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Biological effects of Milan PM: the role of particles dimension and season of sampling

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The biological effects induced in human pulmonary cells by atmospheric particles collected in Milan have been analysed according to their dimension and season of sampling. Summer and winter PM_1, $PM_{2.5}$ and PM_{10} were chemically characterized and used for exposure of pulmonary cultured cell lines. Cell viability, proinflammatory cytokine expression, cell cycle modifications and cytochrome P450 CYP1B1 expression were analysed. Chemical characterization showed that summer fractions had a higher endotoxin content and were enriched in metals while winter PMs in PAHs. In line with this aspect, summer PM_{10} induced stronger cytotoxic and inflammatory effects in comparison with the other fractions. Winter fine PMs provoked a cell cycle arrest in G_2/M phases and remarkable cytochrome P450 CYP1B1 activation, suggesting a potential genotoxic effect.

1. Introduction

Particulate matter (PM) has been unanimously recognized as a hazardous air pollutant whose effects on human health are well known. All the existing epidemiological data agree in describing consistent associations between high PM concentration and increase in mortality and morbidity in the population (Englert, 2004). The Po Valley, and particularly the city of Milan, present high concentrations of air pollutants due to their geomorphological and meteorological characteristic. In Milan the annual mean of PM_{10} is measured in 56 $\mu g/m^3$, with maximum daily peaks over 200 $\mu g/m^3$ (ARPA Lombardia, 2006), clearly exceeding the annual and daily mean EU limits of 40 $\mu g/m^3$ and 50 $\mu g/m^3$ respectively (EU air quality directive 99/30/CE). PM is a complex mixture of particles with different size and chemical composition. According to the criteria of particle size, it can be classified as PM_{10} (particles with an aerodynamic diameter less than 10μm), $PM_{2.5}$ (Ø<2.5μm) and PM_1 (Ø<1μm). The fine fractions ($PM_{2.5}$ and PM_1) are dominated by combustion derived particles, consisting of organic and inorganic compounds adsorbed onto the surface of a carbonaceous core (Zerbi et al., 2008). The coarse fraction (PM_{10}) contains a major part of mineral compounds and some adsorbed elements, such as endotoxins (Pérez et al., 2007). Seasonality is another critical parameter for particle chemical composition, due to the different photochemical and meteorological conditions and to the kind of human emissions. The different toxic

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responses are supposed to be linked to the PM chemical characteristics. For these reasons, the aim of this research is to evaluate the toxicity of Milan PMs, by a comparative study of summer and winter PM_1, $PM_{2.5}$ and PM_{10}, to highlight possible differences in the mechanisms of action that ultimately will be helpful in understanding the effects on human health. After being chemically characterized, the PM fractions were tested on human pulmonary cell lines to analyse PM-related effects on cells. Since the mechanisms elicited by the interaction between particles and pulmonary cells remain an uncertain question, a first attempt to study toxicity pathways was done, by evaluating the expression of the cytochrome P450 (CYP1B1).

2. Methods

2.1 PM sampling and preparation

PM_1, $PM_{2.5}$ and PM_{10} samples were collected at the urban site Torre Sarca (Milan), and processed for cell culture experiments as previously reported (Gualtieri et al., 2009).

2.2 Chemical characterization

PM samples were chemically characterized for inorganic ions, elemental and organic carbon, elements (mineral dust and trace elements), and polycyclic aromatic hydrocarbons (PAHs) as reported in Bolzacchini et al., 2002. Endotoxin content was determined by Limulus amebocyte lysate (LAL) test accordingly to manufacturer instructions (Associates of Cape Cod, Inc., East Falmouth, MA).

2.3 Cell culture and treatment

The human lung epithelial carcinoma cell line, A549, and the human acute monocytic leukemia cell line, THP-1 (American Tissue Type Culture Collection, ATCC, Rockville, MD, USA) were routinely maintained in OptiMEM medium at pH 7.2, 5% CO_2 and 37°C. A549 cells were treated after 24 hours from seeding with 10 $\mu g/cm^2$ of summer and winter PMs. THP-1 cells were treated immediately after seeded with 10 or 25 $\mu g/cm^2$ of summer and winter PMs.

2.4 Cell viability, inflammatory response, cell cycle and cytochrome expression

Particles exposure was extended for 24 hours before performing experiments. Cell viability was determined by lactate dehydrogenase (LDH) assay that provides an indirect evaluation of cell membrane integrity. LDH release in the culture medium has been evaluated accordingly to manufacturer instructions (Lactic Dehydrogenase Assay Kit, Sigma-Aldrich, Inc., St Louis, MO, USA). Cytokine release is a marker of pro-inflammatory response in cells. Interleukine IL-6, IL-8 and IL-1β levels were determined in culture medium by sandwich ELISA according to the manufacturer's guidelines. Absorbance was measured by a Multiskan Ascent multiplate reader (Thermo Scientific, Inc.). Cell cycle analysis were carried out to investigate genotoxicity in cells exposed to PMs. The fluorescent dye propidium iodide (PI) was used to stain DNA of cells and fluorescence was measured by EPICS XL-MCL (Beckman-Coulter) flow cytometer. Quantitative western blot analysis were performed to determine cytochrome P450 CYP1B1 expression (primary CYP1B1 polyclonal antibody, Santa Cruz). Benzo[a]pyrene 14μM was used as positive control. Densitometric quantification was performed with densitometer GEL DOC 2000 (BIO-RAD).

3. Results

3.1 PM characterization

PM composition is strongly affected by seasonality. Water-soluble inorganic ions were the major chemical fraction of PM, Nitrate (NO_3^-) was the most abundant ion in winter season, while sulphate (SO_4^{--}) was prevalent in summer (data not shown). Moreover summer fractions were enriched in metals while winter PMs showed a greater contribution of PAHs (Table 1). Summer PMs presented endotoxin content higher than the winter ones (Table. 1). Furthermore endotoxin content was directly related to the PM fraction analysed, since PM_{10} showed the highest values, while PM_1 the lowest.

3.2 Cell viability

Viability, analysed by LDH test, was not affected in A549 cells treated with summer and winter PMs at 10μg/cm^2 (Fig.1A), while it was significantly reduced in THP-1 cells exposed to PM_{10} (especially summer) and winter $PM_{2.5}$ (Fig. 1B).

Table 1 Chemical characterization of PM fractions. Polycyclic aromatic hydrocarbons (PAHs) and elements (Al, As, Ba, Cd, Cr, Cu, Fe, Mn, Mo, Ni, Pb, V, Zn) values are expressed as mean mass percentage ± SD. Endotoxin content is expressed as mean EU/mg ± SD.

	PM_1		$PM_{2.5}$		PM_{10}	
	Summer	Winter	Summer	Winter	Summer	Winter
PAHs	0.02±0.00	0.37±0.01	0.04±0.00	0.16±0.01	0.03±0.00	0.21±0.02
Elements	2.85±1.52	0.53±0.24	2.26±0.65	0.89±0.52	4.77±1.8	2.52±0.75
Endotoxins	13.6±0.63	2.8±0.15	17.6±0.22	11.29±0.03	25.9±0.63	16.87±0.91

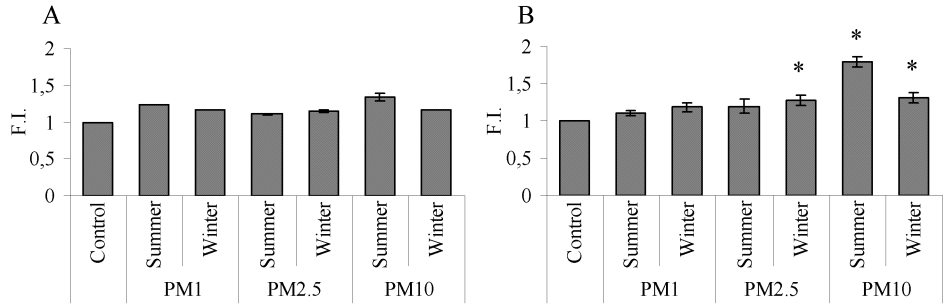

*Fig. 1: A549 (A) and THP-1 (B) cell viability measured by LDH release after 24h exposure to 10μg/cm^2 of PM fractions. Data are expressed as fold increase with respect to control. * Statistical different from control at p<0,05.*

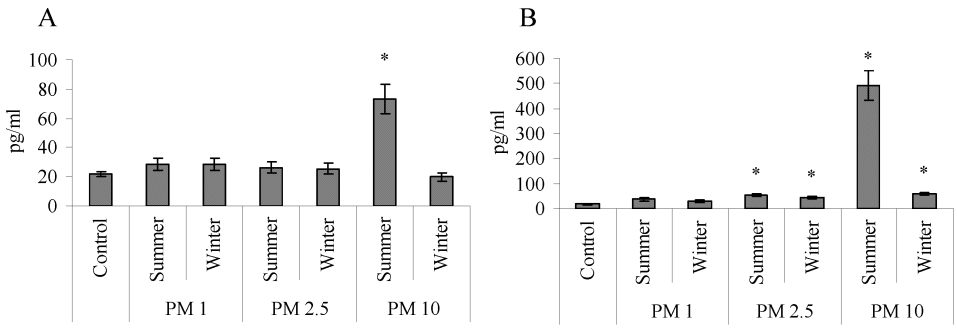

*Fig. 2: IL-8 release in A549 (A) and THP-1 (B) cells after 24h exposure to 10µg/cm² of PM fractions. * Statistical different from control at p<0,05. Note the differences in the Y scales.*

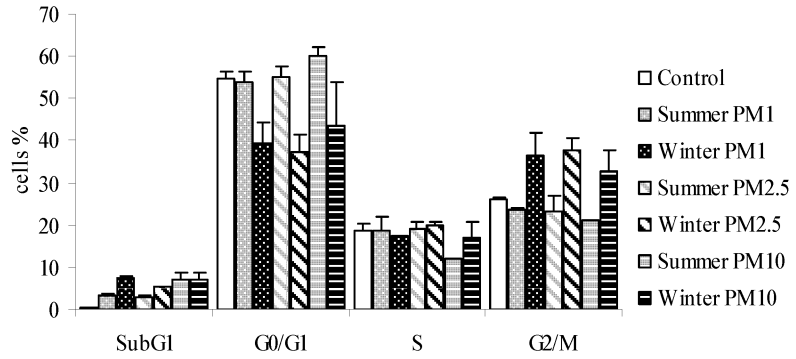

Fig. 3: THP-1 cell cycle after 24h exposure to 25µg/cm² of PM fractions.

3.3 Inflammatory response

In A549 cells, IL-6 and IL-1β production did not increase after treatment with any PM fraction (data not shown), while IL-8 was slightly but significantly increased by summer PM_{10} exposure (Fig. 2A). In THP-1 cells, IL-8 registered a significant increase after exposure to summer and winter $PM_{2.5}$ and PM_{10} (Fig. 2B). In particular, summer PM_{10} triggered the major response inducing IL-8 increase up to 492pg/ml versus 17.4pg/ml of controls. IL-6 and IL-1β behaved similarly, with significant induction after summer PM_{10} treatment, which showed values of 58.7 and 127.5pg/ml respectively versus 0.3 and 0.9pg/ml in controls (data not shown).

3.4 Cell cycle

A549 cells exposed to 10µg/cm² PMs did not show altered cell cycle, while THP-1 did, with a slight decrease in S-phase cell percentage after summer PM_{10} treatment and an increase of cell percentage in G_2/M phases after exposure to winter PM_1 (data not shown). In order to strengthen these findings, THP-1 cells were exposed to a higher PM dose (25µg/cm²). As evidenced in Fig. 3, all winter PMs caused a cell cycle arrest in G_2/M phases. Concomitantly, the effect of summer PM_{10} was amplified at this dose of treatment, with cells in S-phase falling to 12% from 18.7% of control.

Table 2 Quantitative western blot analysis of cytochrome P450 CYP1B1 expression in A549 cells exposed to 10μg/cm^2 of summer and winter $PM_{2.5}$ and to Benzo[a]pyrene (B[a]P) 14μM. Values are reported as ODu/mm^2.

	Control	Summer $PM_{2.5}$	Winter $PM_{2.5}$	B[a]P
CYP1B1	0.52	2.81	3.18	4.81
Tubulin	0.58	0.61	0.58	0.61

3.5 Cytochrome expression

Since PAH-enriched winter PMs seemed to induce genotoxic effects, the expression of the enzyme CYP1B1, involved in the methabolism of such compounds, was analyzed. As shown in Table 2, densitometry of western blot gave positive results, revealing the presence of a major quantity of CYP1B1 in cells exposed to winter $PM_{2.5}$ when compared to both control and summer $PM_{2.5}$.

4. Discussion

Summer and winter PM fractions showed a deep difference in chemical composition and endotoxin content, the latter being higher in summer PM_{10}. These findings can explain the different toxic effects observed. Indeed summer PM_{10} revealed a higher capacity of triggering cytotoxic and pro-inflammatory effects both on THP-1 and A549 cells, since it induced cell death and interleukin release. Other experimental and epidemiological studies suggest that the coarse fraction may have higher pro-inflammatory potential than the fine ones (Camatini et al., 2010; Hetland et al., 2005). Moreover it has been demonstrated that summer and springtime PM induced stronger cytokine responses than winter PM in primary alveolar macrophages and RAW 264.7 cells (Hetland et al., 2005). Endotoxins have been associated with the inflammatory effects of PM both *in vitro* and *in vivo* and it has been demonstrated that the IL-8 release induced by summer PM_{10} was significantly mediated by endotoxins (Gualtieri et al., 2010; Camatini et al., 2010). Moreover chemical characterization showed that PM_{10} contained more crustal elements than fine fractions, and these compounds also can contribute to the inflammatory potential of this PM fraction (Øvrevik et al., 2005). On the other side winter PMs showed a potential genotoxic effect on both THP-1 and A549 cells with cell cycle arrest in G_2/M phases and increased expression of cytochrome CYP1B1 as major events. Gualtieri et al. (2010) already reported an increase in mitotic-arrested BEAS-2B cells after fine PM exposure, supporting the present results obtained in THP-1 cells. Cell cycle arrest has been associated with DNA damage (Huang et al., 2005), that is likely associable to the relatively high PAH concentration in winter fine PMs. Indeed, PAHs are notorious inducers of DNA-adduct and oxidative DNA damages. Since A549 cells have been reported to express inducible forms of cytochrome P450 family proteins, the increased expression of cytochrome CYP1B1, caused by winter PMs, can be considered a valuable molecular marker of exposure. This study showed that the variability of biological effects induced by PM from Milan depends primarily on the season of sampling while the aerodynamic diameter of particles has a lower impact. Summer PM_{10} was more cytotoxic than the other sampled fractions, although winter fine PMs triggered cell cycle arrest in G_2/M phases and

cytochromes P450 activation. The perspectives of this research are oriented to the analysis of the possible involvement of toll-like receptors (TLR-2/TLR-4) in summer PM_{10}-induced inflammation. A further investigation will involve the role of the cell cycle regulatory proteins (Chk and CDK) and the mitotic spindle machinery to explain the winter PMs-induced cell cycle alteration.

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