Fine PM and health: *in vitro* results

M. Gualtieri*, P. Mantecca*,V. Corvaja*, E. Bolzacchini*, G. Fustella†, G. Zerbi†, M. Camatini*

*Centro di Ricerca POLARIS, Dipartimento di Scienze dell'Ambiente e del Territorio, Università degli Studi di Milano Bicocca, Piazza della Scienza 1 - 20126 Milano

† Dipartimento di Chimica, Materiali ed Ingegneria Chimica "Giulio Natta", Politecnico di Milano, Piazza L. Da Vinci 32 - 20133 Milano

Numerous studies on ambient particulate matter provide evidences of the role of its components in release of inflammatory cytokines and cell toxicity in lung cultured cells. However the relative importance of different size fractions to induce such effects is still unclear. We investigated the potency of the fine fraction (PM2.5), collected during winter in Milano at the urban site Torre Sarca, on the human alveolar cell line A549.

Chemical composition and morphology of the collected particles were analysed and the effect evaluated on A549 cells exposed to 1, 10, 25, 50 μg/cm^2 up to 24 h. Cell viability, interleukins (IL-6, IL-8) expression , DNA damage and reactive oxygen species (ROS) induction were investigated. Moreover Raman spectra of particles internalised in A549 cells was analysed.

1 Introduction

The adverse effects of ambient PM on human health have been a concern for decades and attempts to provide correlation between adverse health effects and PM properties have significantly increased (Englert, 2004). At present, even the mechanisms of PM-related adverse health effects are still incompletely understood, a good improvement in the knowledge of the health effect may derives from the evaluation of the mechanisms of action at cell level.

Numerous data exist on the chemical properties of the Po Valley PM pollution (Bolzacchini et al, 2002; Fermo et al., 2003, Marcazzan et al., 2003; Lonati et al., 2007), while no literature is available on its biological activity. Aerosol monitoring reports indicated the Po Valley as one of the most PM polluted area in Europe, outlining enhanced PM levels around Milan (Koelemeijer et al., 2006, Lonati et al.,2007). Recently PM2.5 fraction, which easily reach and interact with the human pulmonary alveoli, has attracted investigations oriented to human pulmonary cell lines (Don Porto Carero et al., 2001; Hetland et al., 2004; Choi et al., 2004; Schwarze et al., 2006; De Vizcaya-Ruiz et al., 2006; Billet et al., 2007; Gualtieri et al., 2008).

The main aim of this research was to investigate for the first time the cytotoxic and genotoxic activity of winter PM2.5 in A549 exposed cells. The results here presented are thus the first, almost at our knowledge, describing the toxicity of the winter PM2.5 fraction chemically and physically characterized collected in Milano on the A549 cell line, successfully used also in particles' toxicity tests

2 Methods

2.1 PM 2.5 collection and preparation

PM2.5 particles were collected from November to February. 2005/2006 with a low volume gravimetric sampler (Teflon filters) in Milan ("Torre Sarca" site University of

Milano-Bicocca) a site mainly influenced by urban traffic. Teflon filters were processed as reported (Fermo et al., 2003).

2.2 PM2.5 chemical characterization

Inorganic ions were determined by ionic chromatography, PAHs were analysed by HPLC-RF, Elemental and Organic carbon were analysed by Thermal Optical Transmission (TOT), elements were detected by X-Ray Fluorescence (XRF) (Bolzacchini et al., 2002).

2.3 Raman spectra

Raman spectra of particles internalised in the cells were recorded using a Horiba Labram 800 spectrometer equipped with an Olympus BX 41 microscope. The 785 nm line of a solid state laser was used for the excitation. The laser beam was filtered in order to prevent damage of the sample. The slide with A549 cells was directly put on the stage of the microscope under a 50X objective.

2.4 Particles' extraction for cell toxicity assay

Detached particles were dried and suspended in sterile water to obtain aliquots at a final concentration of 4µg/µl. These were stored at -20°C until use.

2.5 Cell culture and treatments

A549 cells (American Type Culture collection) were routinely maintained in OptiMEM medium at pH 7.2, according the procedure reported (Gualtieri et al., 2005). After 24h from seeding the cells were treated with PM2.5 suspensions obtained by multiple dilutions of the thawed particle aliquots to reach the final doses of 1, 10, 25 µg/cm^2. Particles exposure was extended for 3h in ROS experiments otherwise 24h. Three independent experiments were carried out.

2.6 Cell viability

MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was used to measure cell proliferation following the standardized procedure. After 24h of treatment cells were incubated with MTT, at the final concentration 0.5 mg/ml for 4h. The absorbance of 300µl of each sample was assessed by Multiskan Ascent (Thermo Scientific Inc) at 565nm.

2.7 Reactive Oxygen Species (ROS) induction

A549 cells were seeded on sterilized glass slips in 8cm^2 plastic Petri, and after PM treatment were incubated with 20µM 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA) in PBS for 30 min. Then cells were processed as reported (Gualtieri et al.2008) The presence of ROS resulted in the oxidation of DCFH to the florescent DCF. Glass slides, were observed under Zeiss Axioplan fluorescence microscope.

2.8 Analysis of cytokines

Quantitative determination of the cytokines IL-6 and IL-8 in the culture medium from exposed A549 cells was performed using ELISA (Hetland et al., 2004). The analyses were performed according to the manufacturers manuals. Increase in colour intensity was quantified using a Multiskan Ascent multiplate reader (Thermo Scientific Inc).

2.9 Comet assay

The alkaline comet assay was performed as previously described (Gualtieri et al., 2005). To assess cell distribution, 400 cells per slide were considered and according to the tail length they were divided in undamaged and damaged cells.

3 Results

3.1 PM2.5 chemical characterization

PM2.5 chemical composition is presented in Fig..1). Nitrate is the most abundant element (26% of the total PM2.5 mass), as usual in winter period. EC is a limited fraction of PM2.5 mass (4%). It is a primary pollutant , deriving from combustion processes mainly from diesel vehicles. OC (22.6 % of the mass) is a wide class, with heterogeneous chemicals. The sum of the eight PAHs analysed is 0.03 % of the mass (Tab..1) indicating a high PAHs content, This aspects have a clear toxicological importance as they indicate a different.

Elements (n= 25) detected by XRF represent 4% of the total mass Al, Si, K, Ca, Ti are elements from mineral dust (Bolzacchini et el., 2004). Their sum constitutes only 0.76 % of total PM2.5 mass, as they are mainly present in the PM coarse fraction (Vecchi et al., 2004). The other elements are influenced by different anthropic sources, traffic combustion oil, ecc., Heavy metal oxides (Fe, Cu, Zn, PB) result to be relevant for their potential toxicity

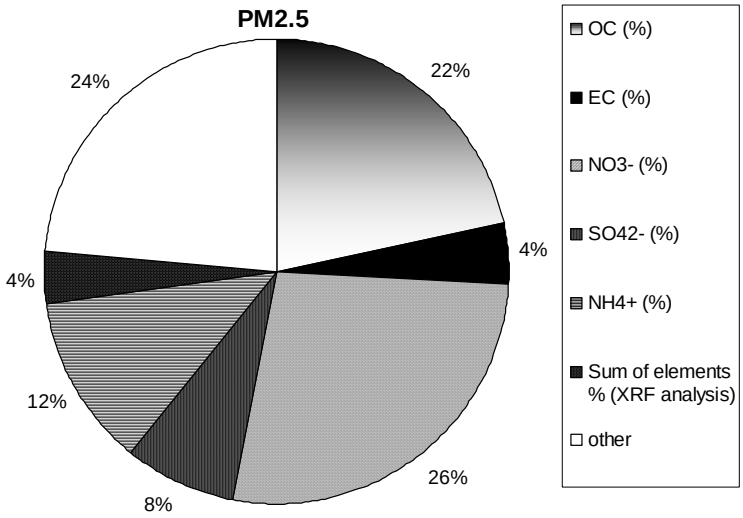


FIG. 1 PM2.5 chemical composition for the Milan urban area during winter season.
Sum of elements (XRF analysis) = Mg, Al, Si, P, Cl, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Br, Sr, Sn, Ba, Pb, Bi

TAB.1 PAHs concentrations

	BaA	Cr	BeP	BbF	BkF	BaP	BghiP	IcdP	Σ 8 PAHs
ng/m^3	2.47	3.71	4.64	3.53	1.58	2.30	3.32	2.68	19.44
% PM2.5 mass	0.003	0.005	0.006	0.005	0.002	0.003	0.005	0.004	0.033

TAB.2 Elements concentrations (XRF analysis)

	Mg	Al	Si	P	Cl	K	Ca	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	As	Se	Br	Sr	Sn	Ba	Pb	Bi
ng/m^3	58.9	137.6	134.4	235.9	427.7	1206.1	126.7	25.8	7.9	7.7	16.9	399.4	6.1	136.4	34.0	125.6	9.0	8.9	3.2	20.1	18.2	13.7	84.2	103.4	5.9
% PM2.5 mass	0.072	0.155	0.160	0.264	0.447	1.334	0.151	0.029	0.009	0.009	0.019	0.467	0.007	0.161	0.039	0.141	0.009	0.009	0.003	0.023	0.017	0.016	0.091	0.108	0.006

3.2 PM 2.5 cytotoxic effect on A549 cells

The cytotoxic effect of PM 2.5 was evaluated by MTT reduction assay (Fig. 2). Treated cells presented a dose dependent reduction in viability and a statistically significant reduction was apparent within 24 h.

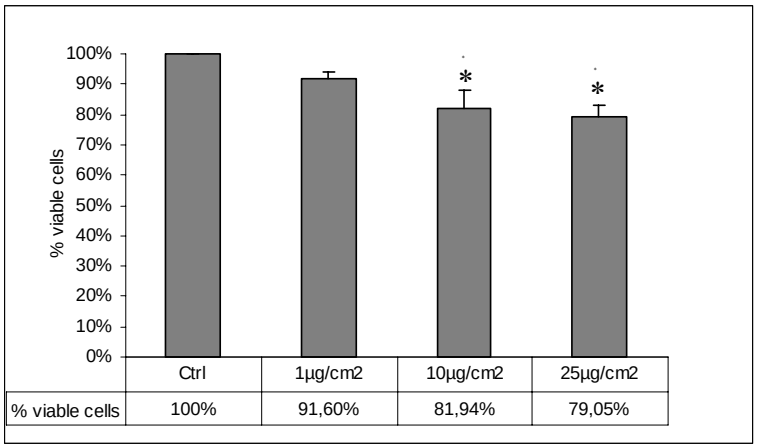


*Fig. 2 Decrease in cell viability, assessed by MTT assay, is evident after 24hrs of treatment. The decrease in dose dependent. * Statistical different from control at p<0,05*

3.3 Release of proinflammatory cytokines

A slight dose-dependent IL-8 induction was observed after winter PM2.5 treatment (Fig. 3) while no IL-6 induction was observed (data not shown).

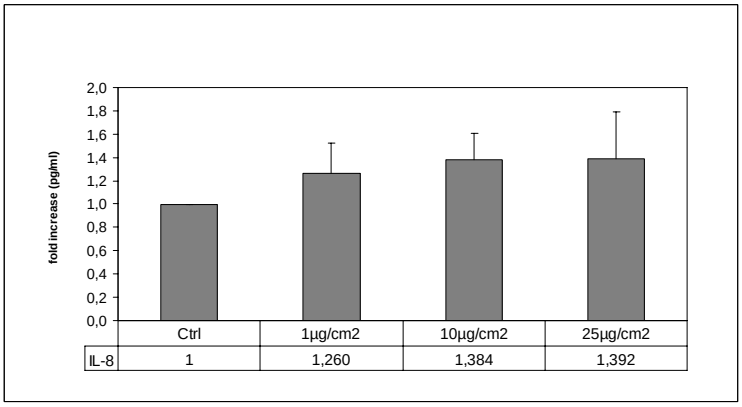


Fig. 3 Winter PM 2.5 induce a slight increase in IL-8 release in A549 cell line. The increase is dose dependent even if there is not statistical differences between treated samples and control.

3.4 ROS generation

Treated cells presented fluorescent spots distributed within the cytoplasm (Fig. 4), which apparently numerically increased at increasing doses. At the highest dose, practically all cells showed ROS derived spots. Control cells did not present fluorescent signals.

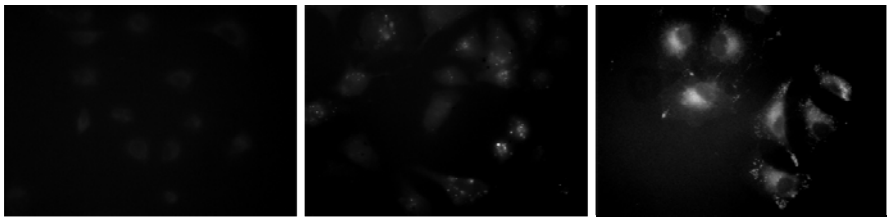
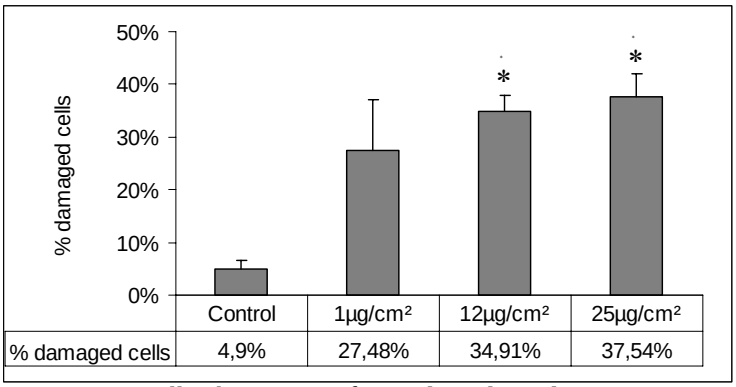


Fig. 4 Winter PM 2.5 induce a dose dependent increase in ROS formation after 3hrs of treatment.

3.5 DNA damage

Results are shown in Fig. 5, where a significant increase in DNA strand breakage correlates with the increase of the doses used. At the dose 25 ug/cm^2 there is a significant increase in % of damaged cells, in comparison with control.



	Control	1µg/cm²	12µg/cm²	25µg/cm²
% damaged cells	4,9%	27,48%	34,91%	37,54%

*Fig 5. A549 cells show a significant dose dependent increase in DNA single strand breaks as evidenced by the Comet Assay. * Statistical different from control at p<0,05*

4 Conclusions

The knowledge of the physical and chemical characteristics of PM, which depend from the sources, the emission intensity and the molecular interactions between inorganic and organic components, constitutes the starting point for the evaluation of its toxicity (Englert,2004) . Nevertheless, the physical and chemical properties of PM air pollution are unsatisfactory to predict its biological reactivity (Baulig et al., 2004). Moreover time and regional differences in PM concentration and composition are well established. In order to fulfill this uncertainness, the present paper evaluates the *in vitro* toxicity of winter PM2.5 collected at an heavy traffic site in Milan. The sampling site is thus representative of an area with a PM 2.5 concentration (Bolzacchini et al.,2002; Marcazzan et al., 2003; Lonati et al., 2007) higher than that reported for other major European cities (Querol et al., 2004). Beside this, the fine PM chemical composition is similar to that reported ,even PAHs levels are higher than the ones usually reported.

The results obtained demonstrate the high potential of PM2.5 to induce *in vitro* cell damage, both in term of viability and lesions at different cell levels. Comet assay evidenced an increase of cells with DNA damage, and within three hours of treatment ROS expression was significantly augmented. IL-8 expression was influenced by PM2,5 even if the results are not statistically significant. It is well known that PM2.5 generates metal-catalyzed reactive oxygen species (Hetland et al, 2004; Schwarze et al., 2006) and thus it is believed to cause oxidative damage to DNA (Sharma et al., 2007). Nevertheless contrasting evidences are reported in the literature concerning the correlation between DNA damage and the role of transition metals and the one of total PAHs (Gutierrez-Castillo, 2006). From our results it can be supposed that both metals and PAHs may contribute equally to DNA damage. In summary particles collected in winter at a traffic site in Milan were citotoxic and genotoxic. Nevertheless the results here presented need a deeper comparison with the data in the literature, presenting chemical and toxicological results in similar *in vitro* systems.
Raman spectra of pristine and internalised particulate matter show some differences (see Zerbi et al., 2008). In particular, a downshift of the frequency of the G peak of the spectrum of internalised PM is observed, whereas the D region in only weakly affected. In order to better understand the possible interactions between the particulate matter and the biological environment more investigations are required.

Acknowledgments
This research has been supported by the project "VESPA" (2007-2009), financed by Lombardia Region, Provincia and Comune of Milan and by the strategic project PROLIFE 2007-2008 of the Comune of Milan to the Research Center POLARIS

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