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Chemical Industry & Chemical Engineering Quarterly 18 (4) 635–641 (2012) CI&CEQ

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SCIENTIFIC PAPER

UDC 577:577.2:616-036.22

DOI 10.2298/CICEQ120206114M

ADVERSE BIOLOGICAL EFFECTS OF MILAN URBAN PM LOOKING FOR SUITABLE MOLECULAR MARKERS OF EXPOSURE•

The results presented summarise the ones obtained in the coordinated research project TOSCA (Toxicity of Particulate Matter and Molecular Markers of Risk), which extensively analysed the impact of Milan urban PM on human health. The molecular markers of exposure and effects of seasonally and size-fractionated PMs (summer and winter PM10, PM2.5) were investigated in in vitro (human lung cell lines) and in vivo (mice) systems. The results obtained by the analyses of cytotoxic, pro-inflammatory and genotoxic parameters demonstrate that the biological responses are strongly dependent upon the PM samples seasonal and dimensional variability, which ultimately reflect their chemical composition and source. In fact, summer PM10, enriched in crustal elements and endotoxins, was the most cytotoxic and pro-inflammatory fraction, while fine winter PMs induced genotoxic effects and xenobiotic metabolizing enzymes (like CYP1B1) production, likely as a consequence of the higher content in combustion derived particles reach in PAHs and heavy toxic metals. These outcomes outline the need of a detailed knowledge of the PMs physicochemical composition on a local scale, coupled with the biological hazard directly associated to PM exposure. Apparently, this is the only way allowing scientists and policy-makers to establish the proper relationships between the respirable PM quantity/quality and the health outcomes described by clinicians and epidemiologists.

Keywords: particulate matter; marker of toxicity; in vitro; in vivo.

There is a large and still growing collection of evidence that air pollutants, and among these airborne particulate matter (PM), represent significant risk factors for respiratory and cardiovascular diseases, like asthma, COPD, myocardial infarction and ischemic stroke, with children and elderly showing the highest sensibility to increasing PM levels [1].

These data, collected by epidemiologists in the last decades and still emerging, stimulated regulatory agencies, national and super-national governments, to undertake policies aimed to contain the PM levels to protect the population health. In fact, at present, atmospheric pollution is considered to be the most significant environmental factor affecting humans.

Italy has promptly adopted the PM10 limits indicated by EU, and has recently concluded the new legislative process that brings to the adoption of the new limits for PM10 and PM2.5 (DL 155/2010).

Besides these efforts that allow national and regional environmental protection agencies adopting modern and efficient air monitoring systems, there still persist hot-spot areas where the levels of PM pollution are greatly above the law limits, thus posing concerns to human health protection. The Po Valley, in the North of Italy, where Milan is located, represents one of the most PM polluted area in Italy, as well as in the European Union. Coupled with the high population density and the large number of industrial activities spread throughout the valley, the peculiar geographic position, enclosed between the Alps at north and the Appennines at south, determines the accu-

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•Part of this paper was presented at the Scientific Meeting Particulate Matter: Research and Management, 3rd WeBIOPATR Workshop & Conference, Belgrade, Serbia, 15-17 November 2011.

Paper received: 6 February, 2012
Paper revised: 8 June, 2012
Paper accepted: 16 July, 2012

mulation of gaseous and particulate pollutants especially during wintertime. As a consequence, the PM10 limits are often exceeded and citizens are constantly alarmed for the possible adverse impact on health. Considering these important premises, the TOSCA project (Toxicity of Particulate Matter and Molecular Markers of Risk) has been developed with the specific aim to provide scientific and multidisciplinary data on the impact of Milan urban PM on human health. The goal was to relate the PM physicochemical properties with its toxicological profile and, finally, with the adverse health effects observed in humans.

Here the focus has been directed to the biological effects elicited by different PM fractions (PM10, PM2.5) collected during summer and winter of the year 2009-2010 in a Milan site (Torre Sarca), representative of an urban background. Torre Sarca was defined as a typical urban station, mainly influenced by vehicular traffic, according to 2001/752/CE. Samplers were located in a fenced area, about 20 m from the nearest roads and 50 m from the nearest traffic light. PM sampling was performed at about 2.5 m from the ground, a height representative of exposure for typical populations. Specific molecular markers, able to describe the different biological responses associated to the different PM fractions, have been investigated. These markers may be helpful in elucidating the influence of PM pollution on human health at a local scale, and in introducing predictive strategies in the management of PM-related health effects linked to the particles' characteristics and sources.

Milan PM fractions were thus sampled and chemically and microbiologically characterized. Particles extracted from filters were used to study the biological responses induced in human lung cell lines and in mouse lungs.

EXPERIMENTAL

PM Sampling and chemical characterization. PMs (PM10 and 2.5) were collected by low volume gravimetric samplers (EU system 38, 33 l min-1, FAI Instruments, Rome, Italy), programmed for daily sampling (24 h, starting time: 00.00 midnight) using Teflon (47 mm, Ø, 2 µm, Pall Gelman, USA) and quartz filters (47 mm, Ø, 2 µm, Whatman). Before and after sampling, filters were equilibrated for 48 h (35% relative humidity, ambient temperature) and weighted with a M5P-000V001 microbalance (Sartorius, Germany) with a precision of 1 µg, to determine the atmospheric particle concentration. All sampled filters were then preserved in the dark at -20 °C for chemical and biological analyses. Ions, elements and organic fraction were analysed by IC, ICP-MS and GC-MS, respectively. The elemental carbon (EC) and organic matter (OM) fractions were analyzed by thermal optical transmission (TOT) using an OM/EC carbon analyzer (Sunset Laboratory Inc., USA).

Microbiological characterization. The total endotoxin content in the PM samples was quantified by the limulus amebocyte lysate (LAL) test according to the manufacturer instruction (Cape Cod Inc., MA, USA). The characterization of the microbial communities associated with the PM was carried out by sequencing the V3 hypervariable region of 16S rRNA gene. DNA was extracted directly from the filters of the different PM samples (for detailed methods, see [2]).

In vitro PM toxicity. Particles for biological analysis were detached from Teflon filters as previously described [3]. Briefly, filters sampled in the same period were pooled and underwent sequential sonications in a Soltec waterbath (SONICA, four cycles of 20 min each) in sterile water. Particle suspensions were dried into a desiccator, weighed and stored at -20 °C until use. Human lung epithelial cells, A549, and human monocytes, THP-1, were routinely maintained in Opti-MEM (Invitrogen, Italy) supplemented with 10% foetal bovine serum and 5% of penicillin/streptomycin, at 37 °C, 5% CO_2. Cell cultures were exposed to 10 µg/cm^2 of PMs for 24 h. Cell viability, pro-inflammatory proteins and cell cycle progression were assessed by LDH assay kit (Sigma-Aldrich, Italy), ELISA technique (Invitrogen, Italy) and differential cell counts after nuclear staining. Nuclear staining was performed with Hoechst 33342 and propidium iodide (H/PI). Cells stained with H/PI and smeared onto glass slide were scored (at least 300 cells per sample) according to the differential nuclear morphology to assess mitotic arrest and apoptotic process activation.

In vivo PM toxicity. The PM aliquots, stored at -20 °C, were reconstituted in isotonic saline. Male BALB/c mice were anesthetized and intratracheally instilled with 100 µg of PMs in 100 µl of isotonic saline, as previously reported [4]. After 3 h, 24 h or 1 week, mice were euthanized with an anesthetic mixture overdose (tiletamine/zolazepam-xylazine and isoflurane), the bronchoalveolar lavage fluid (BALF) collected and lungs excised for biochemical and histological analyses. Routine histology was performed by formalin fixation, paraffin embedding and Haematoxylin - Eosin (HE) staining. HO-1 and CYP1B1 immunohistochemistry was performed on lung sections by an indirect method using primary polyclonal antibodies (Santa Cruz Biotech, Inc, CA, USA) and a peroxidise based antigen retrieval system (Vectastain Elite ABC Kit), following the methodology already reported in [4].

RESULTS AND DISCUSSION

Chemical and microbiological characterization of seasonal- and size-fractionated PMs

The PM10 mean concentrations registered in winter and summer were 73.4 and 31.1 μg/m^3, respectively (data not shown). Figure 1a shows the characteristic variability in the chemical composition of PM10 and PM2.5 collected during summer and winter. Combustion-related primary compounds, such as PAHs, were higher in fine winter fractions (wPMs), while summer PMs (sPMs) were enriched in secondary organic aerosols (Figures 1a and 1b), and in crustal elements such as Fe, Al and Si (data not shown). Similar results were reported for other European cities, such as Dresden, Germany [5], and are explained by the presence of different sources, like the domestic heating in winter, and the meteorological condition, like a lower atmospheric mixing layer in winter and a drier weather in summer, this last factor determining a higher resuspension of crustal elements.

The relative abundance of bacteria in PM did not vary along the seasons, while the community structure did it both in the sampling season and the size fraction. In particular sPM10 samples were largely dominated by gram-negative groups (75% of the total bacterial community *vs*. 51% in winter), and in this fraction the highest endotoxin content (tot 24.7 EU/mg) was registered. The presence of adsorbed endotoxins in the coarse PM fraction has been previously reported [6,7] and some papers in literature suggested this component as responsible for the PM pro-inflammatory and cytotoxic effects [8,9].

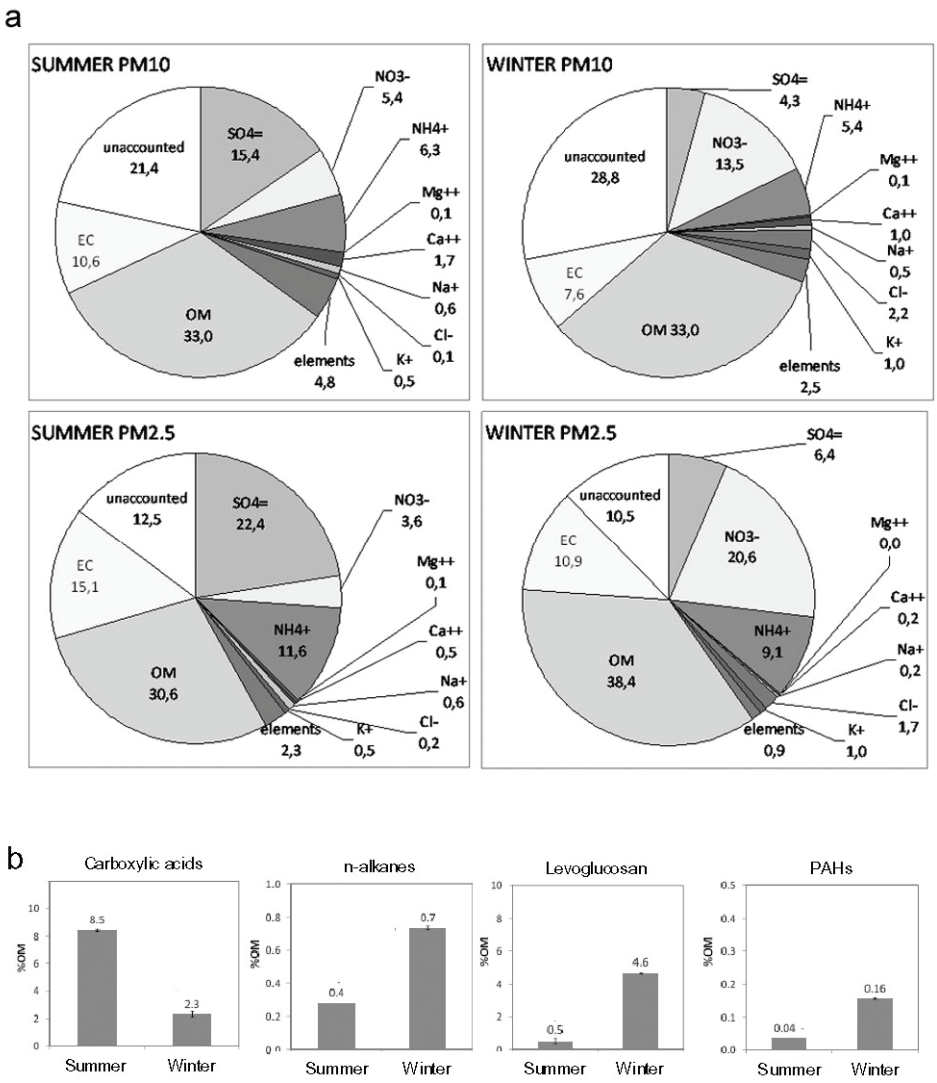

Figure 1. PM Characterization: a) chemical composition of summer and winter PM10 and PM2.5; b) histograms showing some specific primary (PAHs, levoglucosan and n-alkanes) and secondary (carboxylic acids) components of the OM in summer and winter PM2.5 (reported as mass% of OM); OM - organic matter; EC - elemental carbon.

In vitro and *in vivo* PM toxicity

In our study summer PM10, containing abundant endotoxins from gram-negative bacteria, resulted to be the most effective in term of cytotoxic and inflammatory potential both in human lung cells (Figure 2) and in mouse lungs (Figures 3 and 4). This fraction was also enriched by resuspended crustal elements, such as Si and Al, well known pro-inflammatory agents. Lung cells, exposed to summer PM10, actively synthesized pro-inflammatory cytokines, such as TNF-α and IL6, coupled with the expression of the defence protein heme-oxygenase-1 (HO-1). Alveolar macrophages, which clear particles by phagocytosis, activate the inflammatory response [10]. The higher inflammatory potential of summer particles has been previously reported [11] and the associated biological events correlate well with the induction of acute respiratory diseases, described in epidemiological studies [1]. Winter fine PMs did not produce significant inflammatory events.

As a consequence of the very low air circulation in the Po Valley, very high PM concentrations are usually present in Milan during winter. Fine wPMs are dominated by combustion-derived fine particles, enriched in PAHs and heavy toxic metals such as V, Cr, Zn and Cd (data not shown). These PMs induced genotoxic effects, such as cell cycle alteration (mitotic arrest) and apoptotic process activation in THP-1 cells (Figure 5), and a significant increase in the synthesis of xenobiotic metabolizing enzymes (like CYP1B1), in lung tissues (Figure 6). Besides, no acute inflammatory events occurred, suggesting possible genotoxic effects as a consequence of chronic exposure to high wPM concentrations. PAH-enriched fine wPMs resulted in fact more genotoxic than summer ones, as observed in human lung cells, where a large number of genes were modulated after wPM2.5 exposure. The modulated genes are mainly involved in the resistance to xenobiotics and some of these are even pro-oncogenic [12]. Our results are in line with many data in literature, which report a higher genotoxic potential of fine PM, principally originated from combustion processes [13].

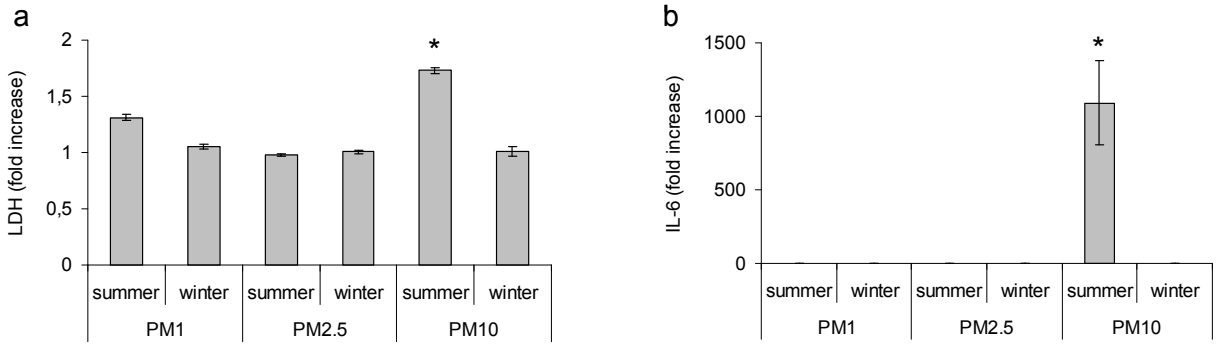

*Figure 2. Cytotoxic and inflammatory effects of PMs in A549/THP-1 co-culture system: a) cell viability measured by LDH release; b) release of the pro-inflammatory cytokine IL-6 (data are expressed as the mean ± SEM * Significant different from control (ANOVA; p <0 .05).*

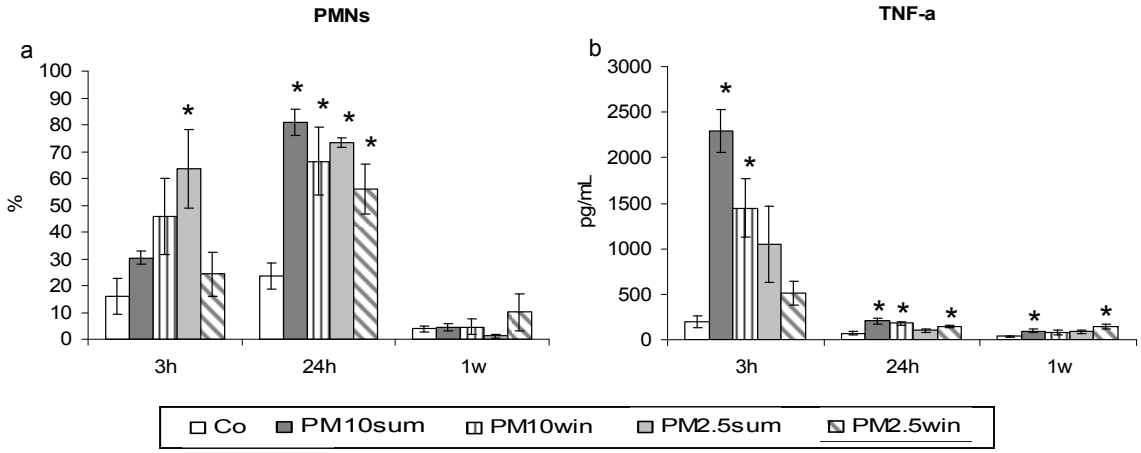

Figure 3. Pro-inflammatory markers in BALF. a) Differential cell count of Polimorphonuclear cells (PMNs), % of the total cells; b) concentration of the pro-inflammatory cytokine TNF-α.

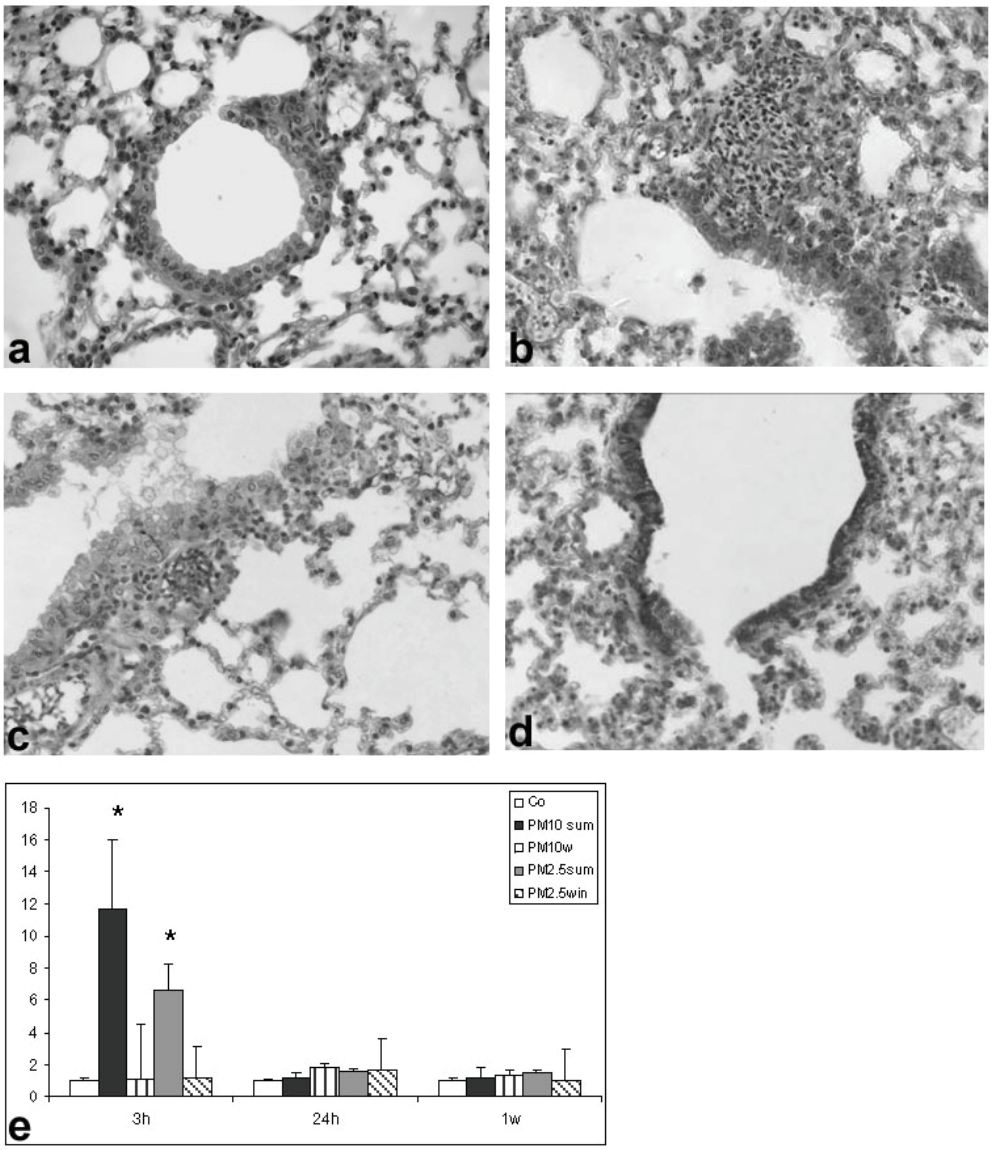

Figure 4. Histology of lungs and HO-1 expression in summer PM10 exposed lungs. a) Control lung section stained with HE; b) PM10 exposed lung showing inflammatory tissue in the peri-bronchiolar region; c) control lung after HO-1 immunohistochemical reaction (no or very weak signal); d) localization of HO-1 expression in bronchiolar epithelial cells (dark precipitate); e) Histogram showing the increased level of HO-1 expression.

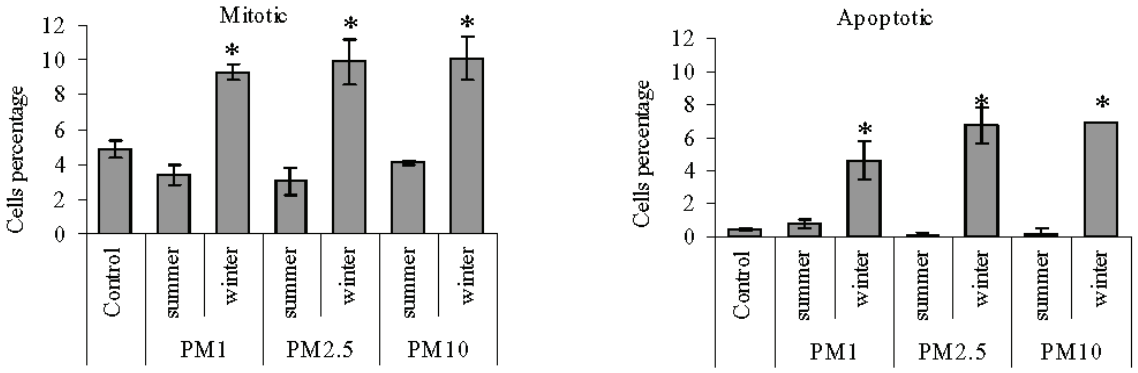

*Figure 5. Mitotic (a) and apoptotic (b) THP-1 cells as scored after Hoechst/PI staining. Data are expressed as mean ± SEM. * - Statistically significant difference from control (ANOVA; $p < 0.05$).*

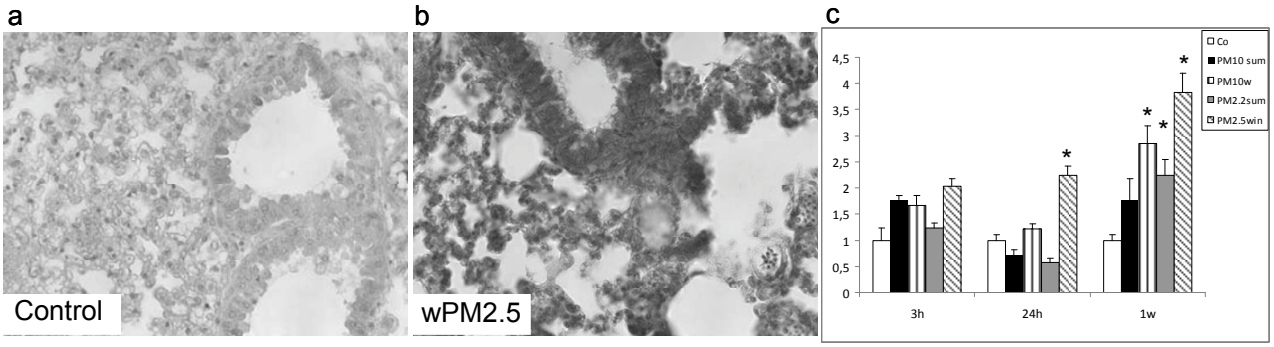

Figure 6. Immunohistochemical localization of the enzyme CYP1B1 in control (a) and winter PM2.5 (b) exposed lungs. c) Histogram showing the increased level of CYP1B1 expression in the lung parenchyma after exposure to winter PMs.

CONCLUSION

The respiratory risk associated with atmospheric PM pollution is still far from being completely clarified. The complex composition of the PMs, their toxic impact and the biological mechanisms evoked may orient the effects to a large number of results. Our data strongly confirm that the toxic potential of urban PM varies greatly according to the sampling period and to the particle size, strictly reflecting the PM chemical composition and sources. PM10 collected during the hot season resulted much more active in inducing acute inflammatory response, and also considering that the atmospheric PM concentration in winter usually is 2 to 4 times higher than in summer, we can affirm that the specific pro-inflammatory events promoted by sPM10 would not be activated (or at least not at similar degree) by a simple increase in the concentration of PM during cold season [14]. Similarly, the genotoxicity of wPMs is likely a result, not simply dependent upon the higher PM (and thus available PAHs) concentration during heating season, but also upon the different sources of particles from combustion processes, like fossil and wood burnings. These data point out the need for an even more detailed knowledge of PM composition and sources, parallel to the investigations of the toxicological profile of particles coming from the different sources contributing to the pollution of a specific region. It could help in the identification of the threshold values and the reliable molecular exposure markers able to describe human health risk.

Acknowledgement

This work was entirely sponsored by CARIPLO Foundation under the TOSCA project.

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NAUČNI RAD

PRETRAGA ODGOVARAJUĆIH MOLEKULARNIH MARKERA EKSPOZICIJE ZA IDENTIFIKOVANJE NEŽELJINIH BIOLOŠKIH EFEKATA RESPIRABILNIH ČESTICA PRISUTINIH U URBANOJ OBLASTI GRADA MILANA

U radu je prikazan rezime rezultata dobijenih u okviru koordinisanog naučnog projekta TOSCA, u kome je intenzivno analiziran uticaj respitabilnih čestica prikupljenih u urbanoj zoni grada Milana na zdravlje. U in vitro (na humanim ćelijama pluća) i in vivo (na miševima) sistemima ispitivani su molekularni markeri izloženosti i efekata frakcija respirabilnih čestica prikupljenih tokom različitih sezona (PM_{10} i $PM_{2.5}$ prikupljenih tokom leta i zime). Rezultati dobijeni analizom citotoksičnosti, proinfamatornih i genotoksočnih parametara pokazuju da je biološki odgovor u strogoj zavisnosti od sezone kada su respirabilne čestice prikpuljene i njihove veličine, od čega ustvari zavisi njihov hemijski sastav i poreklo. PM_{10} prikupljene tokom leta su obogaćene elementima prisutnim u zemljinoj kori i endotoksinima, i predstavljeju frakcije koje su najviše citotoksične i proinfalmatorne, dok $PM_{2.5}$ iz zimskog perioda indukuju genotoksični efekat i produkciju enzima koji metabolišu ksenobiotike (kao što je CYP1B1), što je najverovatnije posledica većeg sadržaja čestica poreklom od sagorevanja supstanci koje su bogate PAH-ovima i teškim metalima. Ovi rezultati ukazuju da je potrebno detaljno poznavanje fizičko-hemijskog sastava respirabilnih čestica na lokalanom nivou, jer to definiše biološki štetne efekte, a koji su pak u direktnoj vezi sa izloženošču respirablnim česticama. Očigledno je da je ovo jedini način koji omogućava naučnicima i kreatorima politike da uspostave logičan odnos između kvantiteta i kvaliteta respirabilnih čestica i zdravstvenog efekta koje opisuju kliničari i epidemiolozi.

Ključne reči: respirabilne čestice, marker toksičnosti, in vitro, in vivo.