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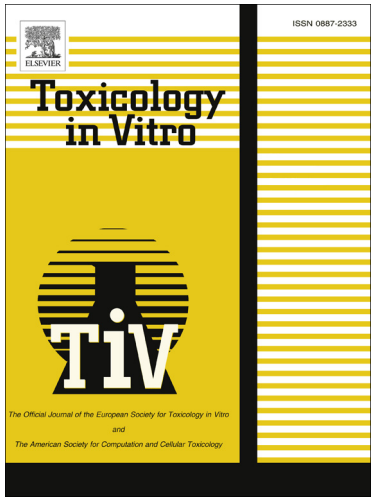

Direct and Indirect Air Particle Cytotoxicity in Human Alveolar Epithelial Cells

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Direct and Indirect Air Particle Cytotoxicity in Human Alveolar Epithelial Cells

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Short running head: Direct and Indirect Air Particle Cytotoxicity

Abstract

Air particulate matter has been associated with adverse impact on the respiratory system leading to cytotoxic and proinflammatory effects. The biological mechanisms behind these associations may be initiated by inhaled small size particles, particle components (soluble fraction) and/or mediators released by particle-exposed cells (conditioned media).

The effect of Urban Air Particles from Buenos Aires (UAP-BA) and Residual Oil Fly Ash (ROFA) a surrogate of ambient air pollution, their soluble fractions (SF) and conditioned media (CM) on A549 lung epithelial cells was examined. After 24h exposure to TP (10 and 100 μg/ml), SF or CM, several biological parameters were assayed on cultured A549 cells. We tested cell viability by MTT, superoxide anion (O_2^-) generation by NBT and proinflammatory cytokine (TNF α , IL-6 and IL-8) production by ELISA.

UAP-BA particles or its SF (direct effect) did not modify cell viability and generation of O_2^- for any of the doses tested. On the contrary, UAP-BA CM (indirect effect) reduced cell viability and increased both generation of O_2^- and IL-8 production. Exposure to ROFA particles, SF or ROFA CM reduced proliferation and O_2^- but, stimulated IL-8. It is worth to note that UAP-BA and ROFA depicted distinct effects on particle-exposed A549 cells implicating morphochemical dependence. These *in vitro* findings support the hypothesis that particle-induced lung inflammation and disease may involve lung-derived mediators.

Keywords: particulate matter, ROS, conditioned media, inflammation, ROFA, A549, cytokines, cytotoxicity

Introduction

Increased levels of air particulate matter (PM) have been associated with adverse effects not only on the respiratory system but on other distant systems such as the cardiovascular and nervous systems [1-3]. Particularly, PM-negative effects in one of its main targets, the respiratory system, may be triggered by inhaled small size particles and/or particle components (soluble fraction). Inhaled fine (micro) or ultrafine (nano) particles and/or its soluble fraction (SF) may interact directly with lung cells [4-6], or induce an indirect response through the release of several pathophysiological mediators such as reactive oxygen species (ROS), reactive nitrogen species (RNS) and proinflammatory cytokines [7, 8].

Airway epithelial cells play critical roles in homeostasis and host defense. These include acting as a physical barrier, removing particles via mucociliary transport, secreting components of the innate immune system, recognizing and responding to pathogen associated molecular patterns and signaling to leukocytes. Recently, the importance of controlled ROS production by non-phagocytic cells, including lung epithelial cells, in the regulation of physiological functions was suggested [9, 10] Therefore, the use of the human lung adenocarcinoma epithelial cell line A549 *in vitro* to study particle toxicology has been selected as one of the major cell lines able to provide valuable insights into the detailed functions and capabilities of *in vivo* airway epithelial cells from humans.

Previously, we characterized Urban Air Particles from downtown Buenos Aires (UAP-BA), a Latin American megacity, and evaluated its effect on the respiratory tract employing an *in vivo* animal model [11]. We demonstrated that UAP-BA are ultrafine particles with no metallic traces, able to generate lung inflammation and an imbalance of the oxidative metabolism probably due to the high content of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) adsorbed to their carbon core [11, 12]. On the contrary, Residual Oil Fly Ash (ROFA), a known surrogate of ambient pollution, widely used to study biological impact in experimental animal models, proved not only to exert an inflammatory response inducing the release of a variety of pro-inflammatory cytokines [13-16] but, due to its high metal content (V, Al and Si), was able to induce the generation of ROS, which in turn could result in an imbalance of the redox metabolism [11, 12].

In the present study we examined the effect of these two very different ambient air particles: Urban Air Particles from Buenos Aires (UAP-BA) and Residual Oil Fly Ash (ROFA), their soluble fraction (**SF**) and mediators released from particle-exposed cells (conditioned media, CM) on the lung epithelia cell line A549.

Materials and Methods

Particle Characterization

Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX) were employed to analyze particle morphology and chemical composition. For SEM observations, collected particles were coated with silver by direct current sputtering. Stub preparations were examined in a quanta SEM FEG-S50 (SEI, Oregon, USA). **Chemical composition was analyzed with a Phillips SEM 505 SEM (Philips Electron Optics, NL, USA) coupled to a EDX dispersion detection unit 4100 with a silicon-lithium detector (EDAX Inc., NJ, USA) used to collect elemental spectra, specifically at energies corresponding to 1.74 and 3.7 keV, representing spectral signals for silicon and calcium, respectively**

Particle Sampling

Urban Air particles from downtown Buenos Aires (UAP-BA) were collected in an area characterized by its intense vehicle traffic with a high exposure to diesel exhaust. A MiniVol™ Portable Air Sampler with 2.5 μm cut-point impactors was employed using a flow rate of 5 l min^{-1} [17]. Teflon filters (0.8 μm pore) were placed in a clean plastic cassette during transport and storage. The filters were weighed (after moisture equilibration) before and after sampling to determine the net particulate mass gain with a microbalance (Mettler M3, weighing accuracy of 0.01 mg), using an alpha source to remove the electrostatic charge. Particle concentration results from sonicate the filters 5 times for 5 min (Astrason, Misonix) in an appropriate volume of phosphate buffer solution (PBS) 1X. This particle suspension is referred as “stock suspension”.

Residual Oil Fly Ash (ROFA) from the Mystic Power Plant, CT, USA was generously provided by J. Godleski (Harvard School of Public Health, Boston, MA, USA).

Lung Epithelial A549 Cell Culture

Human lung carcinoma cell line A549 (American Type Culture Collection, Manassas, VA) was grown and maintained in Minimal Essential Medium Eagle (MEM, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FCS, Internegocios, Argentina), 2 mM glutamine, and 100 IU/ml penicillin, 100μg/ml streptomycin. According to ATCC cells were detached by trypsinization, centrifuged at 800 g for 10 min at 4 °C, resuspended in MEM and seeded at a density of 0.8× 10^6 cells/ml in 24-well culture plates at 37 °C in a

humidified atmosphere with 5% CO_2. Exposure to either UAP-BA or ROFA was always performed after 24 h in culture.

Total Particle (TP)

To prepare Total Particle (TP) suspensions for either UAP-BA or ROFA, aliquots from our "stock suspension" were resuspended in MEM media to the final concentrations desired (10 -100 µg/ml). To disrupt possible particle aggregates or agglomerates, prior to use all suspensions were always sonicated for 10 min.

Soluble Fraction (SF)

Soluble Fraction (SF) was obtained by incubating particles, either UAP-BA or ROFA (10 or 100 µg/ml), in MEM during 24 h on wells free of cells. Supernatant from UAP-BA or ROFA (10 or 100 µg/ml) were centrifuged for 10 min at 12000g in order to get rid of the particles.

Conditioned Media (CM)

Conditioned media were obtained by centrifugation of media from A549 cell cultures exposed to UAP-BA or ROFA (10 or 100 µg/ml) for 24 h. The media were centrifuged for 10 min at 12000g in order to remove particles and cell debris.

***In Vitro* Exposure Protocol**

A549 cells were exposed to:

a) Total Particle (TP) from UAP-BA or ROFA (10 or 100 µg/ml): briefly, cells were incubated with the particle suspension or free-particle media (controls) for 24h. Media were centrifuged for 10 min at 12000g and supernatant aliquots were reserved and stored at -20 ºC until cytokine production was evaluated. Adherent cells were assayed for MTT and NBT. Results are shown in panel A from Figures 3-5.

b) **Soluble Fraction** (SF): briefly, Total Particle (TP) from UAP-BA or ROFA (10 or 100 µg/ml) was incubated with media in cell-free wells. After incubation for 24 h, the media were collected and centrifuged to obtain the SF that were added to fresh A549 cells for 24 h. Media alone incubated in cell-free wells were used as control under

the same experimental conditions. Supernatant aliquots and adherent cells were tested as before for subsequent assays. The results are shown in panel B from Figures 3-5.

c) Conditioned Media (CM): briefly, A549 cells were incubated with Total Particle (TP) from UAP-BA or ROFA (10 or 100 µg/ml) for 24 h. After incubation the media were collected and centrifuged to obtain the conditioned media (CM) that were added to fresh A549 cells for another 24 h. Conditioned media obtained from A549 cells incubated with particle free media were used as control under the same experimental conditions. The experimental design is illustrated in Figure 1. Supernatant aliquots and adherent cells were tested as before for subsequent assays. The results are shown in panel C from Figures 3-5.

Possible microbiological contamination was checked under light microscopy throughout the all experiment. No contaminations were observed neither for TP, SF nor CM cultures.

Spectrophotometric Assays

1. Cell Viability

A549 proliferation was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, as described elsewhere [18, 19]. This assay is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases of living cells. Briefly, medium from control or treated with TP, SF or CM, was removed from cell cultures, washed with PBS 1X two times and then 0.5 ml fresh complete growth medium supplemented with 50 µl MTT (4mg/ml in PBS) was added for 3 h. Immediately after incubation, 10% SDS was added to stop the MTT reaction and to solubilize the formazan precipitate. The optical density (OD) of the final solution was measured at 570 nm in a spectrophotometer (Shimadzu UV-1201V).

2. **Superoxide Anion Generation**

Superoxide anion (O_2^-), a main reactive oxygen species (ROS) generated during the respiratory burst, was evaluated in control and exposed A549 cultures using the NBT (NBT, 2,2-bis(4-nitrophenyl)-5,5-diphenyl-3,3-(3,3-dimethoxy-4,4-diphenylene)(Sigma-Aldrich) reduction test [20]. During the respiratory burst, O_2^- is formed by the reduction of O_2 by NADPH oxidase localized on the surface of the plasma membrane. The intracellular release of this ROS is evidenced by the amount of blue formazan precipitate in the cells after NBT reduction. To measure the generation of O_2^- on A549 cells a modified protocol was employed [21]. A549 cells (0.8×10^6 per

well) were incubated with 1ml, 0.1% in PBS Nitro Blue Tetrazolium chloride in 24-well microplates for 60 min at 37°C, 5%CO_2. After incubation, cells were washed sequentially with warm PBS (x2) and methanol. It is critical to remove all extracellular NBT since the presence of the blue intracellular formazan precipitate is considered indicative of a positive reaction. Intracellular NBT was dissolved by adding 120ml of 2 M KOH and 140ml of DMSO and shaked for 10 min at room temperature. Each dissolved NBT solution was transferred to a 96-well plate and the intensity of the blue color was spectrophotometrically evaluated on a microplate reader at 655 nm [21].

3. **Cytokine Production**

Tumor Necrosis Factor alfa (TNF-α), Interleukin-8 (IL-8) and Interleukin-6 (IL-6) levels were detected in supernatants from the three experimental conditions described above using a commercially available specific enzyme linked immunosorbent assay (ELISA) kit, following the manufacturer's instructions. ELISA plates (Nalge Nunc Int.Corp., NY) were coated with 1:125 cytokine-specific capture antibody diluted in coating buffer (0.1 M Na_2CO_3, pH 9.5) at 4ºC overnight. Wells were blocked with PBS containing 10% FCS for 1h at RT. Cytokine standards and A549 supernatants were added to wells and incubated for 2h. Following three washes, biotinylated cytokine-specific detection antibody 1:250 was added for 1h. After washing, streptavidin-peroxidase/TMB detection system was employed for 30 min. Absorbance was measured on a microplate reader (Bio-Rad, bench mark) at 655 nm. All samples were run in triplicates.

Statistical Analysis

Results for control and exposed cultures were compared employing a multiple comparison procedure, the Tukey-Kramer test. Statistical significance was set at $p<0.05$. All data is expressed as the mean±SD; from samples (n=12-16) of at least three independent experiments. Statistical analysis was performed with Graphpad Prism, Inc (Graphpad Software).

Results

UAP-BA and ROFA morphochemical characterization

The morphology of UAP-BA from downtown Buenos Aires (UAP-BA) and ROFA are shown

in figure 2. UAP particle size and composition revealed small homogeneous spherical ultrafine particles (< 0.2 μm) with no detectable inorganic composition by EDX (Fig 2 A and B). On the contrary, ROFA particles proved to be heterogeneous both in size and shape and the spectral composition confirmed that ROFA is mainly composed of inorganic material (90%), mostly sulfur (S), vanadium (V), sodium (Na), silica (Si), aluminum (Al) and calcium (Ca) (Fig 2 C and D).

UAP-BA Conditioned Media (CM) decreases cell viability

First, we examined the direct effect of ROFA or UAP-BA Total Particle (TP) on the cell viability of A549 cells in culture. We found that ROFA (used as a positive control), affected cell viability at the highest dose employed. On the contrary, no significant differences on this parameter were observed when A549 cells were exposed to UAP-BA (10 or 100 μg/ml) (Fig **3**A). Furthermore, significant differences were found between ROFA 100 μg/ml and UAP-BA.

When epithelial cell cultures were incubated for 24 h with either ROFA or UAP-BA Soluble Fraction (SF), the biological response showed a similar pattern to that obtained when cells were incubated with the total particle. Only ROFA-SF 100 μg/ml induced a significant reduction on MTT values while neither 10 nor 100 μg/ml UAP-BA-SF were able to provoke any change on the cell viability (Fig **3**B).

Surprisingly, when A549 cells were exposed to 100 μg/ml UAP-BA-CM, cell viability significantly diminished, behaving as with ROFA-CM **(Fig 3C).**

UAP-BA Conditioned Media increases superoxide anion generation

We examined the ability of 10 and 100 μg/ml ROFA or UAP-BA particles and their Soluble Fraction (SF) on A549 superoxide generation. As shown in figure 4 both ROFA-TP (Fig 4A) and SF (Fig 4B) at the highest dose employed (100 μg/ml) significantly changed the generation of superoxide generation. However, it is worth to note that A549 cells exposed to UAP-BA-TP or its SF were unable to modify this parameter (Fig 4 and B). On the contrary, incubation with CM from the two particles assayed in this study showed a different cellular response. ROFA-CM, as observed with ROFA-TP or ROFA-SF, reduced superoxide anion generation while, UAP-BA-CM induced a marked increase of this oxygen radical (Fig **4**C)

UAP-BA Conditioned Media increased IL-8 production

The cytotoxic effect of proinflamatory cytokines like TNFα, IL6 and IL8 was investigated in A549 cell cultures exposed to TP, SF or CM from either ROFA or UAP-BA.

No release of TNFα or IL-6 was found for the TP, SF or CM of the air particles assayed (data not shown).

On the other hand, for both particles at the highest concentration assayed, a significant increase ($p < 0.05$) on IL-8 production was observed in A549 cells exposed to the TP (Fig 5 A), SF (Fig 5 B) and CM (Fig 5 C) . We found that only ROFA (TP, SF and CM), showed a dose response regarding IL-8 production In addition, when cells were exposed to CM from both particles, IL-8 values reached the highest values as compared to controls.

Discussion

It is widely accepted that many environmental particles are associated with increased mortality and morbidity [22] and the underlying mechanism(s) by which air pollution causes their adverse effects are subject of intense research.

It is known that particulate matter (PM) or their soluble fraction can directly interfere with different cellular processes affecting cells and tissues locally or distantly. In this study, we explore the possibility that some PM effects on cells are mediated by cellular factors released post-PM exposure.

Herein we used A549 cells, a widely used model cell line for the toxicity study and two distinct PM as regards of their morphology and composition. ROFA is an industrial PM rich in metal traces displaying an heterogeneous size while UAP-BA, is an ultrafine spherical urban PM with no metal traces in its composition and a high content of HAPs and PCBs (Fig.2) [11].

Indeed, our results showed that, A549 cells exposed to the media conditioned (CM) by cells pre-exposed to PM (indirect effect) but not directly to UAP-BA particles or to its soluble fraction (direct effect) have a reduced cell viability (Fig 3), and an increased O_2- generation (Fig 4).

Our study opens interesting questions about the role of secreted factors by cells exposed to UAP-BA**.** First, we observed that 24 h exposure to either UAP-BA or its soluble fraction does not affect the viability of A549 cells (Fig **3**A-B). Interestingly, exposure to CM for 24 h moderately but significantly decreased the viability of these cells (Fig **3**C). Therefore, we can speculate that the ultimate effect of UAP-BA actually depend on the contribution of secreted cellular factors acting via an autocrine loop.

Second, cellular factors released as the result of PM exposure may reach the general circulation and affect distant cell types and may be responsible for the known adverse effects associated with air pollution in other systems (e.g. cardiovascular) believed to be caused by PMs [23] . These possibilities are summarized in figure 6.

Third, our results clearly suggest that the effect may be dependent on the nature of the PM since ROFA (used as a positive control in this study) showed different effects (particularly at 100 µg/ml) compared to UAP-BA on the parameters evaluated in Figs 3-5.

A possible role for secreted cellular factor induced by PM may involve the regulation of signaling pathways associated with the defense against oxidative stress as an attempt for self-defense (via autocrine loop) as well as for the protection of distant cells. In this context, it was recently reported that PM2.5 activate the PIK3/AKT

signaling pathway and induces Nrf2-mediated defense mechanisms against oxidative stress [24]. The Nrf2/ARE has been previously shown to be modulated by extracellular signals such as ROS and cytokines [25]. Oxidative stress events linked to inflammation could be an adaptative response to environmental factors as suggested in other systems [26]. It is important to point out that in the present study we showed a significant increase in the production of IL-8 by CM (Fig **5**C). In agreement with our studies increased IL-8 production has been shown in A549 cells by traffic-related particulate matter [27] suggesting that this cytokine may be an important and ubiquitous factor induced by PMs.

It is worth to note that UAP-BA and ROFA depicted distinct effects on particle-exposed A549 cells implicating morphochemical dependence. These *in vitro* findings support the hypothesis that particle-induced lung inflammation and disease may involve lung-derived mediators.

Future studies aimed at characterizing the nature and functions of the secreted factors induced by PM will significantly expand our knowledge on the mechanism of action of air pollution.

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Legends to Figures

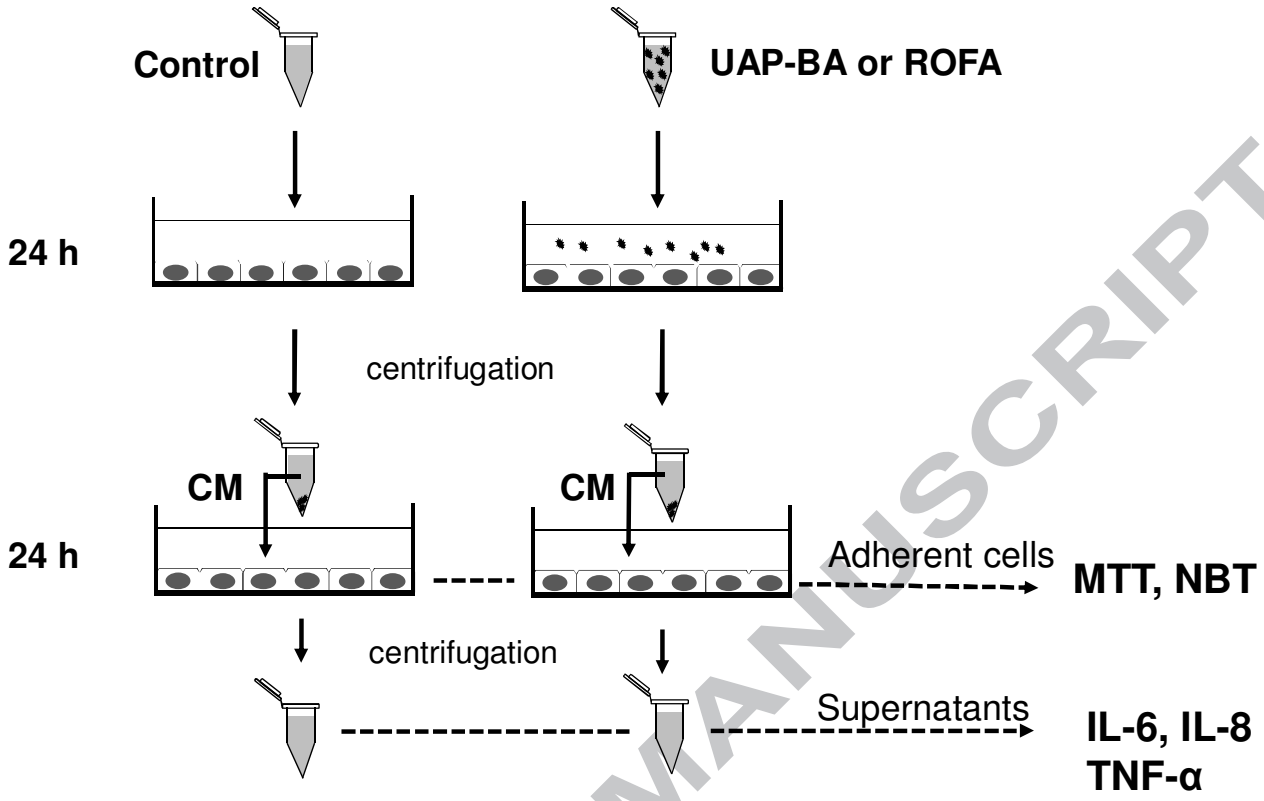

Figure 1. Experimental procedure for cultured A549 cells exposed to conditioned media (CM, panels C in **Figures 3-5**). Cultured A549 cells were incubated with media alone (control) or media containing total particles (UAP-BA or ROFA). After 24 hours of incubation the media were collected, centrifuged and the supernatants (conditioned media, CM) were added to fresh A549 cells and reincubated for another 24 h. After incubation with CMs, the media were collected and centrifuged for IL-6, IL-8 and TNF-α determinations and the adherent cells were assayed for MTT and NBT.

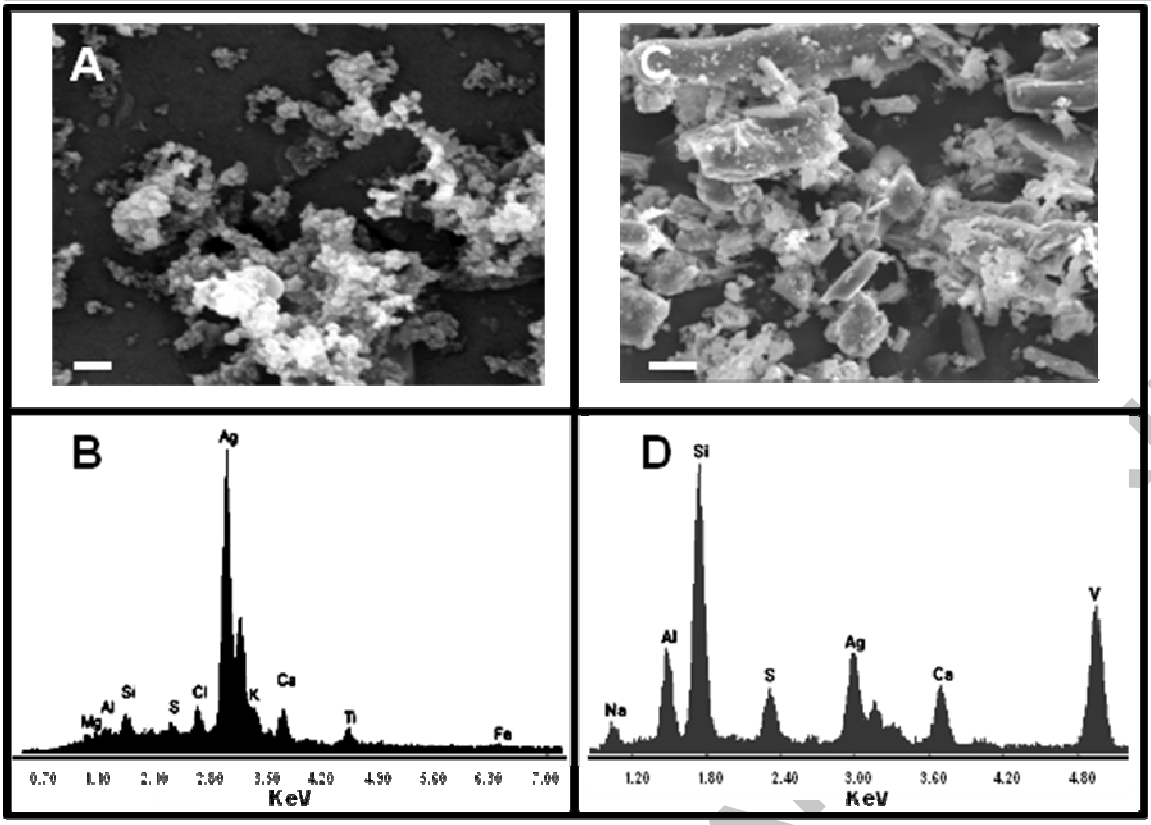

Figure 2. Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Analysis (EDX) from UAP-BA and ROFA.

A and C: Microphotographs from UAP-BA (Mag 80 000, scale bar = 1µm) or ROFA (Mag. 1 000, scale bar = 10µm) samples respectively.

B and D: Elemental analysis by EDX from UAP-BA or ROFA samples respectively.

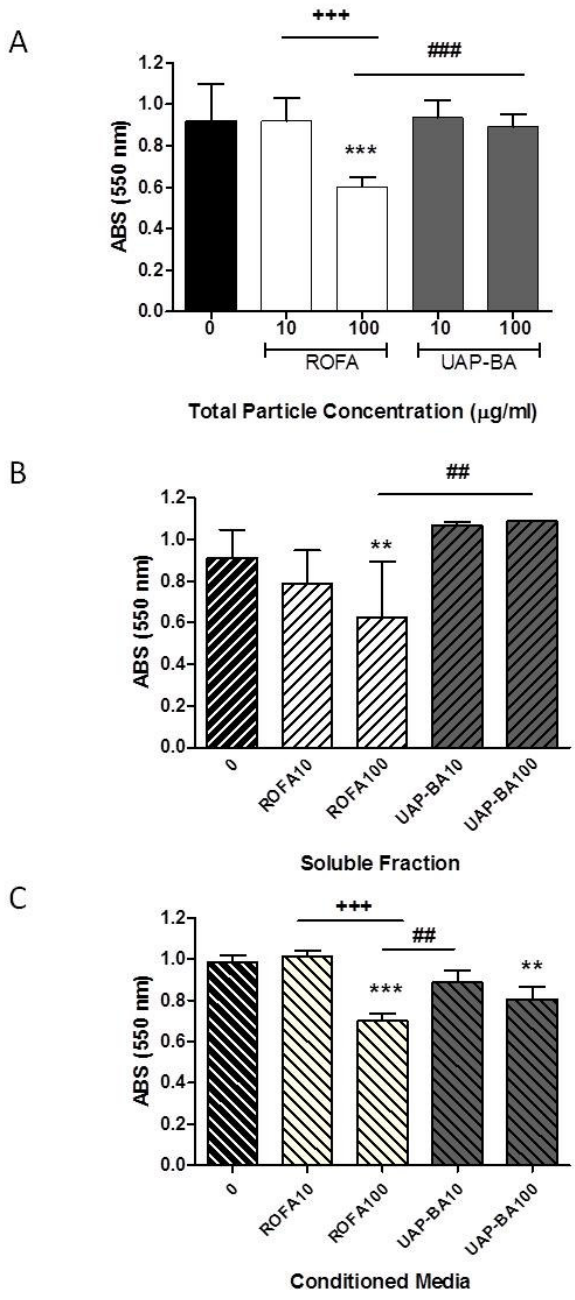

Figure 3. Cell viability of A549 cells exposed to Total Particle, Soluble Fraction or Conditioned Media from both air particulate matter. A549 cells were exposed 24h to a) ROFA or UAP-BA (10 or 100 µg/ml), b) their Soluble Fraction or c) Condition Media obtained from 24h PM pre-exposed cultures. Cell viability was spectrophotometrically measured by the MTT colorimetric assay. Each bar represents the mean ± SD, n = 12-15 from 3-5 independent experiments with triplicates or quadruplicates replicas. Statistically significant differences between exposed and control cells (**) $p < 0.01$, (***) $p < 0.001$, ROFA and UAP-BA exposed cells (##) $p < 0.01$, (###) $p < 0.001$ and ROFA exposed cells (+++) $p < 0.001$.

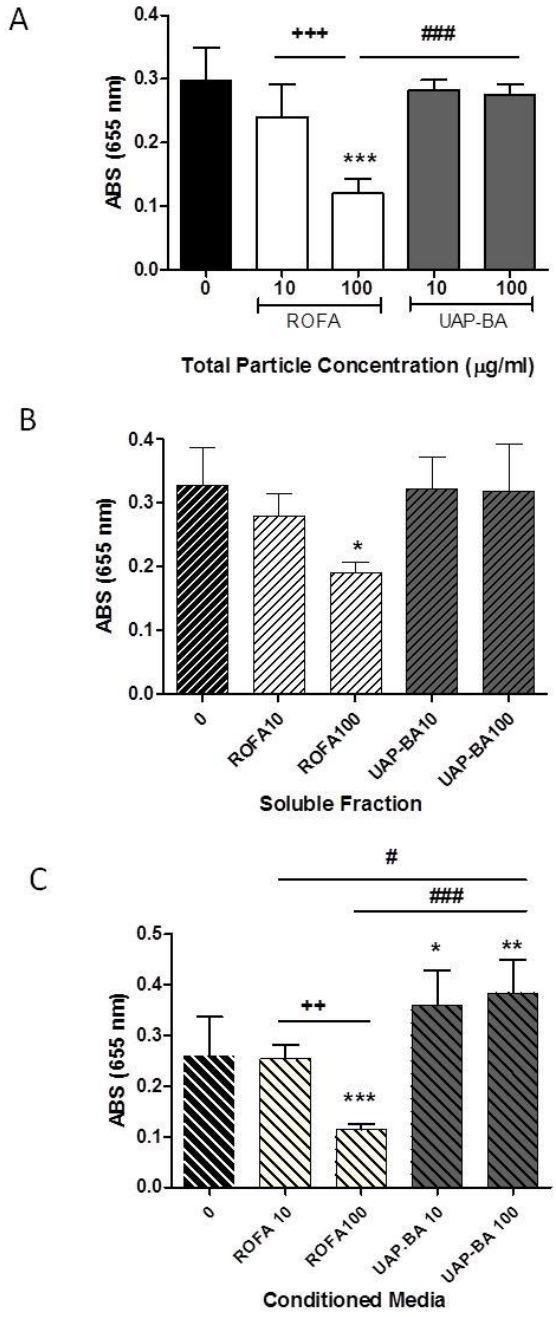

Figure 4. Superoxide anion generation in A549 cells exposed to Total Particle, Soluble Fraction or Conditioned Media from both air particulate matter. A colorimetric NBT assay was used to qualitatively detect the superoxide anion generated in A549 exposed and non-exposed cultures. Each bar represents the mean ± SD, n = 12-16 from 3-4 independent experiments with triplicates or quadruplicates replicas. Statistically significant differences between exposed and control cells (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$,ROFA and UAP-BA exposed cells (#) $p < 0.05$, (###) $p < 0.001$ and ROFA exposed cells (++) $p < 0.01$, (+++) $p < 0.001$.

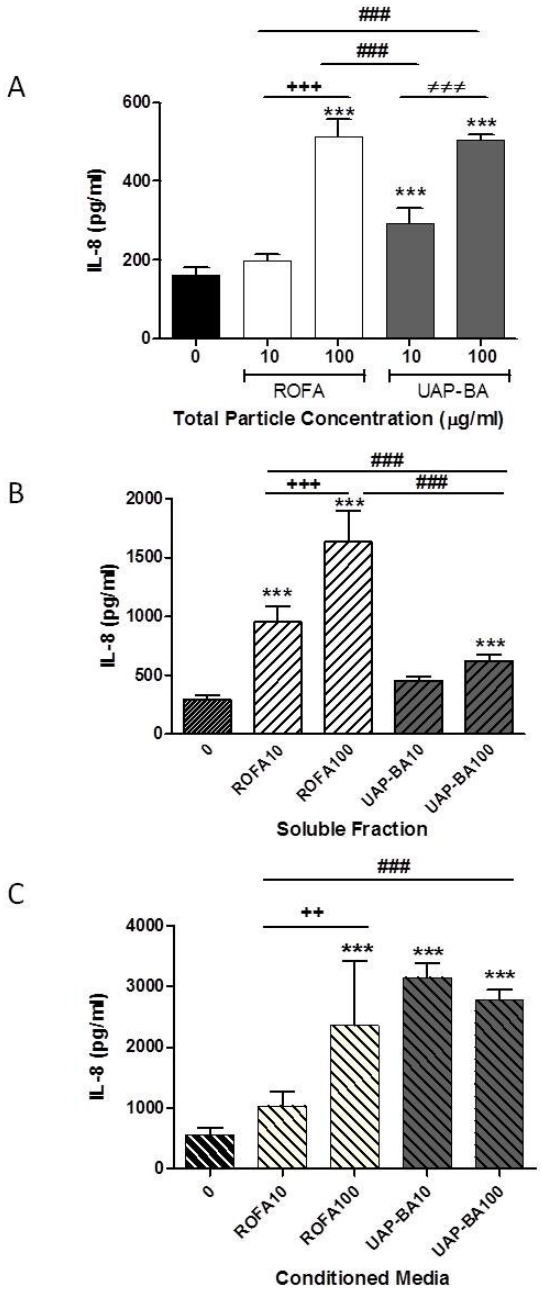

Figure 5. Interleukin-8 (IL-8) production in A549 cells exposed to Total Particle, Soluble Fraction or Conditioned Media from both air particulate matter. IL-8 release [pg/ml] of A549 cultured cells after 24 h exposure to a) ROFA or UAP-BA (10 or 100 µg/ml), b) their Soluble Fraction or c) Condition Media obtained from 24h PM pre-exposed cultures. Each bar represents the mean ± SD, n = 12-15 from 3-5 independent experiments with triplicates or quadruplicates replicas. Statistically significant differences between exposed and control cells (***) $p < 0.001$, ROFA and UAP-BA exposed cells (###) $p < 0.001$, UAP-BA exposed cells ($^{\neq\neq\neq}$) $p < 0.001$and ROFA exposed cells (++) $p< 0,01$, (+++) $p < 0.001$.

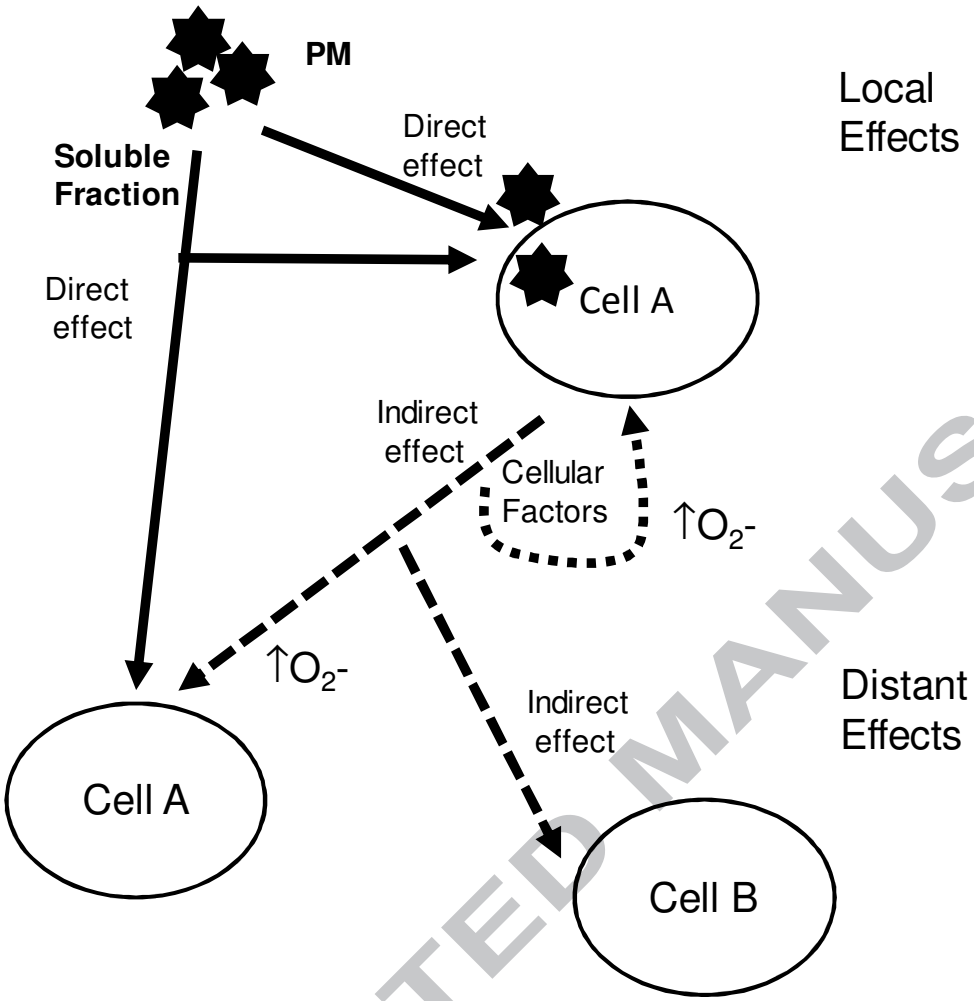

Figure **6.** Schematic model for UAP-BA effect. PM can have effect on the cellular function directly by physical interaction (local effect) or through their soluble fraction (local or distant effect) and/or indirectly by inducing the release of cellular factors. These can exert their action locally by an autocrine loop (Cell A) or systemically (distant effect) on different cells (e.g. Cell A or Cell B).

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A549 cells exposed to the media conditioned by pre-exposed cells (indirect effect) to urban air particles elicited:

- Reduced cell viability
- Increased generation of superoxide anion.
- increased IL-8 production

This behavior was not observed when A549 cells were exposed to the total particle or its soluble fraction (direct effect)

Even more air particle direct and indirect cytotoxicity on human epithelial cells depend on its morphology and/ or chemical composition