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In vitro evaluation of organic extractable matter from ambient PM_{2.5} using human bronchial epithelial BEAS-2B cells: Cytotoxicity, oxidative stress, pro-inflammatory response, genotoxicity, and cell cycle deregulation

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1 ***IN VITRO* EVALUATION OF ORGANIC EXTRACTABLE MATTER FROM AMBIENT PM_{2.5} USING**
2 **HUMAN BRONCHIAL EPITHELIAL BEAS-2B CELLS: CYTOTOXICITY, OXIDATIVE STRESS, PRO-**
3 **INFLAMMATORY RESPONSE, GENOTOXICITY, AND CELL CYCLE Deregulation.**

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1 **ABSTRACT**

2
3 A particular attention has been devoted to the type of toxicological responses induced by particulate matter (PM),
4 since their knowledge is greatly complicated by the fact that it is a heterogeneous and often poorly described
5 pollutant. However, despite intensive research effort, there is still a lack of knowledge about the specific chemical
6 fraction of PM, which could be mainly responsible of its adverse health effects. We sought also to better investigate
7 the toxicological effects of organic extractable matter (OEM) in normal human bronchial epithelial lung BEAS-2B
8 cells. The wide variety of chemicals, including PAH and other related-chemicals, found in OEM, has been rather
9 associated with early oxidative events, as supported by the early activation of the sensible NRF-2 signaling pathway.
10 For the most harmful conditions, the activation of this signaling pathway could not totally counteract the ROS
11 overproduction, thereby leading to critical oxidative damage to macromolecules (lipid peroxidation, oxidative DNA
12 adducts). While NRF-2 is an anti-inflammatory, OEM exposure did not trigger any significant change in the
13 secretion of inflammatory cytokines (*i.e.*, TNF α , IL-1 β , IL-6, IL-8, MCP-1, and IFN γ). According to the high
14 concentrations of PAH and other related organic chemicals found in this OEM, *CYP1A1* and *IB1* genes exhibited
15 high transcription levels in BEAS-2B cells, thereby supporting both the activation of the critical AhR signaling
16 pathway and the formation of highly reactive ultimate metabolites. As a consequence, genotoxic events occurred in
17 BEAS-2B cells exposed to this OEM together with cell survival events, with possible harmful cell cycle
18 deregulation. However, more studies are required to implement these observations and to contribute to better
19 decipher the critical role of the organic fraction of air pollution-derived PM_{2.5} in the activation of some sensitive
20 signaling pathways closely associated with G1/S and intra-S checkpoint blockage, on the one hand, and cell survival,
21 on the other hand.

22
23 **Keywords:** organic extractable matter; BEAS-2B cells; oxidative stress; inflammation; genotoxicity; cell survival
24

1 1 - INTRODUCTION

2
3 Nowadays, air-pollution-derived particulate matter (PM) is fully acknowledged to be a major public health
4 problem, responsible for several hundreds of thousands of premature deaths and the loss of millions of dollars yearly
5 in health care (AQiE 2016). Fine fraction of the PM (particles with an aerodynamic diameter $\leq 2.5 \mu\text{m}$, $\text{PM}_{2.5}$)
6 generally corresponds to a very complex and heterogeneous mixture of both inorganic and organic chemicals, from
7 natural and anthropogenic sources, and its composition varies as a function of location and season depending on the
8 diversity of sources and the effects of atmospheric processes (Abbas *et al.* 2018). Due to its size, $\text{PM}_{2.5}$ constitutes a
9 serious health hazard. After its penetration, deposition and absorption deeply in the tracheobronchial and alveolar
10 regions, it can also either exert its toxicity within the lungs or, after its translocation through the bronchoalveolar
11 barrier, be distributed through the blood circulation into the whole body (Cao *et al.* 2014; Liu *et al.* 2016; Pui *et al.*
12 2014; Van Winkle *et al.* 2015). Epidemiological studies have shown a significant correlation between long-term
13 exposure to $\text{PM}_{2.5}$ and the initiation and/or exacerbation of a wide range of adverse health effects, including asthma,
14 chronic obstructive pulmonary disease, lung cancer, other respiratory and cardiovascular diseases, and premature
15 death (Kim *et al.* 2016; Weber *et al.* 2016; Xing *et al.* 2016). In 2013, the international agency for research on cancer
16 (IARC) has classified outdoor air pollution and its fine fraction as carcinogenic to humans, based on sufficient
17 evidence of carcinogenicity in humans and experimental animals (Loomis *et al.* 2013).

18 Over the last two decades, *in vitro* toxicology methods have been heavily used as they constitute a relevant
19 approach for determining and describing the underlying mechanisms involved in the toxicity triggered by air
20 pollution-derived $\text{PM}_{2.5}$, at cellular and molecular scales. However, despite intensive research effort, there is still a
21 lack of knowledge about the specific chemical fraction within airborne $\text{PM}_{2.5}$, which could be mainly responsible of
22 its adverse effects on human health. A particular attention has also been devoted to the type of toxicological
23 responses induced by PM, since this knowledge is greatly complicated by the fact that PM is generally a very
24 complex and heterogeneous, regrettably often poorly described, pollutant (Perrone *et al.* 2013). Hence, a great deal
25 of effort has been done to identify some characteristic patterns of *in vitro* toxicological responses which could be
26 specifically related to airborne PM. In this respect, Lonkar and Dedon (2010) supported the current hypothesis that
27 airborne PM with higher participation of crustal elements was predominantly associated with some molecular and
28 cellular effects participating in severe health processes like chronic inflammatory lung diseases. Moreover, PM with
29 larger anthropogenic participation may have a higher impact on lung and systemic cardiovascular diseases, due to its
30 stronger oxidative and proinflammatory potentials. Gualtieri *et al.* (2010) and Perrone *et al.* (2013) reported that
31 some coarse PM showing the presence of crustal elements, ions and metals, contributed to oxidative stress and,
32 thereafter, inflammation, but, on the contrary, other coarse PM triggered inflammation through a reactive oxygen
33 species (ROS)-independent pathway, as ROS levels were not significantly altered. Their results pertinently supported
34 the growing evidence that PM-size, composition and origin interact in an intriguing manner together with the own
35 responsiveness of the cell models to induce the adverse health effects of airborne PM. Hence, while the inclusion of
36 inorganic constituent grouping provided useful information about the differential effects of PM components and
37 sources, it is worthy to extend such approach to organic constituents. Towards this end, Delfino *et al.* (2010) stated
38 that, despite a higher presence of metals within fine and quasi-ultrafine particles, polycyclic aromatic hydrocarbons
39 (PAH) were the major redox-active components, and thereafter, inflammatory response inducers. Longhin *et al.*

1 (2013) indicated that low doses of PM_{2.5} with high quantities of PAH induced an early cell cycle arrest with a
2 subsequent inhibition of cytokinesis and, an induction of DNA damage. Against this background, Dergham *et al.*
3 (2012, 2015) have already intended to gain information on the toxicological effects induced by chemical variations
4 of airborne PM_{2.5} samples produced during different seasons in rural-, urban-, or industrial-surroundings. Taken
5 together, their results clearly supported that some inorganic, ionic and organic components can act as exogenous
6 source of ROS and the possible role of other factors that remain to be determined (*e.g.*, interaction of PM_{2.5} physical
7 characteristics and cell uptake efficiency, role of other non-investigated chemicals and/or biological materials).
8 Accordingly, PM contains a wide variety of organic chemicals, which could, directly or indirectly, through their
9 metabolic activation, possibly participate to the induction of oxidative and/or proinflammatory events in human
10 bronchial epithelial cell models (Boublil *et al.* 2013; Dergham *et al.* 2012, 2015; Longhin *et al.* 2013, 2016; Zhou *et al.*
11 *et al.* 2014). Other studies supported that organic extractable matter (OEM) prepared from PM can contain a mixture of
12 carcinogenic and/or toxic compounds; they evaluated its roles in the overall toxicity but failed to clearly decipher the
13 underlying mechanisms (Akhtar *et al.* 2010; Alessandria *et al.* 2014; De Brito *et al.* 2013, Healey *et al.* 2006, Oh *et al.*
14 *et al.* 2011, Xiang *et al.* 2018).

15 In view of the literature supporting the critical role of the organic chemical fraction of airborne PM in its
16 harmful effects on human health, further toxicological researches are requested to gain more knowledge about the
17 crucial role played by the organic chemical fraction in the activation of some classical underlying mechanisms of
18 toxicity. The objectives of this pilot work were also to better investigate the toxicological effects of OEM prepared
19 from PM_{2.5} collected in Beirut (Lebanon), in an urban surrounding, on normal human bronchial epithelial cells
20 (BEAS-2B). After having chemically characterized this OEM, we sought to better investigate some underlying
21 mechanisms possibly involved in its cellular and molecular toxicological effects, with regards to specific endpoints
22 of cytotoxicity, oxidative stress, inflammation, metabolic activation, genotoxicity, and cell cycle deregulation.

23

2 - MATERIALS AND METHODS

2.1. Chemicals

All the cell culture reagents, the Vybrant Cytotoxicity Assay Kit, the FxCycle PI/RNase Staining Solution, the High Capacity cDNA Reverse Transcription Kit, the Taqman fast advanced Master Mix, Taqman gene expression assays, and the Pierce™ BCA Protein Assay Kit were provided by Thermo-Fisher scientific (Villebon-sur-Yvette-France). CellTiter-Glo® Luminescent Cell Viability was from Promega (Charbonniere-les-Bains, France). Benzo[*a*]Pyrene (B[*a*]P), superoxide dismutase (SOD) determination kit, and other chemical reagents were from Sigma Aldrich (France). AllPrep DNA/RNA/miRNA Universal Kit was from QIAGEN (Beirut - Lebanon). TransAM® NRF2 was from Active Motif (La Hulpe, Belgium). 8-isoprostane (8-Isop) ELISA kit was from Abcam (Paris, France). Highly-sensitive 8-hydroxy-2'-deoxyguanosine (8-OHdG) Check was from Gentaur France SARL (Paris, France). MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead Panel- Immunology Multiplex Assay, MILLIPLEX® map 7-plex DNA Damage/Genotoxicity Kit, and the 7-Plex Early Apoptosis Magnetic Bead Kit 96-well Plate Assay were from Merck-Millipore (St Quentin-en-Yvelines, France).

2.2. Sampling site description

Air derived-PM samples were collected in the old airport avenue situated in south Beirut suburb (33° 50' 59" N; 35° 29' 57" E) from the 9th of November 2015 to the 28th of January 2016 (See supplemental material: Figure S1-A, B, and C). The sampling site was placed close to one of the busiest area of the city, characterized by huge road traffic, large residential areas, the proximity of three important hospitals, and several commercial centers. About at 2 km from the study site is located the Rafic Hariri International airport, characterized by an important aerial traffic. Beirut is characterized by a high transport density and this sector is known to be one of the main causes of air pollution in Lebanon, due to the absence of organized public transport resulting in the duplication, each year, of the number of private cars, mainly gasoline engines. Moreover, Beirut suffers from a major problem that relies on the use of private electric generators diesel powered, in order to fill the gap of the shortage in electricity production supplied by the public sector. The production of electricity in industrial and residential sectors provided by electric generators diesel powered is estimated to 500 MW (MOEW, 2010). It is also important to note that Lebanon has been subjected to a household waste crisis and wild open burning of waste occurred randomly in Beirut agglomeration during the sampling period.

2.3. Sampling of PM_{2.5}

PM_{2.5} was sampled using a high-volume cascade sampler TE-231 single (TISCH®, 40 CFM PM_{2.5} single stage), on a daily basis. The first stage was covered with a slotted glass fiber filter (TISCH, TE-230-WH) to retain particles with aerodynamic diameter higher than 2.5 µm. After this, impaction stage, a Whatman® cellulose-base filter (230 mm x 254 mm) was used as back-up filter to collect PM with aerodynamic diameter smaller than 2.5 µm (PM_{2.5}). After each sampling period, filters were dried under laminar flow hood during 48 h and then stored at -20°C until further preparation. At the end of the collection period, 15 filters were selected for both global chemical characterization and toxicological endpoint study. Corresponding wind conditions were mainly from the East

1 pointing to the East residential areas (See also supplemental material: Figure S1-C). Indeed, wind speed was quite
2 low ($<2 \text{ m.s}^{-1}$) and would not have favored the dispersion of pollutants during the collection period.

4 **2.4. Chemical characterization of PM_{2.5}**

5 PAH quantification was done using gas chromatography-mass spectrometry (GC-MS) after extraction of the
6 pristine filters using dichloromethane as described in section “2.5. Preparation of the OEM”. Polychlorodibenzo-p-
7 dioxins (PCDD), polychlorodibenzofurans (PCDF), and polychlorobiphenyls (PCB) were quantified using high
8 resolution/GC-MS, after acid treatment of the pristine filters, followed by soxhlet extraction using toluene,
9 purification on silica and alumina columns, and elution with toluene for PCB quantification, and
10 dichloromethane/hexane mixture for PCDD and PCDF quantification. All the PAH, PCDD, PCDF and PCB
11 quantifications were performed by MicroPollutants Technologie S.A. (Saint Julien les Metz, France). The whole
12 analytical procedure has been already described in details in Borgie *et al.* (2015b) and Cazier *et al.* (2016).

14 **2.5. Preparation of the OEM**

15 Briefly, OEM was prepared from PM_{2.5} using a quarter of each of the 15 filters. Pieces of filters were placed in
16 3 glass tubes (5 pieces per tube) to which 40 mL of dichloromethane were added before ultrasonic treatment (50 min,
17 100 W, 20°C). Then, dichloromethane solutions were gathered, filtered using cellulose acetate filters (Sartorius@,
18 0.45 µm) and slowly evaporated under nitrogen flow to obtain a final volume of 5 mL. Indeed, 1 mL of this
19 dichloromethane solution was dedicated to the organic chemical characterization whereas the remaining 4 mL were
20 totally evaporated under nitrogen flow before being resuspended in 4 mL dimethyl sulfoxide (DMSO) as described
21 by Borgie *et al.* (2015a). This solution was considered as OEM and used for the toxicological study. Considering the
22 15 pieces of filters, 212.5 mg of PM_{2.5} were used to extract the organic fraction and the final concentration of the
23 final OEM was 42.5 mg PM-equivalent /mL (PM-eq/mL). This concentration was used to express as µg PM-eq/cm²
24 the increasing concentrations of OEM to which BEAS-2B cells were exposed in the toxicological part of this work.

26 **2.6. Toxicity of OEM**

28 **2.6.1. Cell culture and exposure**

29 BEAS-2B cells (ATCC® CRL-9609™, ECACC, Wiltshire, UK) were purchased from Sigma-Aldrich (Saint-
30 Quentin Fallavier, France). This cell line, originally derived from normal human bronchial epithelial cells, was
31 obtained from autopsy of non-cancerous individuals. Cell culture methods have already been published elsewhere
32 (Leclercq *et al.* 2017b). Briefly, BEAS-2B cells were cultured in 95% humidified air with 5% CO₂ at 37°C in 75 cm²
33 CellBIND® surface plastic flasks (Corning; Sigma-Aldrich) in LHC-9 medium. Before reaching 80% of confluence,
34 cells were subcultured by using 0.25% trypsin/0.53mM versene solution containing 0.5% (w/v) polyvinylpyrrolidone
35 (Sigma-Aldrich). Medium was refreshed every 2-3 days. All the BEAS-2B cells we used derived from the same cell
36 culture. Just before reaching the confluence (<80%), BEAS cells were exposed to control solution (*i.e.*, LHC-9 with
37 DMSO at 0.025% v/v) or OEM (*i.e.*, concentrations ranging from 1 to 30 µg PM_{2.5}-eq/cm², suspended in LHC-9,
38 with DMSO ≤ 0.025% v/v). After 6, 24 and/or 72 h, aliquots of cell-free culture media were collected and quickly
39 frozen at - 80°C. Cell free-culture supernatants were dedicated to assess cytotoxicity and inflammatory endpoints.

1 Adherent cells were washed twice with 1mL-aliquots of cold sterile PBS, and either quickly frozen at - 80°C or fixed
2 for flow cytometry analysis. The study of the toxicological endpoints (*i.e.*, cytotoxicity, gene expression, oxidative
3 stress, genotoxicity, apoptosis, and cell cycle) needed the preparation of various cell matrices, as previously
4 described in details by Leclercq *et al.* (2016).

6 **2.6.2. Cytotoxicity**

7 The cytotoxicity of OEM was evaluated after cell exposure to increasing concentrations, ranging from 1 to 30
8 µg PM_{2.5}-eq/cm² during 6, 24 or 72 h through the determination of intracellular ATP concentration (CellTiter-Glo®
9 Luminescent Cell Viability, Promega) and extracellular G6PD activity (Vybrant Cytotoxicity Assay Kit, Thermo
10 Fisher Scientific). It should be noted that the concentration of DMSO in the cell culture medium did not exceed
11 0.025% (v/v), which avoided the occurrence of its specific toxicity.

13 **2.6.3. Oxidative stress**

14 Oxidative stress was evaluated firstly by the DNA binding activity of nuclear factor erythroid 2-related factor 2
15 (NRF2) and the associated gene expression of heme oxygenase 1 (*HMOX*) and NAD(P)H quinone dehydrogenase 1
16 (*NQO1*), and secondly by SOD activity, 8-Isoprostane (8-Isop) concentration, and 8-hydroxy-2'-deoxyguanosine (8-
17 OHdG) concentration. NRF2 binding activities were studied using TransAM® NRF2 from Active Motif (Leclercq *et al.*
18 *et al.* 2018). Gene expressions of *HMOX* and *NQO1* were evaluated using quantitative RT-PCR. Briefly, total RNA
19 were isolated using the RNeasy Mini Kit (Qiagen). After the reverse transcription of 1 µg of total RNA in single-
20 stranded cDNA using the High Capacity cDNA Reverse Transcription Kit, the relative quantitation of the gene
21 expressions was carried out using specific Taqman™ gene expression assays (*HMOX*: Hs0110250_m1, *NQO1*:
22 Hs1045993_g1, ARN18S: Hs99999901_s1), a StepOnePlus™ Real-Time PCR System, and the Expression Suite
23 Software (Thermo Fisher Scientific). The relative change in gene expression was calculated by the $\Delta\Delta CT$ method,
24 normalized against endogenous ribosomal 18S. In other respects, SOD activities were carried out using a
25 commercially available kit (SOD determination kit, Sigma-Aldrich) as described elsewhere (Garçon *et al.* 2000). 8-
26 Isop concentrations were determined using 8-isoprostane ELISA kit from Abcam (Leclercq *et al.* 2016). Oxidative
27 DNA adduct 8-OHdG concentrations were studied using a commercially available enzyme immunoassay (Highly
28 Sensitive 8-OHdG Check, Gentaur France SARL), as published elsewhere (Leclercq *et al.* 2016).

30 **2.6.4. Inflammation**

31 Several inflammatory cytokines (tumor necrosis factor, TNF- α ; interleukin-1 beta, IL-1 β , interleukin-6, IL-6;
32 interleukin-8, IL-8; monocyte chemoattractant protein-1, MCP-1; interferon-gamma, IFN- γ) were determined in cell-
33 free culture supernatants using a MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead Panel-
34 Immunology Multiplex Assay (Merck-Millipore) according to the manufacturer's instructions.

36 **2.6.5. Xenobiotic-metabolizing enzyme gene expression**

37 Expressions of some of the genes closely involved in the AhR signaling pathway and, therefore, in the
38 metabolic activation of the PAH (*i.e.*, *aryl hydrocarbon receptor*, *AHR*; *aryl hydrocarbon receptor nuclear*
39 *translocator*, *ARNT*; *cytochrome P450 1A1*, *CYP1A1*; *cytochrome P450 1B1*, *CYP1B1*) were evaluated using

1 quantitative RT-PCR, as above-described, using specific Taqman™ gene expression assays (*AHR*: Hs00169233_m1,
2 *ARNT*: Hs01121918_m1, *CYP1A1*: Hs01054797_m1, *CYP1B1*: Hs00164383_m1, *ARNI8S*: Hs99999901_s1).

3 4 **2.6.6. Cell cycle**

5 Just after their exposure, BEAS-2B cells were washed twice with DPBS, carefully fixed in 70% (v/v) ethanol,
6 washed again twice with DPBS, and thereafter resuspended in FxCycle PI/RNase stain for 30 min at room
7 temperature. The FxCycle™ PI/RNase Staining Solution, provided as ready-to-use by Thermo-Fisher scientific, was
8 formulated with DNase-free RNase A and a permeabilization reagent in DPBS. Propidium iodide (PI) bind to DNA
9 by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4-5
10 base pairs of DNA. Once the dye has been bound to nucleic acids, its fluorescence was analyzed using 488 nm
11 excitation, and a 585/42 nm bandpass filter emission by flow cytometry (Attune® Acoustic Focusing Cytometer;
12 Life Technologies, France). The flow cytometric analysis of the stained cell populations was then used to produce a
13 frequency histogram that reveals the proportion of the cells in each of the different phases of the cell cycle (Attune™
14 NxT Flow Cytometer Software).

15 16 **2.6.7. Genotoxicity and apoptosis**

17 Genotoxicity and apoptosis were respectively evaluated using the MILLIPLEX® map 7-plex DNA
18 Damage/Genotoxicity Kit (phosphorylated checkpoint kinase 1, pChk1 (Ser345); phosphorylated checkpoint kinase
19 2, pChk2 (Thr68); phosphorylated H2A histone family member X, γ H2AX (Ser139); phosphorylated protein 53,
20 pP53 (Ser15); total serine/threonine protein kinase, ATR; total mouse double minute 2 homolog, MDM2; and total
21 protein 21, p21), and the 7-Plex Early Apoptosis Magnetic Bead Kit (phosphorylated AKT/protein kinase B,
22 pAKT/PBK (Ser473); phosphorylated c-Jun N-terminal kinase/Serine/threonine-protein kinase, pJNK/SAPK1
23 (Thr183/Tyr185); phosphorylated BCL-2-associated death promoter, pBAD (Ser112); phosphorylated B-cell
24 lymphoma 2, pBCL-2 (Ser70); phosphorylated protein 53, pP53 (Ser46); active Caspase-8 (Asp384) and active
25 Caspase-9 (Asp315)), according to the manufacturer's recommendations (Merck-Millipore). After their exposure to
26 EOM, BEAS-2B cells were collected and centrifuged (10,000 g for 10 min at 4°C), before the preparation of protein
27 extracts. Protein concentrations were determined by bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit,
28 Thermo Fischer Scientific) before the dilution of the samples to a concentration of 20 μ g/well. Analyses were then
29 led scrupulously following the manufacturer's instructions.

30 31 **2.6.8. Statistical analysis**

32 Results are expressed as mean values and standard deviations. For each incubation time, results from BEAS-2B
33 cells exposed to OEM at its different PM_{2.5}-eq concentrations or B[a]P were compared to those obtained from non-
34 exposed cells. Statistical analyses were performed by the non-parametric Mann-Whitney U-test with correction for
35 multiple comparisons (Software: SPSS for windows). Significant differences were reported with *p* values < 0.05.
36

3. RESULTS

3.1. Chemical characteristics of PM_{2.5}

The mean concentration of PM_{2.5} was calculated by considering the total mass of particles collected on each filter during the whole sampling period and the total volume of filtered air. This concentration was estimated at 48 µg/m³. The 16 PAHs listed by the US-EPA were quantified and their concentrations are given in Table 1. The total PAH concentration was 9.7 ng/m³. Benzo[*b*]fluoranthene (B[*b*]F), chrysene (CHR), benz[*a*]anthracene (B[*a*]A), pyrene (PYR) and benzo[*ghi*]perylene (B[*ghi*]P) were the most abundant with values between 989 and 1519 pg/m³ (or 20.6 and 31.6 µg/g PM_{2.5}). Moreover, there were several polychlorinated highly persistent compounds such as PCDD, PCDF and DL-PCB within the sampled particles. Indeed, total concentrations of PCDD, PCDF and PCB were about 459 fg/m³ (or 9,587 pg/g PM_{2.5}), 560 fg/m³ (or 12,255 pg/g PM_{2.5}), and 949 fg/m³ (or 19,818 pg/g PM_{2.5}) respectively, as shown by Table 2. According to the respective toxicity of the congeners, as defined by Van den Berg *et al.* (2005), their total concentration, as expressed by international toxic equivalent quantity (I-TEQ) was about 58 fg I-TEQ/m³ (or 1,217 pg I-TEQ/g).

3.2. Cytotoxicity of OEM

As shown in Figure 1, significant decreases of ATP levels were observed in BEAS-2B cells 72 h after their exposure to increasing concentrations ranging from 5 to 30 µg PM_{2.5}-eq/cm² *versus* control ($p < 0.05$). Concerning G6PD activity, significant increases were seen in BEAS-2B cells only 72 h after their exposure to OEM at 15 and 30 µg PM_{2.5}-eq/cm² *versus* control ($p < 0.01$).

3.3. Oxidative effects of OEM

Figure 2 showed the dose-dependent increases of NRF-2 binding activity observed in BEAS-2B cells 6 h after their exposure to [OEM] eq PM_{2.5} at 15 and 30 µg/cm², and to B[*a*]P, and 24 h after their exposure to [OEM] eq PM_{2.5} at 3, 15 and/or 30 µg/cm², and to B[*a*]P *versus* control ($p < 0.05$). Significant inductions of both *HMOX* and *NQO1* gene transcription were reported in BEAS-2B cells, 6 h after their exposure to OEM at 3, 15 and/or 30 µg PM_{2.5}-eq/cm², and to B[*a*]P ($p < 0.05$), and 24 h after their exposure to OEM at all the tested concentrations, and to B[*a*]P ($p < 0.05$). With regards to SOD activity, statistically significant increases were observed in BEAS-2B cells 6 h after their exposure to OEM at 30 µg PM_{2.5}-eq/cm², and to B[*a*]P, and 24 h after their exposure to OEM at 15 and 30 µg PM_{2.5}-eq/cm², and to B[*a*]P *versus* control ($p < 0.05$). Whatever the incubation times and exposure conditions, significant increases of 8-Isop concentrations were reported in BEAS-2B cells ($p < 0.05$). Moreover, dose-dependent increases of 8-OHdG were seen in BEAS-2B cells 6 h and 24 h after their exposure to OEM and to B[*a*]P *versus* control ($p < 0.05$).

3.4. Inflammatory effects of OEM

As shown in Figure 3, no statistically significant change of the secretion of all the cytokines under study (TNF- α , IL-1 β , IL-6, IL-8, MCP-1, IFN- γ) was reported in OEM-exposed BEAS-2B cells, whatever the exposure

1 conditions and incubation times. A significant increase of the concentration of IFN- γ was observed in the
2 supernatants of BEAS-2B cells 24 h after their exposure to B[a]P *versus* control ($p < 0.05$).

3 4 **3.5. Xenobiotic-metabolizing enzyme gene induction by OEM**

5 Whatever the incubation times and the exposure conditions, no statistically significant change of *AHR* and
6 *ARNT* gene expression was observed in BEAS-2B cells (Figure 4). In contrast, a statistically significant induction of
7 *CYP1A1* and *CYP1B1* gene transcription was reported in BEAS-2B cells whatever the incubation times (6 and 24 h)
8 and exposure conditions (3, 15 and 30 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$, or to B[a]P) *versus* control ($p < 0.05$).

9 10 **3.6. Genotoxic effects of OEM**

11 Figure 5 showed slight but statistically significant increases of phosphorylated-P53 (pP53) concentrations in
12 BEAS-2B cells 72 h after their exposure to increasing concentrations of OEM, and to B[a]P *versus* control ($p <$
13 0.05). With regards to P21 concentrations, significant increases was reported only in BEAS-2B cells 6 h after their
14 exposure to B[a]P *versus* control ($p < 0.05$). Whatever the incubation times and exposure conditions, slight but
15 significant decreases of phosphorylated-H2AX (γ H2AX) concentrations, most often statistically significant, were
16 reported in BEAS-2B cells ($p < 0.05$). In contrast, no significant change of total MDM-2 concentrations was
17 observed. There were slight but significant increases of phosphorylated-CHK-1 (pCHK-1) concentrations in BEAS-
18 2B cells 72 h after their exposure to OEM from 3 to 30 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$ *versus* control ($p < 0.05$). Similarly, slight
19 but significant increases of phosphorylated CHK-2 (pCHK-2) concentrations were observed in BEAS-2B cells 72 h
20 after their exposure to OEM at the two highest concentrations, and to B[a]P *versus* control ($p < 0.05$).

21 22 **3.6. Cell cycle effects of OEM.**

23 As shown in Figure 6, most of control and exposed BEAS-2B cells were in the S phase after 24 h: from $40.07 \pm$
24 4.57% for the control cells to $49.48 \pm 4.75 \%$ for 15 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$ exposed cells. Indeed, there were slight
25 increases of the percentages of BEAS-2B cells in the S phase in BEAS-2B cells 24 after their exposure to OEM,
26 particularly at the two highest concentrations ($49.48 \pm 4.75 \%$ for 15 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$ and $48.53 \pm 0.75 \%$ for 30 μg
27 $\text{PM}_{2.5}\text{-eq/cm}^2$), *versus* control ($40.07 \pm 4.57 \%$). In contrast, the majority of control and exposed BEAS-2B cells were
28 in the G1 or S phases after 72 h. There were also slight increases of the percentages of BEAS-2B cells in the G1
29 phase 72 h after their exposure to OEM at the highest concentration ($29.39 \pm 2.93 \%$ for 30 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$) and
30 B[a]P ($28.71 \pm 2.74 \%$), *versus* control ($23.25 \pm 2.45 \%$). Moreover, very slight increases of the percentages of
31 BEAS-2B cells in the S phase were reported 72 h after their exposure to OEM at the different concentrations (30.98
32 $\pm 0.95 \%$ for 3 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$ and $30.00 \pm 0.71 \%$ for 30 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$), *versus* control ($28.97 \pm 0.47 \%$).

33 34 **3.7. Apoptotic effects of OEM.**

35 Generally relatively low but statistically significant increases in phosphorylated-AKT (pAKT) concentrations
36 were observed in BEAS-2B cells 24 and 72 h after their exposure to OEM at the two highest concentrations, and to
37 B[a]P *versus* control ($p < 0.05$) (Figure 7). However, no significant phosphorylation of JNK was detected in BEAS-
38 2B cells after their exposure to OEM nor to B[a]P. In contrast, for all the incubation times and exposure conditions
39 to OEM, slight but significant increases of phosphorylated-BAD (pBAD) concentrations but not phosphorylated-

1 BCL-2 (pBCL-2) concentrations were reported in BEAS-2B cells *versus* control ($p<0.05$). Only a 72 h incubation of
2 the BEAS-2B cells with B[a]P lead to a statistically significant increase of pBAD concentrations *versus* control
3 ($p<0.05$). The active forms of caspase-8 and -9 were not statistically modified in BEAS-2B cells 24 h nor 72 h after
4 their exposure to each of the tested conditions.

5

4. DISCUSSION

Despite the high number of *in vitro* approaches reported in the respiratory toxicology literature, researchers are still far from having a complete detailed mechanistic explanation of the causal relation between PM and health effects (Longhin *et al.* 2016). There is still a lack of knowledge about the specific chemical fraction within particles, which could be mainly responsible of its adverse effects on human health. Hence, this work aimed to better decipher the toxicological effects of the OEM from ambient PM_{2.5} on normal human bronchial epithelial cells (BEAS-2B).

Fine particles were collected close to one of the busiest area in the southern suburb of Beirut (Lebanon). Their average concentration (48 µg/m³), 1.9 times higher than the daily PM_{2.5} World Human Organization guideline (25 µg/m³), was in line with those recently reported in Beirut (41 µg/m³) by Borgie *et al.* (2015a) but also higher than others observed few years ago in Beirut (20 µg/m³) by Massoud *et al.* (2011), and in the suburb (28 µg/m³) by Saliba *et al.* (2010). The mean total concentration of PAH (9.7 ng/m³) found in these particles was higher (8.3 and 28-times, respectively) than those reported in 2011 in the Sin El Fil district of Beirut (1.16 ng/m³) and in Bejje (0.35 ng/m³), a rural area located in the north of Lebanon. Benzo[*b*]fluoranthene (B[*b*]F), the most abundant congener, generally reflects the contribution of diesel power generator and diesel trucks and buses as personal cars only runs with gasoline. The fleet of vehicles in Beirut is generally old and use low quality fuel oil, which is problematic considering the daily density of traffic. Chrysene (CHR), the second most abundant congener, is often generated during oil distillation process or waste incineration (Borgie *et al.* 2015a). To better determine the sources of PAH, ratios were calculated referring to Ravindra *et al.* (2008) (Table 3). Diesel exhaust was also incriminated as a PAH contributing source with ratios of indeno[1,2,3-*cd*]pyrene (I[*cd*]P)/(I[*cd*]P + benzo[*ghi*]perylene (B[*ghi*]P)) = 0.33 and Benzo[*a*]pyrene (B[*a*]P)/(B[*a*]P + Chrysene (CHR)) = 0.3. These emissions could be related to the use of generators, to compensate the lack of electricity provided by the Lebanese state. In addition, vehicular emissions and the use of gasoline engines were evidenced by Fluoranthene (FLT)/Pyrene (PYR) = 0.6 and PYR/B[*a*]P = 2, which were in agreement with the huge road traffic occurring in Beirut. Reported levels of PCDD (449 fg/m³) and PCDF (560 fg/m³) were higher (2.1 and 6.5 times, respectively) than those obtained in 2011 in the Sin El Fil district of Beirut (214 and 87 fg/m³, respectively) (Borgie *et al.* 2015a). The high concentrations of dioxins and furans we found could be correlated with the recent worrying problem of waste occurring in Lebanon since July 2015, resulting in uncontrolled open waste burning. Indeed, in 2016, Baalbaki *et al.* found PCDD and PCDF levels near an open-air waste burning site in Beirut higher (2.2 and 16,4-fold, respectively) than those above-reported. Taken together, these data supported that the PM_{2.5} samples collected in this work contained a wide variety of chemicals including PAH and other related-chemical compounds (*e.g.*, PCDD, PCDF, DL-PCB).

This work was carried out on normal human bronchial epithelial BEAS-2B cells, a dedicated cell model, mainly due to its relatively high homology to human lung tissues and primary cells (Courcot *et al.* 2012). In BEAS-2B cells, the dose- and time-dependent cytotoxicity induced by OEM was used to choice the concentrations (from 1 to 30 µg PM_{2.5}-eq/cm²) and the kinetic (from 6 to 72 h) to apply for the further study of the other toxicological endpoints.

PAH and other related-chemical compounds found in the PM_{2.5} samples under study have been rather associated with early oxidative events and inflammation in human bronchial epithelial cell models (Boublil *et al.* 2013; Dergham *et al.* 2012, 2015; Gualtieri *et al.* 2010, 2011; Longhin *et al.* 2013, 2016; Zhou *et al.* 2014). Because of its key role as master regulator of the cell redox homeostasis, NRF-2 is well-equipped to counteract the cytosolic

1 ROS production and is critical for maintaining the redox balance in the cell (Wardyn *et al.* 2015). Following
2 exposure to oxidants or electrophiles, NRF2 accumulates in the nucleus where it binds to antioxidant response
3 element in the upstream regulatory regions of genes encoding NRF-2 targets including antioxidant enzymes, proteins
4 involved in xenobiotic metabolism and clearance, protection against metal toxicity, inhibition of inflammation, repair
5 and removal of damaged proteins, as well as other transcription and growth factors (Suzuki *et al.* 2016). In this work,
6 dose- and time-dependent significant increases of NRF-2 binding activity and NRF-2 target gene transcription (*i.e.*,
7 *HMOX1* and *NQO1*) or target protein (*i.e.*, SOD) were seen in OEM-exposed BEAS-2B cells. Indeed, some
8 transcription factors, such as NRF2, can be activated by electrophilic metabolites arising through the metabolic
9 activation of PAH (Nguyen *et al.* 2009; Lawal, 2017). Despite the clear activation of the NRF-2 signaling pathway,
10 somewhat surprisingly, there were anyway dose- and time-dependent significant lipid peroxidation and oxidative
11 DNA adduct formation. Indeed, 8-isoP is a lipid peroxidation product of arachidonic acid which has been identified
12 as one of the most useful biomarkers of oxidative damage (Erve *et al.* 2017). In DNA, 8-OHdG is considered as one
13 of the predominant forms of ROS-induced oxidative lesions and has also been widely used as a biomarker for
14 oxidative stress (Valavanidis *et al.* 2009). Taken together, these data closely supported that OEM actively
15 contributed to redox imbalance, thereby activating the NRF-2 signaling pathway; however, for the most harmful
16 conditions, chemical-catalyzed ROS overproduction, notably through the metabolic activation of organic chemicals,
17 was not totally counteracted, thereby leading to oxidative damage to some critical macromolecules (Boublil *et al.*,
18 2013; Dergham *et al.*, 2012, 2015; Leclercq *et al.* 2018; Longhin *et al.* 2013; Zhou *et al.*, 2014).

19 While NRF-2 is an anti-inflammatory, the inhibition of the inflammation by NRF-2 is generally associated with
20 the inhibition of the NF- κ B signaling pathway and, therefore, that of the inflammatory cytokine production (Fischer
21 *et al.* 2015; Ma *et al.* 2013). According to the above-reported activation of the NRF-2 signaling pathway, OEM
22 exposure did not trigger any significant change in the secretion of inflammatory cytokines (*i.e.*, TNF α , IL-1 β , IL-6,
23 IL-8, MCP-1, and IFN γ). However, the proinflammatory properties of various types of air pollution-derived PM have
24 already been largely described (Boublil *et al.* 2013; Ghio *et al.* 2013; Gualtieri *et al.* 2010, 2011; Leclercq *et al.*
25 2016, 2017a, 2017b, 2018; Longhin *et al.* 2013; Loxham *et al.* 2015). There seemed also to be a striking disconnect
26 between the proinflammatory properties of whole PM, on the one hand, and of OEM, on the other hand. This
27 apparent discrepancy could possibly relies on the specific roles played by particles themselves, as carrier of various
28 coated-organic chemicals, in the efficient absorption of these organic chemicals by epithelial cells and/or in the
29 induction of some of the underlying mechanisms clearly related to the initiation and/or exacerbation of the
30 inflammatory response (*e.g.*, endocytosis mechanisms) (Garçon *et al.*, 2000, 2004; Gosset *et al.*, 2003).

31 In general, metabolic activation is beneficial in helping lung cells to reduce the potential toxicity of inhaled
32 pollutants. However, sometimes, it transforms harmless substances into electrophile metabolites that are highly
33 reactive towards endogenous macromolecules, thereby producing toxicity (Saint-Georges *et al.* 2008, 2009). In this
34 work, CYP1A1 and 1B1 isoforms, constitutively poorly or not expressed in bronchial tissues, exhibited high
35 transcription levels in BEAS-2B cells already 6 h until 24 h after their exposure to OEM, enriched in PAH, PCDD,
36 PCDF, and PCB. Gualtieri *et al.* (2011) reported that, due to their relatively high contents of PAH, PM₁₀ and PM_{2.5},
37 representative of background urban air pollution, diesel exhaust particles, and wood smoke were all able to
38 significantly activate the AHR signaling pathway in BEAS-2B cells. Oh *et al.* (2011) indicated that OEM which
39 contain aliphatic chlorinated hydrocarbons, PAH/their alkyl derivatives, and nitro-AH/ketones/quinones, induced

1 CYP1A1-dependent EROD activity, through AHR signaling pathway activation, which is specifically related to the
2 critical role of the organic chemical fraction of ambient PM.

3 Despite the alkyl and oxidative DNA adducts generally reported in human bronchial epithelial cells exposed to
4 whole PM or OEM, significant changes in primary DNA single-strand breaks (SSB), DNA double-strand break
5 (DSB), and chromosomal aberrations were not systematically reported (Andre *et al.* 2011, Borgie *et al.* 2015b,
6 Leclercq *et al.* 2017a, Lepers *et al.* 2014, Oh *et al.* 2011). There is now overwhelming evidence that ambient air
7 contains hundreds of genotoxic compounds; however, some apparent difference in the genotoxic potential might
8 probably rely on the use of different materials, cell models, and exposure strategies. There is also still an urgent need
9 to better investigate the genotoxic potential of OEM. It was reported several years ago, that in mammalian cells the
10 phosphorylation of the subtype of histone H2A, called H2AX, in the position of Ser139 occurs in response to DSB
11 formation (Podhorecka *et al.* 2010). Due to the fact that γ H2AX appears early and forms foci at damage sites, it is
12 widely considered to be a sensitive biomarker for DSB (Tu *et al.* 2013). However, in this work, OEM induced slight
13 but significant decreases of H2AX phosphorylation, thereby indicating an important default in the repair of genetic
14 material. Bouquet *et al.* (2006) reported that γ H2AX loss correlates with DSB repair activity only at low cytotoxic
15 doses, when less than 100-150 DSB breaks per genome are produced, independently of the method used. Their
16 results suggested that the dephosphorylation step of γ H2AX may be limiting and that the loss of foci is mediated not
17 only by γ H2AX dephosphorylation but also through its redistribution towards the chromatin. Since the
18 phosphorylation of H2AX is linearly related to the dose used, although dependent on the cell line, the phosphatase
19 activity might represent the limiting step leading to a persistent signal. Finally, it is conceivable that the γ H2AX
20 might also be targeted for degradation, a step that might be saturable, as observed in this work. The γ H2AX
21 decreases also reported within BEAS-2B cells after their exposure to low cytotoxic doses of OEM can therefore be
22 related to the higher levels of phosphorylated P53, CHK-1 and CHK-2 proteins, generally closely associated with
23 cell cycle arrest (Fragkos *et al.* 2009). The tumor suppressor protein P53 plays a critical role in cell fate
24 determination. Especially after DNA damage, P53 plays an anti-apoptotic role through activation of P21, which
25 induces cell cycle arrest, and facilitates DNA repair. However, when DNA damage exceeds a threshold or is so
26 severe that it cannot be repaired, P53 promotes apoptosis (Shin *et al.* 2015). It might be expected that the OEM-
27 induced P53 phosphorylation reported in this work, concurrent with DNA damage, would lead to cell cycle arrest, as
28 reported by Gualtieri *et al.* (2011). These authors indicated that BEAS-2B cell exposure to whole PM_{2.5} resulted in
29 DNA damage, giving DNA-damage response characterized by increased phosphorylation of ATM and CHK2, but,
30 contrary to the present results, increased phosphorylation of γ H2AX, with an apparent lack of G2-arrest.

31 To go further in this work, the cell cycle was analyzed. After 24 h, the cell cycle profile of control cells clearly
32 indicated that they were already stalled in S-phase. There were also slight increases of the percentages of BEAS-2B
33 cells in the S phase after their exposure to OEM, particularly at the two highest concentrations. In contrast, after 72
34 h, the cell cycle profile of control cells showed that the majority of cells were in G1 and S phases. There were also
35 only very slight increases of the percentages of BEAS-2B cells in both these phases 72 h after their exposure to
36 OEM. The response to DNA damage, as those previously reported through the excessive 8-OHdG formation in
37 OEM-exposed BEAS-2B cells, is an inherent part of the cell cycle and its main action is to halt cell cycle progression
38 and establish a checkpoint (G1/S, intra-S, G2/M, and M). At these positions a cell can stop or slow down in case of
39 unfavorable conditions, stress or DNA damage. Chen *et al.* (2016) also reported that different sizes and shapes of

1 TiO₂ nanomaterials (AFDC and cNRs) could inhibit the growth of BEAS-2B cells and allow a considerable
2 proportion of them to remain in the G1/G0 phase: in the low-concentration exposure, the cell cycle was terminated
3 during the G1/G0 phase without apparent cell death, whereas in the high-concentration exposure, the dramatic
4 decrease in cell viability was contributed by apoptotic cell death. To take care of DNA damage, repair mechanisms
5 are put in place and if possible, a cell eventually continues proliferation or exits the cell cycle (Bertoli *et al.* 2013;
6 Iyer *et al.* 2017). In particular, cells activate the intra-S checkpoint in response to damage during S phase to protect
7 genomic integrity and ensure replication fidelity. Accordingly, in this work, most of the OEM-exposed BEAS-2B
8 cells were in the S phase after 24 h. The checkpoint prevents genomic instability mainly by regulating origin firing,
9 fork progression, and transcription of G1/S genes in response to DNA damage. Despite having specific repair
10 pathways dedicated to each kind of DNA lesion, the cell relies on a single checkpoint to mediate the DNA damage
11 response during S phase. The cell has two main checkpoint kinases, Rad3-related (ATR) and ataxia telangiectasia
12 mutated (ATM), both of which are critical for maintaining genomic integrity. ATR and ATM activate two effector
13 kinases, CHK1 and CHK2, in response to damage to relay the checkpoint signal across the cell. Accordingly, in this
14 work, there were slight but significant increases of phosphorylated-CHK-1 and/or CHK-2 concentrations in BEAS-
15 2B cells 72 h after their exposure to OEM. Although CHK1 is primarily activated by ATR in response to various
16 kinds of lesions and CHK2 by ATM in response to DSB, there is substantial cross-talk between the two pathways
17 making it difficult to unambiguously assign CHK1 and CHK2 to a single checkpoint pathway (Iyer *et al.* 2017).
18 Relatively low but statistically significant increases in the phosphorylation of AKT were observed in BEAS-2B cells
19 24 and 72 h after their exposure to OEM at the two highest concentrations. Zhang *et al.* (2013) investigated the role
20 of CYP2A13, an extrahepatic enzyme that mainly expresses in human respiratory system, in the metabolic activation
21 of aflatoxin G1, in BEAS-2B cells that stably express CYP2A13 (B-2A13). They reported that low concentrations of
22 aflatoxin G1 induced S phase arrest and DNA damage in B-2A13 cells, and the proteins related to DNA damage
23 responses, such as ATM, ATR, Chk2, p53, BRCA1, and cH2AX, were activated. For all the incubation times and
24 exposure conditions we tested, slight but significant increases of phosphorylated-BAD concentrations were reported
25 in BEAS-2B cells. Taken together, these results clearly indicated the survival of the exposed cells with possible
26 blockages of their cell cycle in G1/S and intra-S checkpoints. In response to replication stress, CHK1, reported as
27 slightly but significantly phosphorylated in BEAS-2B cells 72 h after their exposure to OEM, could be responsible
28 for inactivating G1-S transcription (Bertoli *et al.* 2013). This transcriptional response seems to be specific for
29 replication stress, which, in contrast to the DNA damage response, does not induce apoptosis, according to the results
30 of the present work. Accordingly, there were also slight increases of the percentages of BEAS-2B cells in the G1
31 phase 72 h after their exposure to OEM. Gualtieri *et al.* (2011) reported that, in accordance with the observed DNA
32 damage, whole PM_{2.5} also induced activation of the ATM/CHK2 pathway in BEAS-2B cells, without any apparent
33 arrest of cells in G2 suggesting that the G2/M checkpoint failed. In contrast, they did not observe any increase in the
34 activation of the ATR/CHK1 pathway, which would be expected following DNA DSB and stalled DNA replication.
35 Wu *et al.* (2017) hypothesized that exposure to whole PM could trigger cell cycle arrest in G2/M phase and DNA
36 damage in BEAS-2B cells, thereby activating the expression of related proteins. According to these results, Zhuang
37 *et al.* (2011) reported increases of the levels of phosphorylated AKT, which plays an important role in survival
38 pathways by inactivating downstream pro-apoptotic factors in many cell systems. According to the results reported
39 in the present work, ROS-activated AKT could participate in ultrafine carbon black-induced cell proliferation, and

1 mediates pro-carcinogenic effects induced by diesel exhaust particle and smoking compounds, respectively (Park *et*
2 *al.* 2013, Weissenberg *et al.* 2010). Moreover, Zhou *et al.* (2016) observed continuous elevation of ROS and
3 concomitant phospho-activation of AKT in BEAS-2B cells during exposure to whole PM_{2.5}. These changes were
4 blocked by pretreatment with the free-radical scavenger NAC, suggesting that PM_{2.5} activates AKT by increasing
5 ROS; this latest result was closely related to our own observations supporting that OEM induced a noticeable
6 oxidative stress. Furthermore, in this work, for all the incubation times and exposure conditions, slight but significant
7 increases of phosphorylated-BAD concentrations were reported. According to Datta *et al.* (1997), AKT
8 phosphorylates BAD *in vitro* and *in vivo*, and may thereby suppress BAD-induced cell death and promote cell
9 survival. It is also noteworthy that, in this work, both caspase 8 and 9 activities were not significantly changed in
10 BEAS-2B-exposed to OEM, thereby supporting the absence of clear apoptotic events. In contrast, Abbas *et al.* (2010,
11 2016), studying the sequential occurrence of molecular abnormalities from TP53-RB gene signaling pathway
12 activation in the L132 target human lung epithelial cell model, reported that relatively high doses of PM_{2.5} altered the
13 gene expression and/or the protein concentration of several key cell cycle controllers from TP53-RB gene signaling
14 pathway, thereby leading to the occurrence of cell proliferation and apoptosis together. Taken together, these results
15 might ask the critical question whether there is a balance or, in contrast, there will rather be an imbalance between
16 the cell survival, cell proliferation and cell death possibly occurring in whole PM or OEM-exposed cells, depending
17 of the dose applied. However, the results reported in this work, despite the fact that only the active forms of the
18 different key endpoints were studied without correction to their respective total forms, clearly indicated that exposure
19 to EOM induced cell deregulation through blockages of their cell cycle in G1/S and intra-S checkpoints, and even
20 cell survival.
21

1 **5. CONCLUSION**

2
3 In this work, we demonstrated that the OEM derived from ambient PM_{2.5} collected in urban surroundings was
4 enriched in PAH and other related chemical compounds, which could also trigger some underlying mechanisms of
5 toxicity, and a combination thereof may be same or different compared with those induced by whole PM. Indeed,
6 acute exposure to OEM at relatively low concentrations activated the AHR and NRF2 signaling pathways but,
7 contrary to whole PM, failed to trigger an inflammatory response. Moreover, these results closely supported the
8 capacity of OEM to trigger cell deregulation notably through blockages of their cell cycle in G1/S and intra-S
9 checkpoints, and even cell survival. In view of the original data reported in this preliminary work, future
10 complementary works are also needed to better decipher the critical role of OEM on the cell cycle regulation. In
11 these future complementary works, it could be more insightful to investigate the toxicity of OEM together with those
12 of whole PM_{2.5}, to which it has been extracted, and with non-organic fraction (*i.e.*, residual PM_{2.5} after OEM
13 extraction), to allow to compare the adverse effects from the OEM fraction with both total and residual PM_{2.5}
14 fractions.

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2

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34

FIGURE LEGENDS

Figure 1: Adenosine triphosphate (ATP) concentrations in BEAS-2B cells and glucose-6-phosphate dehydrogenase (G6PD) activities in cell-free culture supernatants, 6, 24 and 72 h after their exposure to increasing concentrations of organic extract matter (OEM: 1, 3, 5, 15 and 30 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$). Results, normalized to control, are described by their means and their standard deviations (6 replicates for controls, and 6 replicates for exposed cells; Mann-Whitney U-test; vs. controls; a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$).

Figure 2: Gene and/or protein expression of nuclear factor erythroid 2-related factor 2 (NRF-2), heme oxygenase 1 (HMOX), NAD(P)H quinone dehydrogenase 1 (NQO1), superoxide dismutase (SOD), 8-isoprostane (8-Isop) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in BEAS-2B cells 6 and 24 h after their exposure to organic extract matter (OEM: 3, 15 and 30 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$) and B[a]P (25 μM). Results, normalized to control, are described by their means and their standard deviations (6 replicates for controls, and 6 replicates for exposed cells (Mann-Whitney U-test; vs. controls; a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$).

Figure 3: Concentrations of tumor necrosis factor (TNF- α), interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and interferon-gamma (IFN- γ), in the supernatants of BEAS-2B cells 6 and 24 h after their exposure to organic extract matter (OEM: 3, 15 and 30 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$) and B[a]P (25 μM). Results, normalized to control, are described by their means and their standard deviations (6 replicates for controls, and 6 replicates for exposed cells (Mann-Whitney U-test; vs. controls; a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$).

Figure 4: Gene expression of the *aryl hydrocarbon receptor (AHR)*, *aryl hydrocarbon receptor nuclear translocator (ARNT)*, *cytochrome P450 1A1 (CYP1A1)* and *cytochrome P450 1B1 (CYP1B1)* in BEAS-2B cells 6 and 24 h after their exposure to organic extract matter (OEM: 3, 15 and 30 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$) and B[a]P (25 μM). Results, normalized to control, are described by their means and their standard deviations (6 replicates for controls, and 6 replicates for exposed cells (Mann-Whitney U-test; vs. controls; a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$).

Figure 5: Concentrations of phosphorylated protein 53 (pP53), total protein 21 (P21), phosphorylated H2A histone family member X (H2AX), total mouse double minute 2 homolog (MDM-2), phosphorylated checkpoint kinase 1 (CHK-1), and phosphorylated checkpoint kinase 2 (CHK-2) in BEAS-2B cells 24 and 72 h after their exposure to organic extract matter (OEM: 3, 15 and 30 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$) and B[a]P (25 μM). Results, normalized to control, are described by their means and their standard deviations (6 replicates for controls, and 6 replicates for exposed cells (Mann-Whitney U-test; vs. controls; a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$).

Figure 6: Percentage of BEAS-2B cells in G1, S, and G2M cell cycle phases 24 and 72 h after their exposure to organic extract matter (OEM: 3, 15 and 30 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$) and B[a]P (25 μM). Results, normalized to control, are described by their means and their standard deviations (6 replicates for controls, and 6 replicates for exposed cells (Mann-Whitney U-test; vs. controls; a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$).

Figure 7: Concentrations of phosphorylated protein kinase B (AKT), phosphorylated c-Jun N-terminal kinase (JNK), phosphorylated Bcl-2-associated death promoter (BAD), phosphorylated B-cell lymphoma 2 (BCL-2), and phosphorylated Caspase-8 and 9, in BEAS-2B cells 6 and 24 h after their exposure to organic extract matter (OEM: 3, 15 and 30 $\mu\text{g PM}_{2.5}\text{-eq/cm}^2$) and B[a]P (25 μM). Results, normalized to control, are described by their means and their standard deviations (6 replicates for controls, and 6 replicates for exposed cells (Mann-Whitney U-test; vs. controls; a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$).

Figure 8: Underlying mechanisms involved in the toxicity of air pollution-derived organic extract matter (OEM) in BEAS-2B cells (8-Isop: 8-Isoprostaglandin, 8-OHdG: 8-hydroxy-2'-deoxyguanosine, AhR: aryl hydrocarbon receptor, CYP1A1: cytochrome P450 1A1, CYP1B1: cytochrome P450 1B1, G6PD: glucose-6-phosphate dehydrogenase, H2AX: phosphorylated H2A histone family member X, HMOX: heme oxygenase 1, NQO1: NAD(P)H quinone dehydrogenase 1, NRF-2: nuclear factor erythroid 2-related factor 2, PAH: polycyclic aromatic

1 hydrocarbon, pAKT: phosphorylated protein kinase B, pBAD: phosphorylated Bcl-2-associated death promoter,
2 PCB: polychlorobiphenyl, PCDD: polychloro dibenzo-p-dioxin, PCDF: polychlorodibenzofuran, pCHK-1:
3 phosphorylated checkpoint kinase 1, pCHK-2: phosphorylated checkpoint kinase 2, pP53: phosphorylated protein 53,
4 ROS: reactive oxygen species, SOD: superoxide dismutase).

5

6 **Figure S1:** Location of sampling site (A and B) and the concerned recorded wind rose

Figure 1

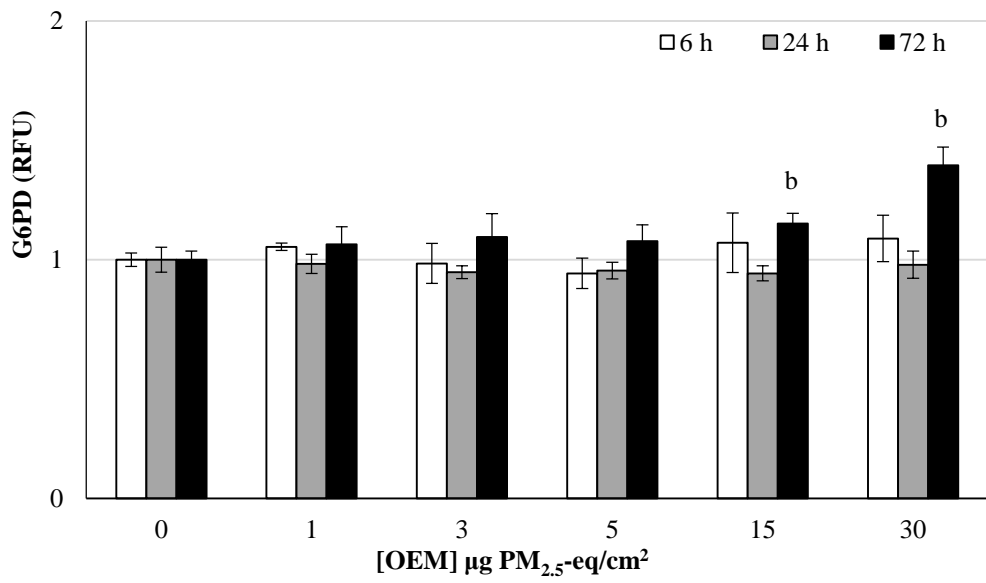
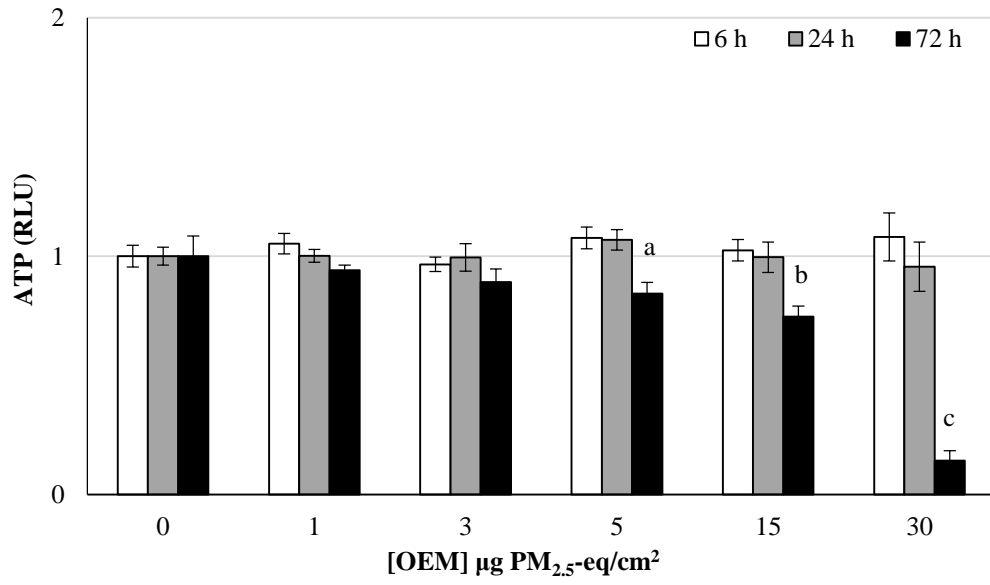


Figure 2

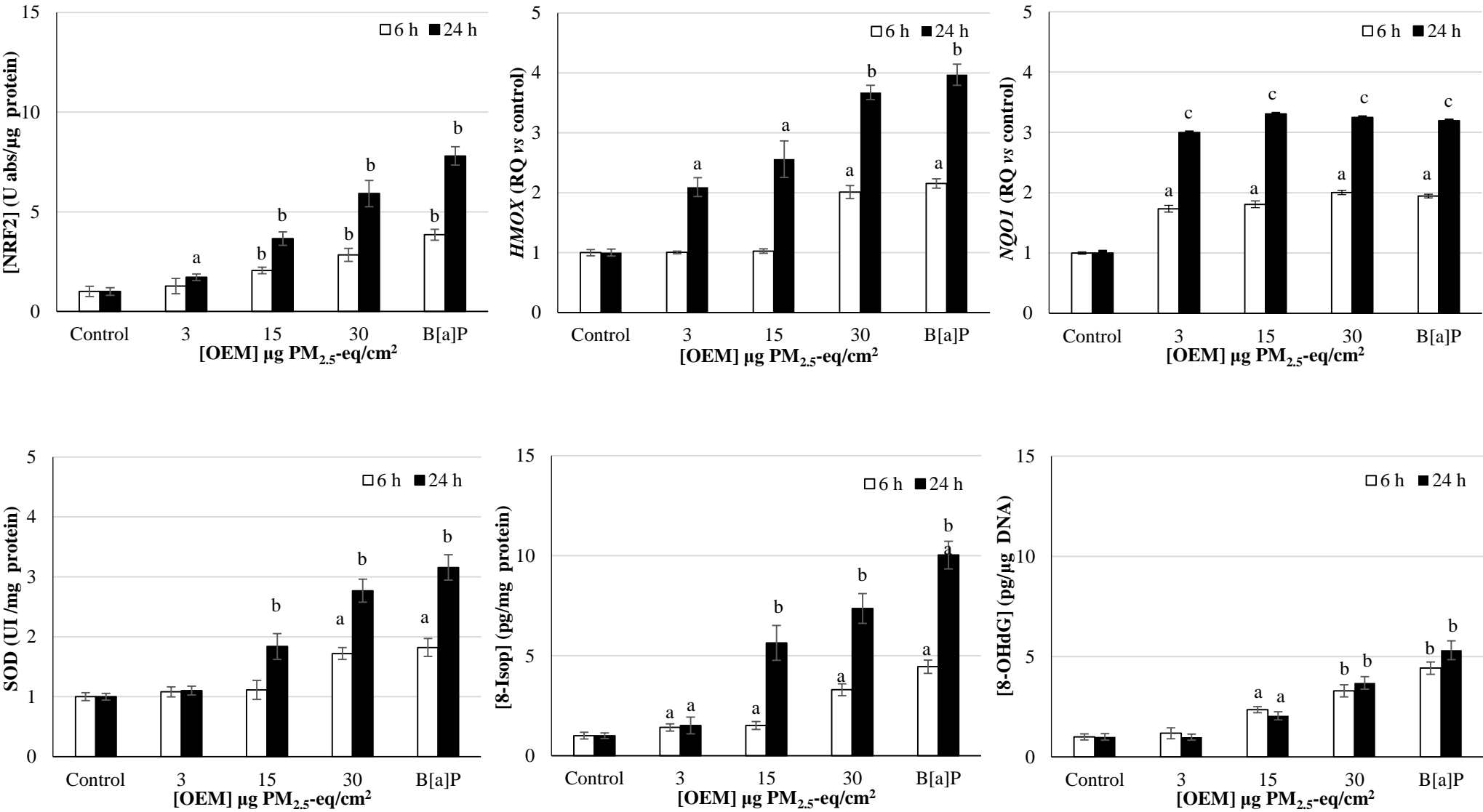


Figure 3

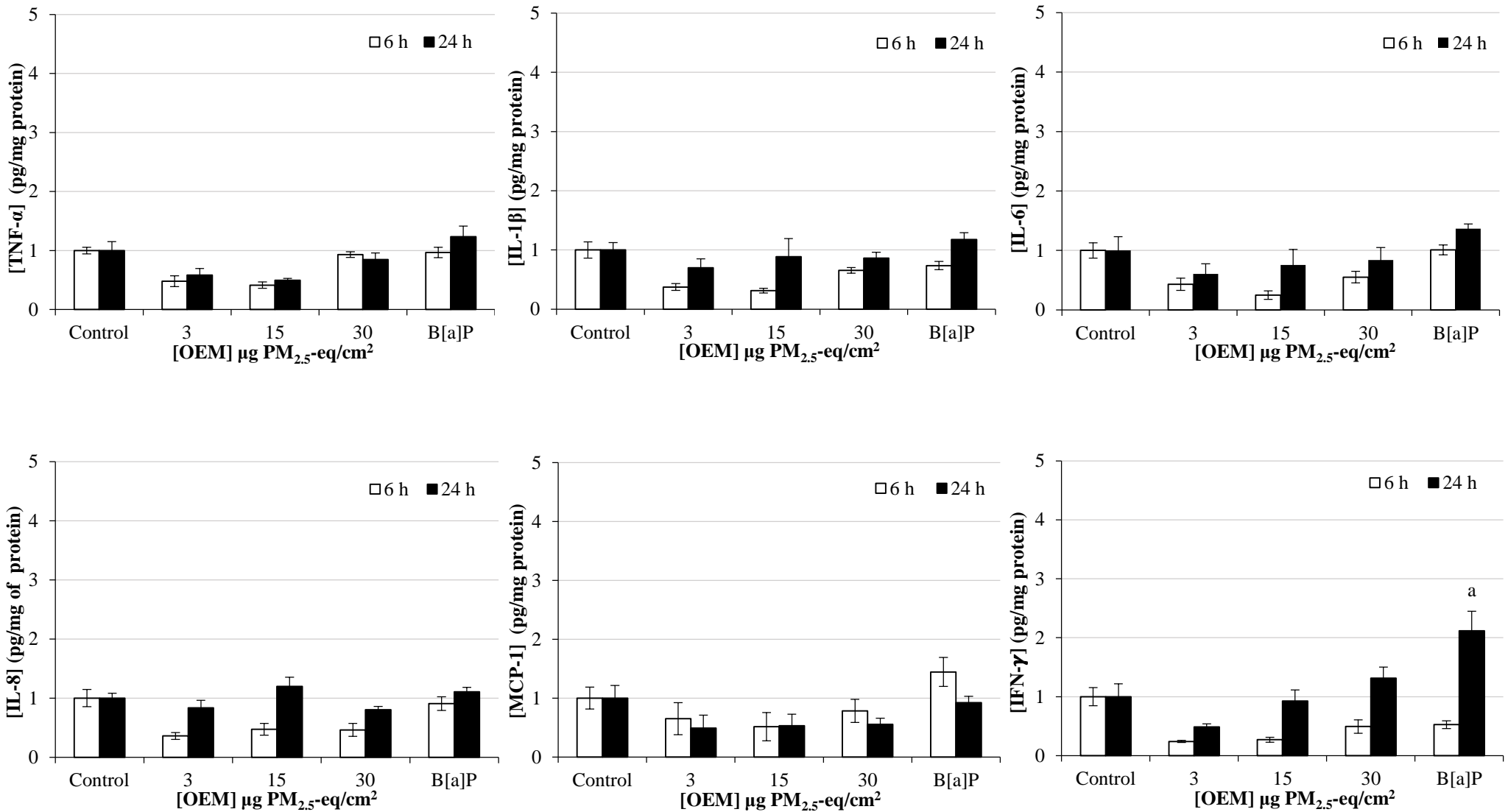


Figure 4

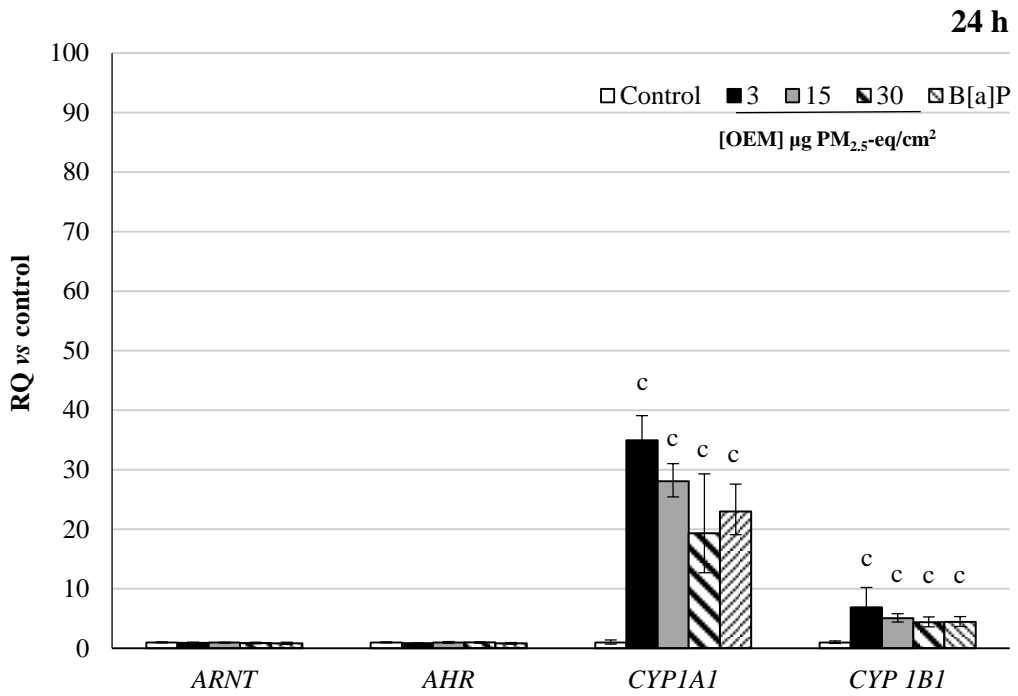
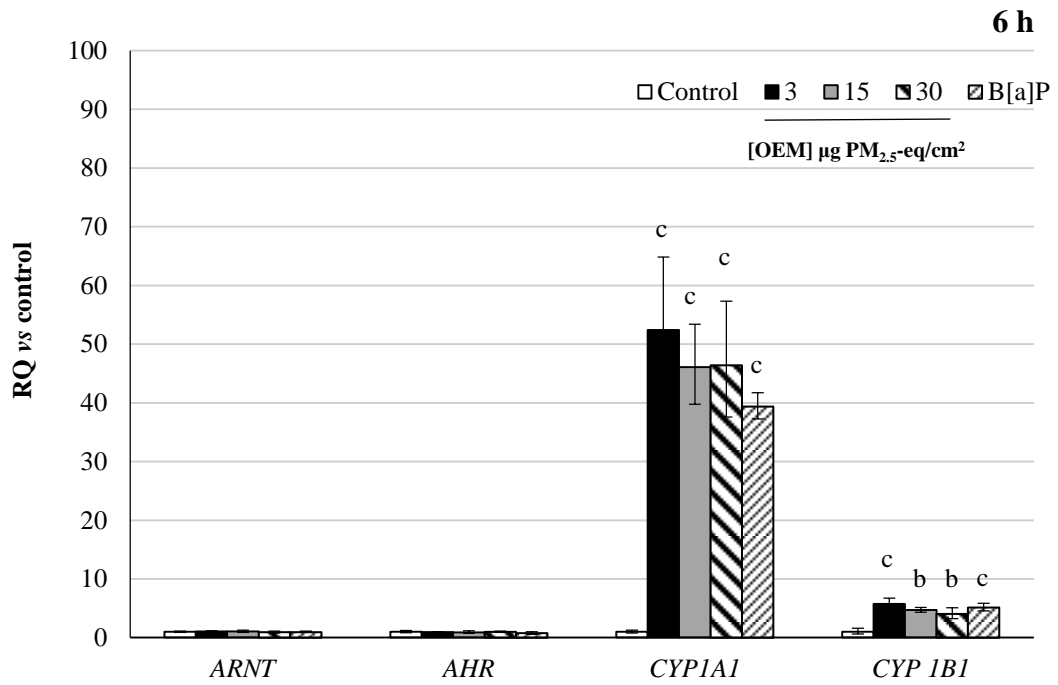


Figure 5

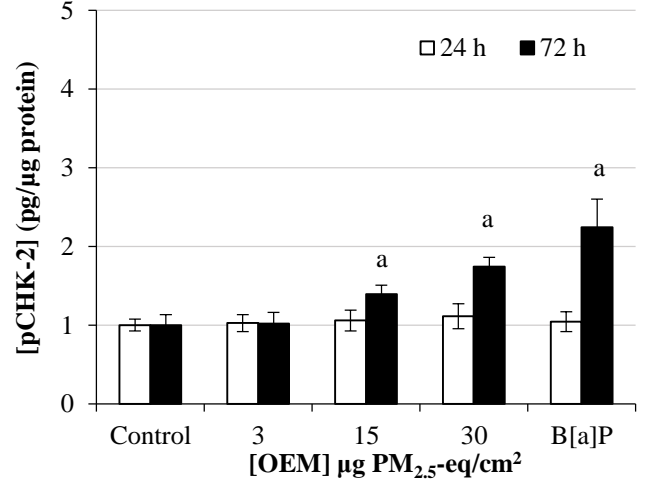
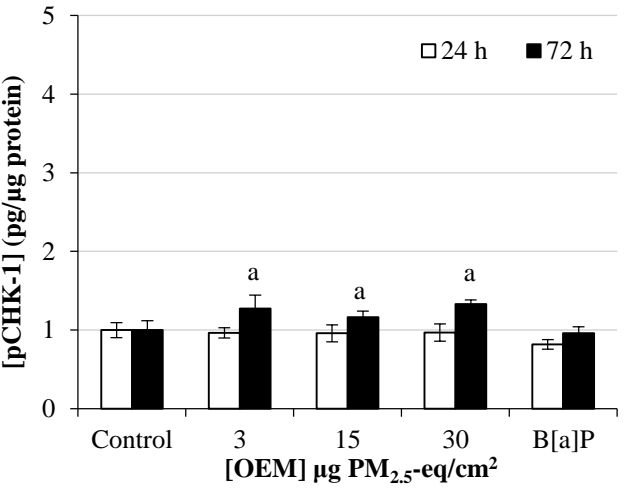
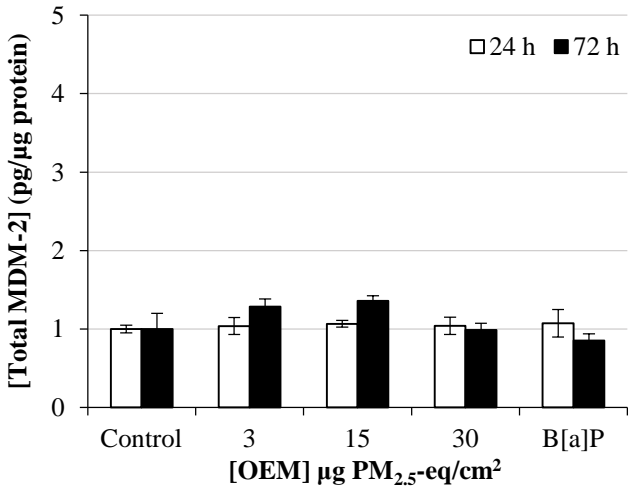
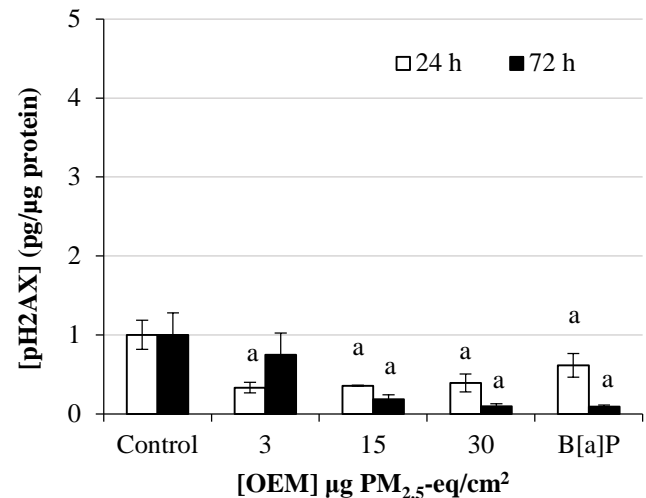
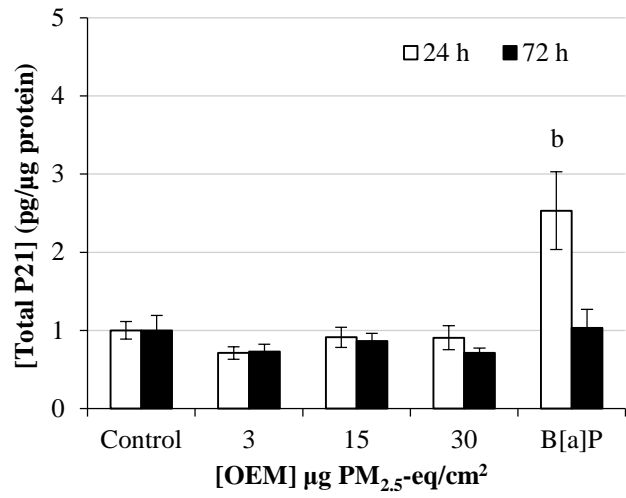
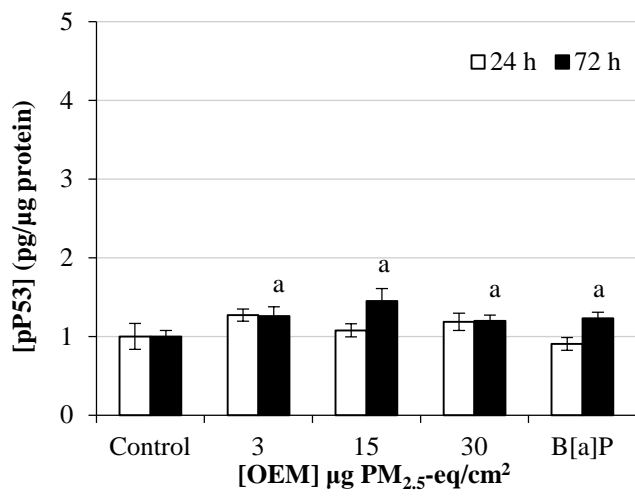


Figure 6

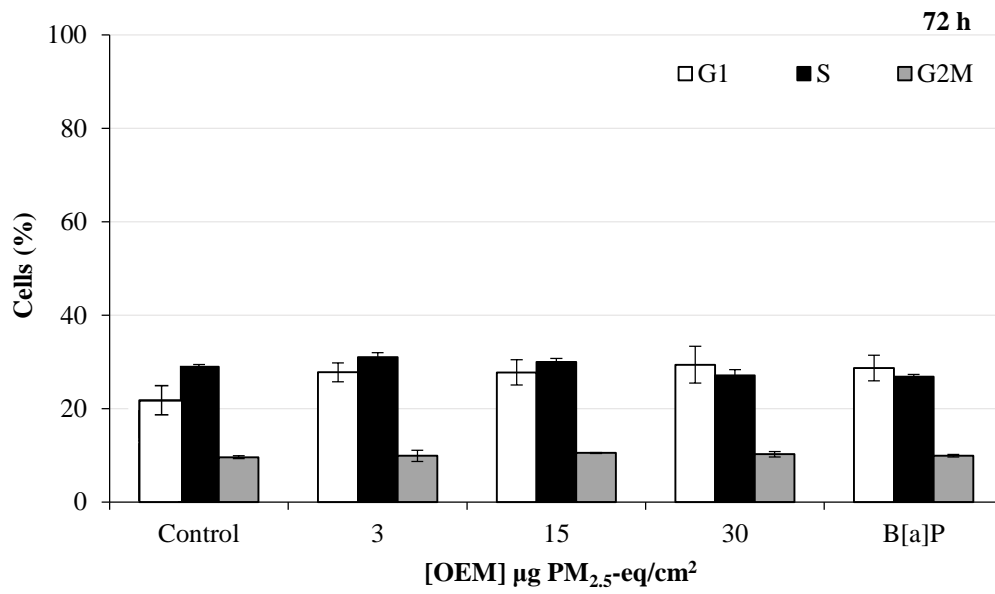
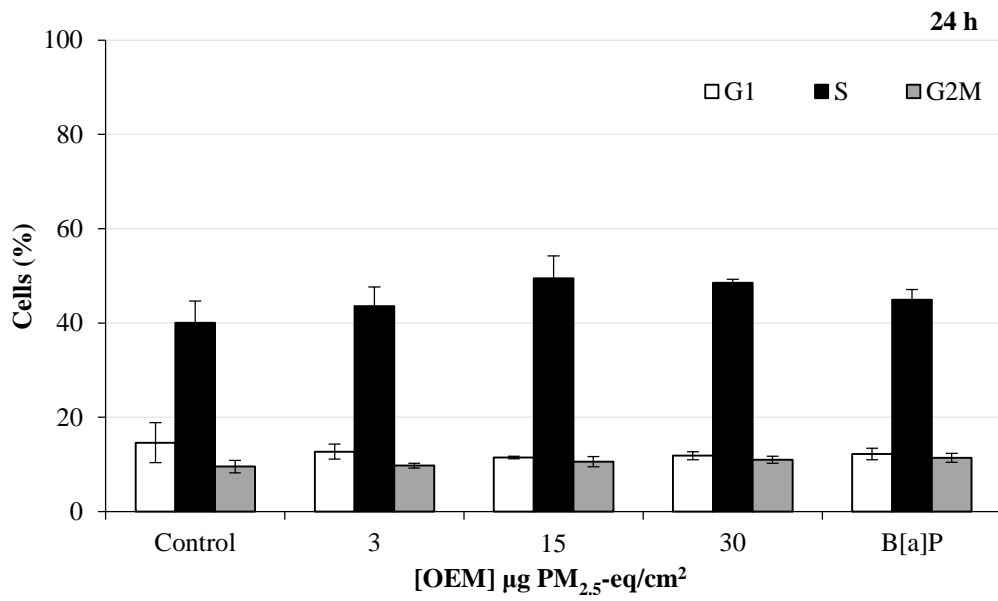


Figure 7

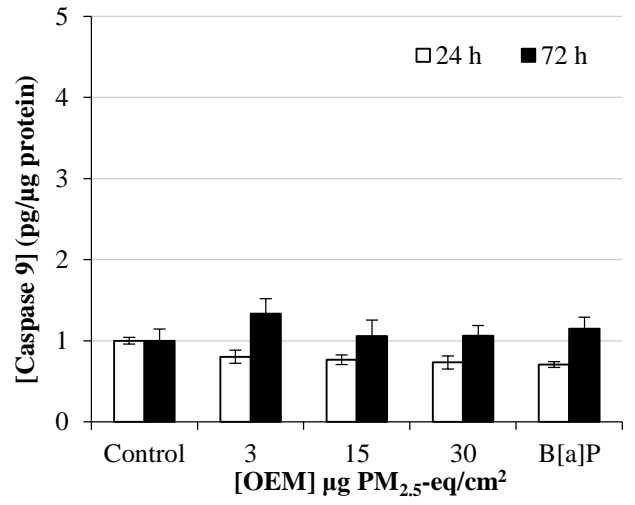
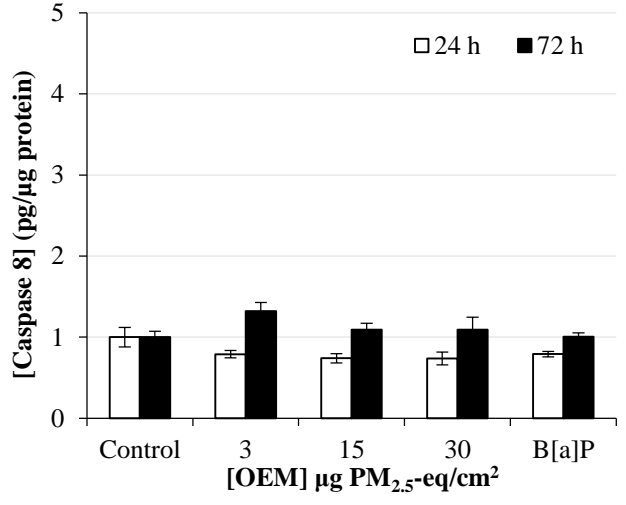
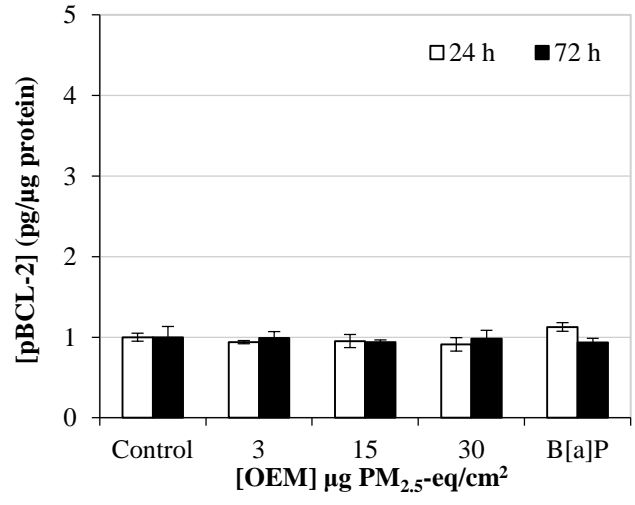
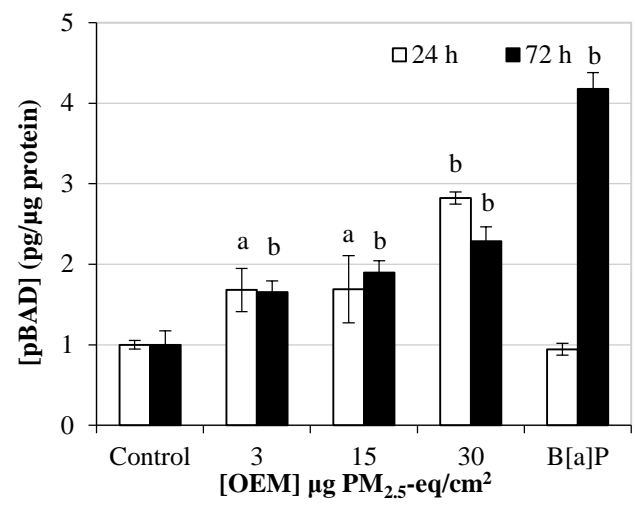
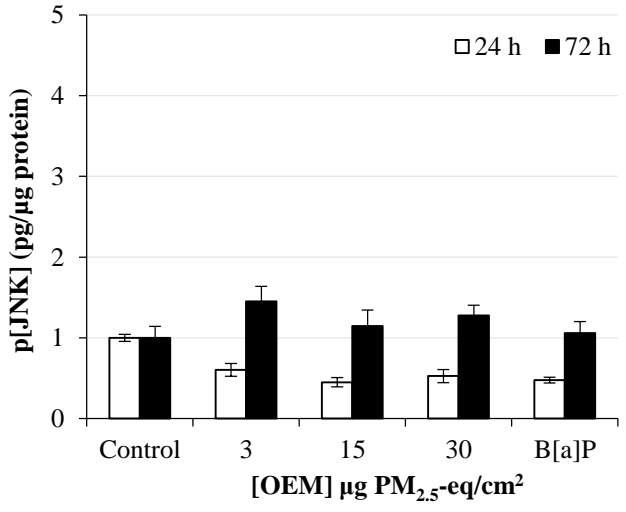
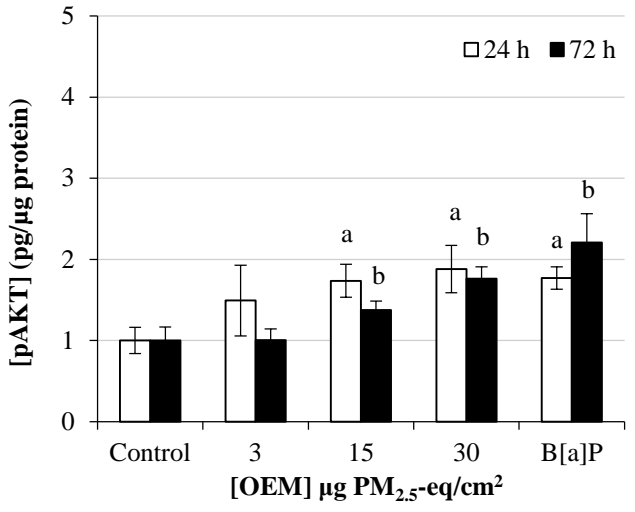
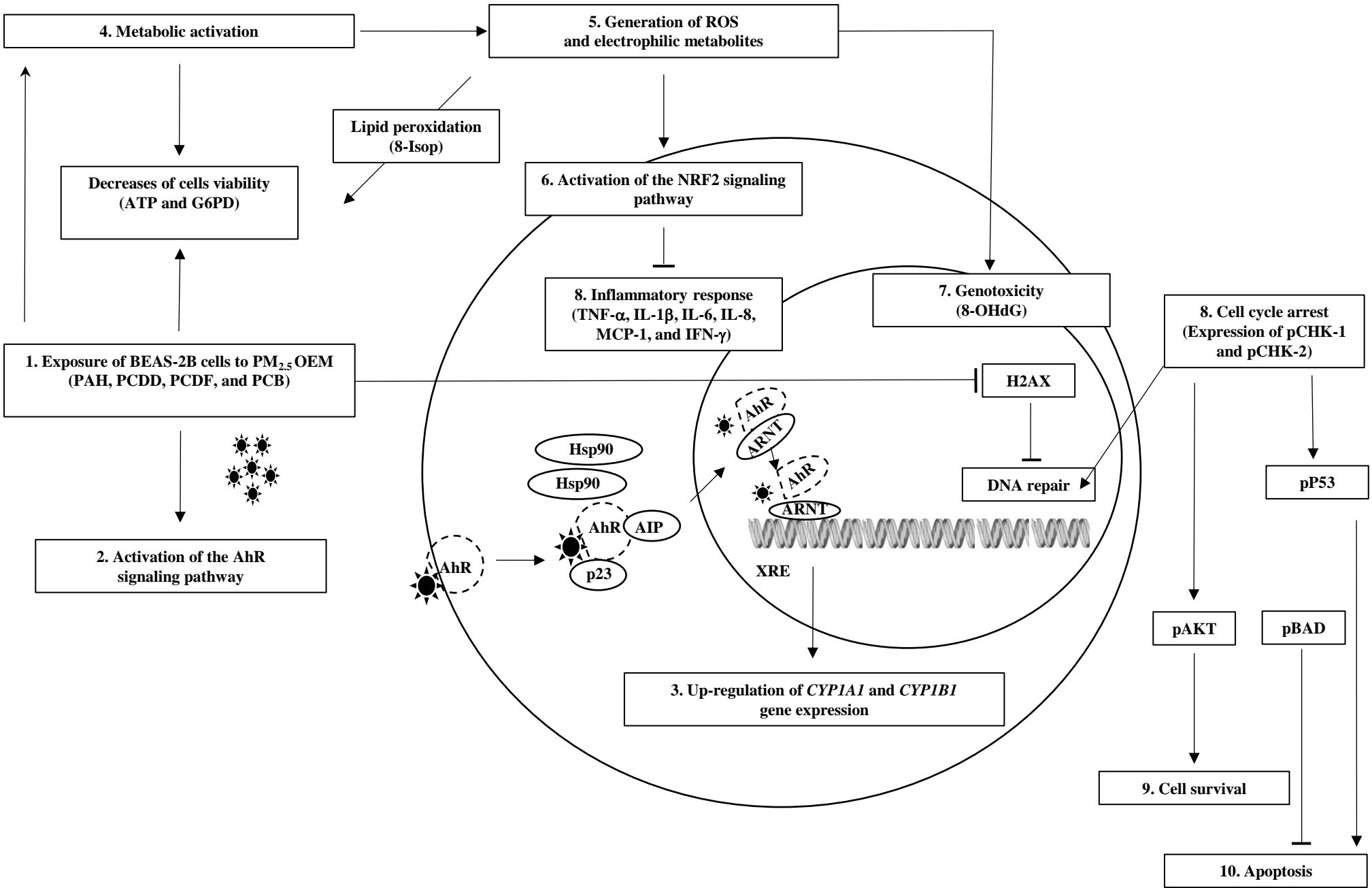


Figure 8



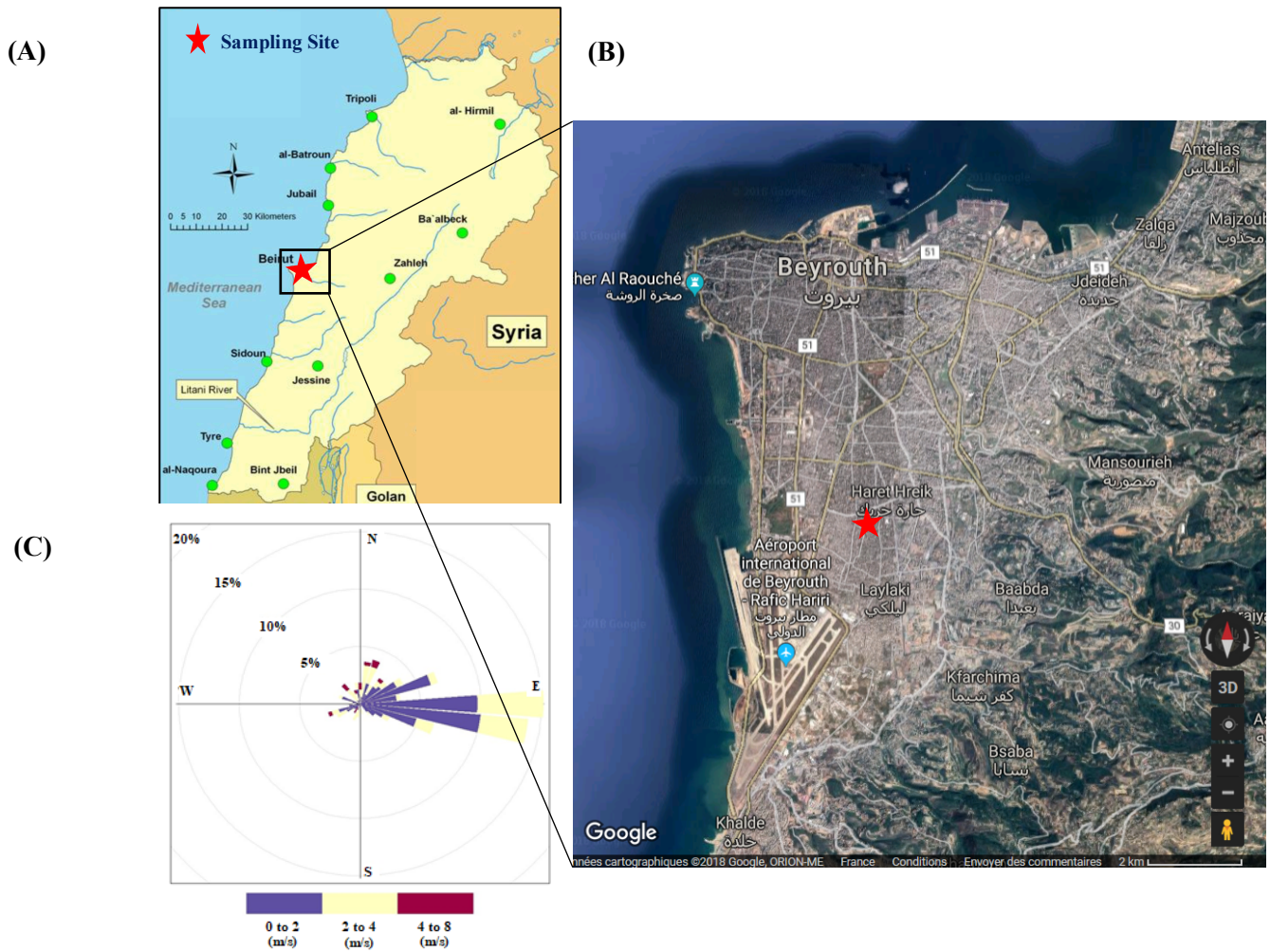


Figure S1: Location of sampling site (A and B) and the concerned recorded wind rose (C)

Table 1: Polycyclic aromatic hydrocarbons (PAH) concentrations in the ambient PM_{2.5} collected in Beirut (Lebanon).

Congener ^a	Concentration (µg/g)	Concentration (pg/m ³)
NAP	2.7	131
ACY	4.5	218
ACE	2.3	112
FLU	<0.2	<11
PHE	9.6	458
ANT	2.8	134
FLT	15.4	736
PYR	25.9	1243
B[a]A	25.7	1231
CHR	28.8	1379
B[b]F	31.7	1519
B[k]F	8.2	394
B[a]P	12.9	618
D[ah]A	<0.2	<11
I[cd]P	10.3	495
B[ghi]P	20.6	989
Total PAH	202	9655

^a Naphthalene: NAP, Acenaphthylene: ACY, Acenaphthene: ACE, Fluorene: FLU, Phenanthrene: PHE, Anthracene: ANT, Fluoranthene: FLT, Pyrene: PYR, Benz[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, Dibenz[ah]anthracene: D[ah]A, Indeno[123cd]pyrene: I[cd]P, Benzo[ghi]perylene: B[ghi]P

Table 2: PolyChloro Dibenzo-p-Dioxins (PCDD), PolyChloroDibenzoFurans (PCDF) and PolyChloroBiphenyls (PCB) concentrations in the ambient PM_{2.5} in Beirut (Lebanon)

Congener ^a	Concentration (pg/g)	Concentration (fg/m ³)
2,3,7,8 TCDD	39	2
1,2,3,7,8 PeCDD	184	9
1,2,3,4,7,8 HxCDD	220	11
1,2,3,6,7,8 HxCDD	480	23
1,2,3,7,8,9 HxCDD	380	18
1,2,3,4,6,7,8 HpCDD	3,170	152
OCDD	5,114	245
Total PCDD	9,587	459
2,3,7,8 TCDF	401	20
1,2,3,7,8 PeCDF	431	20
2,3,4,7,8 PeCDF	919	40
1,2,3,4,7,8 HxCDF	1,076	50
1,2,3,6,7,8 HxCDF	983	50
2,3,4,6,7,8 HxCDF	1,341	50
1,2,3,7,8,9 HxCDF	304	10
1,2,3,4,6,7,8 HpCDF	3933	200
1,2,3,4,7,8,9 HpCDF	477	20
OCDF	2,390	100
Total PCDF	12,255	560
PCB81	214	10
PCB77	791	38
PCB123	156	7
PCB118	7,330	351
PCB114	156	7
PCB105	4,213	202
PCB126	483	23
PCB167	2,124	102
PCB156	2,573	123
PCB157	317	15
PCB169	279	13
PCB189	1,182	57
Total DL-PCB	19,818	949

^a Tera- (T), Penta- (Pe), Hexa- (Hx), Hepta- (Hp), and Octa (O)-chlorinated Dibenzo-p-Dioxin (PCDD) and Dibenzo-Furans (PCDF). Dioxin-Like PolyChlorinated Biphenyls: DL-PCB.

Table 3: PAH ratios used as source indicators

Diagnosis Ratio ^a	Literature ^b		Current Study
	Values	Sources	
I[cd]P/(I[cd]P + B[ghi]P)	0.18	Gasoline	0.33
	0.37	Diesel	
	0.56	Coal	
	0.62	Wood burning	
	0.35-0.70	Diesel emissions	
B[a]P/(B[a]P + CHR)	0.5	Diesel	0.3
	0.73	Gasoline	
B[b]F/B[k]F	<0.5	Diesel	3.8
B[a]P/B[ghi]P	0.5-0.6	Traffic emission	0.6
	>1.25	Brown coal	
PYR/B[a]P	≈10	Diesel Engine	2
	≈1	Gasoline engine	
FLT/PYR	0.6	Vehicular emissions	0.6

^a Fluoranthene: FLT, Pyrene: PYR, Chrysene: CHR, Benzo[b]fluoranthene: B[b]F, Benzo[k]fluoranthene: B[k]F, Benzo[a]pyrene: B[a]P, Indeno[123cd]pyrene: I[cd]P, Benzo[ghi]perylene: B[ghi]P

^b Adapted from Ravindra *et al.* (2008) and Borgie *et al.* (2015a)

