PM_{10}$ OE (−) control; (*g*) PAHs Mix (+) control; (*h*) NOX-PAHs and (*i*) XO-PAHs $O_2^-$ production when inhibited and exposed to PAHs Mix standard. *Bars* with the same letter means no significant difference between treatments (LSD Fisher)

(Fig. 1(iii)), the trend was similar to the one observed in liver tissue, despite the differences between XO and NOX were not statistically significant.

To assess the degree of DNA damage produced by $O_2^-$ production, we performed the comet assay. In all tissues, type 4 comets tend to decrease and type 0 tend to increase when PAHs or OE are not present or the enzymes are inhibited (Fig. 2). The same trend was observed when analyzing the TMV (Table 1). This parameter is the product between the mean tail length and its percentage of DNA and shows how scattered is DNA in the tail, which refers to the size of the DNA fragments that were detached from the cell. Therefore, as smaller DNA migrates further, a high TMV implies more DNA fragmentation. Particularly, heart TMV tends to be higher in baseline treatment and in tissues with no exposition to OE. Considering that DNA percentage in comet tail is similar to the one observed in other tissues, the high heart TMV is probably due to smaller DNA fragments, which migrates further and increase tail length and therefore TMV. Specifically, in the liver, we observed a significantly higher percentage of cells with comets when they were exposed to $PM_{10}$ OE or PAHs standard (Fig. 2(i)). The DNA damage diminished when the enzymes were inhibited. In every case, the main type of comet cell was type 3, followed by type 2 (Fig. 3(i)). We observed the same trend in heart cells (Fig. 2(ii)), although the percentages were half the ones observed in liver cells (Fig. 3(ii)). In addition, there were no significant difference between the XO treatment and the negative control. The kidney tissue (Fig. 2(iii)) exhibited the highest percentages of cells with comets when exposed to OE or PAHs, with prevalence of types 2, 3, and 4 comets (Fig. 3(iii)). The inhibition of NOX showed fewer comets than the XO inhibition, though in both cases the type 3 comets predominated. In all the tissues, negative control and blank treatments showed significantly lower percentage of damaged cells with no significant difference between them.

## Discussion

Our findings support the hypothesis that NOX and XO enzymatic pathways are involved in $O_2^-$ production in different tissues when exposed to organic extracts of $PM_{10}$. In fact, we demonstrated that when one or both enzymes are inhibited, the $O_2^-$ generation decays in mammal liver, heart, and kidney tissues and the DNA damage is significantly reduced, as well.

A good correlation between the oxidative effects of real organic mixtures and PAHs standard was observed, even though the concentration of PAHs was higher in the real samples that in the standard mix, as shown in Fig. 4. This result indicates that the damaging effect is mainly due to the presence of PAHs than to any other compounds that could be present in the organic extracts. Our data allows us to assume that NOX and XO play an important role in the process of cell stress by PAHs and that there is a mechanism that involves them (Laing et al. 2010; Pereira et al. 2013; Pinel-Marie et al. 2009).

Regarding the $O_2^-$ production by NOX complexes, the effect can be explained by the enzymes isoforms functionality and the types of subunits involved. Except for NOX5, the activation of the remaining NOX occurs through a series of protein interactions. In resting cells, the NOX subunits remains in cytoplasm, but when a stimulus arrives, they assemble to form the active enzyme. One of the critical subunits is the p22phox, which stabilizes all NOX complexes. However, each complex is activated differently: the NOX2 gp91phox subunit couples with the p22phox subunit and this complex interacts with p47phox, the "activator subunit" p67phox, the small subunit p40phox, and the GTPase Rac, while for NOX1 and NOX3, p22phox recruits the NOXO1 and NOXA1 proteins. NOX4 only requires the p22phox protein for its activation. All isoforms generate $O_2^-$ by transferring an electron from NADPH from the cytosol to extracellular oxygen (Bedard and Krause 2007; Silva and Garvin 2010). In

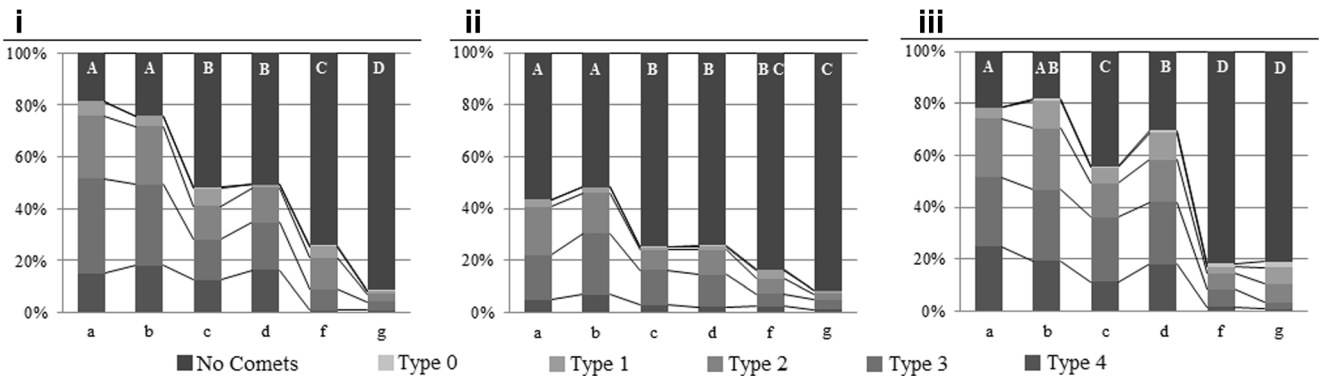


**Fig. 2** Percentages of cells with comets in the liver (*i*), hearth (*ii*), and kidney (*iii*) tissues. Mean frequency of nuclei with comet in human peripheral blood lymphocytes exposed in vitro to neonicotinoid insecticides. The *bars* represent the mean values ± SEM of the comet from the three independent experiments: (*a*) $PM_{10}$ OE; (*b*) NOX and (*c*) XO $O_2^-$ production when inhibited and exposed to $PM_{10}$ OE sample; (*d*) $PM_{10}$ OE with total inhibition (+) control (baseline); (*f*) $PM_{10}$ OE (−) control and (*g*) PAHs Mix (+) control. *Bars* with the same letter means no significant difference between treatments (LSD Fisher)

**Table 1** Mean tail moment values in baseline and treated cells from the liver, heart, and kidney tissues

| Treatment | Liver (%) | Heart (%) | Kidney (%) |
|---|---|---|---|
| (a) $PM_{10}$ OE (+) | 59.72 | 53.15 | 33.18 |
| (b) $NOX_{inh}$ | 46.61 | 39.74 | 31.42 |
| (c) $XO_{inh}$ | 39.77 | 41.20 | 38.90 |
| (d) Baseline | 13.09 | 46.82 | 15.87 |
| (f) $PM_{10}$ OE (−) | 12.53 | 32.86 | 7.82 |
| (g) PAHs Mix | 34.57 | 33.83 | 51.51 |

Finally, to analyze the relationship between both experiments, we calculated a Pearson correlation coefficient between chemiluminescence data and comet assay results. A close relationship for all tissues was found (liver $R^2 = 0.8983$; heart $R^2 = 0.8112$; kidney $R^2 = 0.9753$), implying that the DNA damage could be a result of the $O_2^-$ generated by the enzymes studied (Fig. 4)

agreement, we found that in the presence of an inhibitor (apocynin), PAHs failed to induce $O_2^-$ production in the liver, heart, and kidney cells. As mentioned, the NOX2 enzyme activity, which prevails in the liver and heart tissue, rely on a subunit recruitment (Bedard and Krause 2007). Therefore, when apocynin inhibits p47phox subunit translocation to the plasma membrane, the $O_2^-$ production decreases. The data obtained in these experiments suggests that PAHs associated to $PM_{10}$ can induce activation of the NOX2 isoenzyme, showing a full agreement with the mechanism proposed by Pinel-Marie et al. (2009) and Wada et al. (2013). These authors observed that a PAH complex interacts with the NOX complex encoding gene and induces the expression of NOX2 subunits, resulting in an increment of the $O_2^-$ production. On the other hand, the isoform NOX4, which prevails in the kidney tissue, only needs p22phox subunit to be active, which according to Olukman et al. (2010) is efficiently inhibited by apocynin. This fact might imply that apocynin not only inhibits the NOX2 complex but also interfere with the activity of the other isoenzymes. Since PAHs leads to reticulum stress (Laing et al. 2010), the expression of NOX4 mRNA could be induced (Bedard and Krause 2007), contributing to the $O_2^-$ production. Actually, recent studies showed that quinone compounds, like the intermediates of PAHs metabolism, regulate the $O_2^-$ production by NOX4 (Nguyen et al. 2013). Regarding the NOX1 isoform, also expressed in the kidney, it could be possible that the PAH-receptor complex stimulates the expression of the activator NOXO1. This factor shares about 25 % sequence identity to p47phox with a high degree of similarity in their functional domains (Bedard and Krause 2007). Our results showed that $O_2^-$ decays in PAHs stimulated kidney cells when apocynin is added, suggesting that PAHs are also implicated in $O_2^-$ generation through NOX1 and NOX4 pathways. Since NOX1 is also found in liver tissue (Bedard and Krause 2007), it may contribute to the production of $O_2^-$ in that tissue, as well.

The data about XO treatments showed similar trend to the observed in the NOX cases, which suggest the presence of direct or indirect activators of the enzyme. A previous study (Harris et al. 1999) showed that the XO complex (xanthine oxireductase, XOR) can be converted from its dehydrogenase form (XDH) to its oxidase form (XO) by oxidation of punctual cysteines. Therefore, it is possible that NOX-generated ROS may act as oxidants of this complex and convert it to the XO form, increasing $O_2^-$ levels. In accordance, another study (McNally, et al. 2003) showed that the inhibition of the p47phox-NOX subunit decreases the XO levels and $O_2^-$ levels in endothelial cells. These two findings are consistent with our results, indicating that XO might be indirectly implicated in the ROS production when exposed to PAHs. In contrast, direct activation of the enzyme has not been reported, and it should be further studied to fully confirm this hypothesis.

Since the studied enzymes produce $O_2^-$ and ROS produce DNA damage, they were involved in the genetic damage observed when cells were exposed. The results of the comet assay proved that the radicals generated by NOX and XO produce damage to nuclear DNA. However, while inhibition reduces the number of comets, DNA damage remains high. This allows us to deduce the presence of another damaging mechanism, probably related to one or several of the classical pathways such as dihydrodiol epoxides, radical cation, and activation through PAH-*o*-quinone (Gurbani et al. 2013; Muñoz and Albores 2011; Xue and Warshawsky 2005).

Despite the fact that other non-specific and enzymatic superoxide sources, like mitochondrial, were not determined, the superoxide production by this two enzymes when exposed to PAHs not only represents an important pathogenesis way by

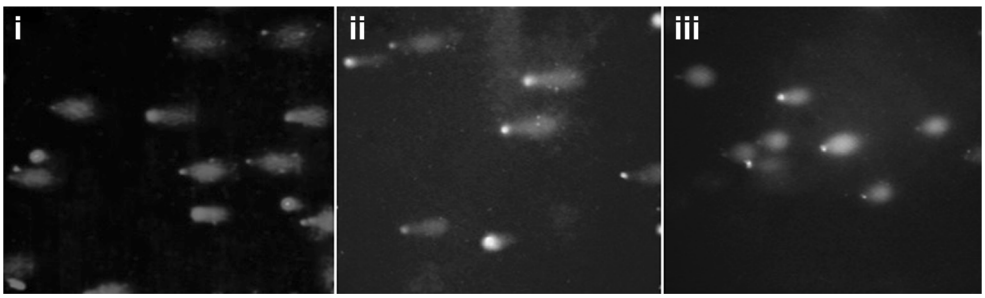


**Fig. 3** Comet assay photographs of the liver (*i*), hearth (*ii*), and kidney (*iii*) cells exposed to $PM_{10}$ OE (×800)

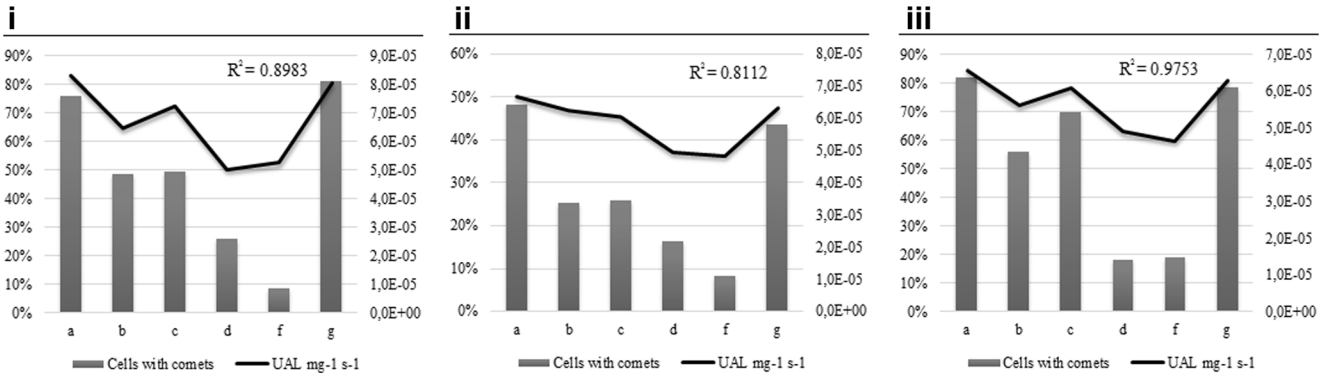


**Fig. 4** Percentage of cells with comets and corresponding chemiluminescence of treated cells from the liver (*i*), hearth (*ii*), and kidney (*iii*) tissues. (*a*) $PM_{10}$ OE; (*b*) NOX and (*c*) XO $O_2^-$ production when inhibited and exposed to $PM_{10}$ OE sample; (*d*) $PM_{10}$ OE with total inhibition (+) control (baseline); (*f*) $PM_{10}$ OE (−) control; (*g*) PAHs Mix (+) control

itself but also synergizes with the already known ones (Behndig et al. 2006; Topinka et al. 2008; Xue and Warshawsky 2005), all of them leading to cell metabolic disorders, mutagenesis, and cancer (André et al. 2011; Carreras et al. 2013; Goulaouic et al. 2008; Gurbani et al. 2013; Muñoz and Albores 2011; Xue and Warshawsky 2005).

## Conclusions

The outcomes of the present study imply that the enzymatic ROS production by non-classical PAHs pathways (NOX and XO) is an important contribution to oxidative cellular stress and DNA damage in the liver, heart, and kidney tissues.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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