

Particles and Health: State of the Research

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1. State of the art

Evidence of the health effects of air pollution at levels currently common in Europe has grown stronger over the past few years, and suggests to recommend further policy action to reduce emissions of particulate matter, ozone, and nitrogen dioxide (Pope, 2000; Pope et al., 2009; Anderson, 2009). Particle pollution is made up of a number of components, including acids (such as nitrates and sulfates), organic chemicals, metals, and soil or dust particles. The size of particles is directly linked to their potential for causing health problems and research evidences that particle size is an important factor that influences how particles deposit in the respiratory tract and affect human health (Anderson et al., 2005; Davidson et al., 2005; Donaldson et al., 2005a; Englert, 2004; Graff et al., 2009). "Inhalable coarse particles" larger than 2.5 micrometers and smaller than 10 micrometers in diameter (PM₁₀), such as those found near roadways and dusty industries are deposited almost exclusively in the nose and throat; "Fine particles," 2.5 micrometers in diameter (PM_{2.5}) and smaller (ultrafine, UFP), such as those found in smoke and haze can be directly emitted from sources such as forest fires, combustion processes, incinerators or they can form when gases emitted from power plants, industries and automobiles react in the air, are able to penetrate to deep areas of the lung. Fine and ultrafine particles are present in greater numbers, have greater surface area than coarse particles, and they are generally considered to be more toxic as they can affect the heart and lungs and cause serious health effects. These effects are likely to depend on several factors, including the size and composition (the presence of metals, PAHs, other organic components, or certain toxins, the level and duration of exposure, and age and sensitivity of the exposed person. The documented toxicity of ultrafine particulates serves as an indicator for the potential hazards associated with engineered nanoparticles (NPs) (Kahru and Dubourguier, 2010). Indeed in the literature the term "ultrafine particles" can be somehow exchanged for nanoparticles, as these particles are manufactured industrially while the UFP should be generally considered as environmental pollutant. Nanotechnology has generated substantial interest for NPs to be incorporated into a wide variety of products including electronics, food, clothing, medicines, cosmetics and sporting equipment. While there is general recognition that nanotechnology has the potential to advance science, quality of life and to generate substantial financial gains, NPs potential toxicity should be considered in order to allow the safe and sustainable development of such products. The release of NPs into the

environment including air, water, and soil, has obvious health implications (Stone et al., 2007), considering the consistent epidemiological findings on respirable particles such as PM and UFP toxicity. Research on respirable air pollution particles has started to focus on the role of UFP (diameter less than 100 nm) in inducing adverse effects such as oxidative stress leading to inflammation and resulting in exacerbation of pre-existing respiratory and cardiovascular disease. In vivo and in vitro toxicology studies confirm that ultrafine particles of titanium oxide (TiO₂) and carbon black are more toxic and inflammogenic than fine particles of the same material. These particles generate reactive oxygen species (ROS) to a greater extent than larger particles leading to increased transcription of pro-inflammatory mediators via intracellular signalling pathways.

2. Milan PM

2.1 *In vitro/in vivo* approach

The Lombardy region is heavily polluted with PM and the yearly average PM 10 concentration measured is $44 \pm 4 \mu\text{g}/\text{m}^3$ breaching the yearly average EU Air Quality Limit of $40 \mu\text{g}/\text{m}^3$. PM10 is characterized by the presence of inorganic pollutants (such as nitrates and sulphates) and organic matter. During frequent peak pollution episodes in the heating season, the mass of these substances alone is sufficient to infringe the 24 h EU Air Quality Limit of $50 \mu\text{g PM}_{10}/\text{m}^3$. Risk assessment indicated that nitrate and organic carbon at the present levels may cause adverse health effects in the living population. The biological effects of PM10, PM2.5 and PM1, sampled in Milan in 2009 during summer and winter seasons, were analysed on in vitro (the alveolar epithelial cell line A549 and in THP-1 derived macrophages) and in vivo (intratracheal instillation of mice) systems. The microbiological and chemical characterization performed (Perrone et al., 2010) evidenced substantial differences mainly related to the season of sampling (Camatini et al., 2009; Gualtieri et al., 2009, 2010). A correlation between these differences and the biological effects produced on the systems investigated has been found.

Chemical characterization (Fig. 1) revealed specific differences between the summer and winter PM samples. Summer PMs have a higher content of elements and sulphates (SO₄). The total organic matter is not different among winter and summer samples, nevertheless winter PMs have a higher content of PAHs, specifically related to the contribution of the combustion processes, and nitrates (NO₃-).

The quantification of endotoxins (Fig. 2) on the other hands revealed a higher content of these components in summer samples and especially in summer PM10.

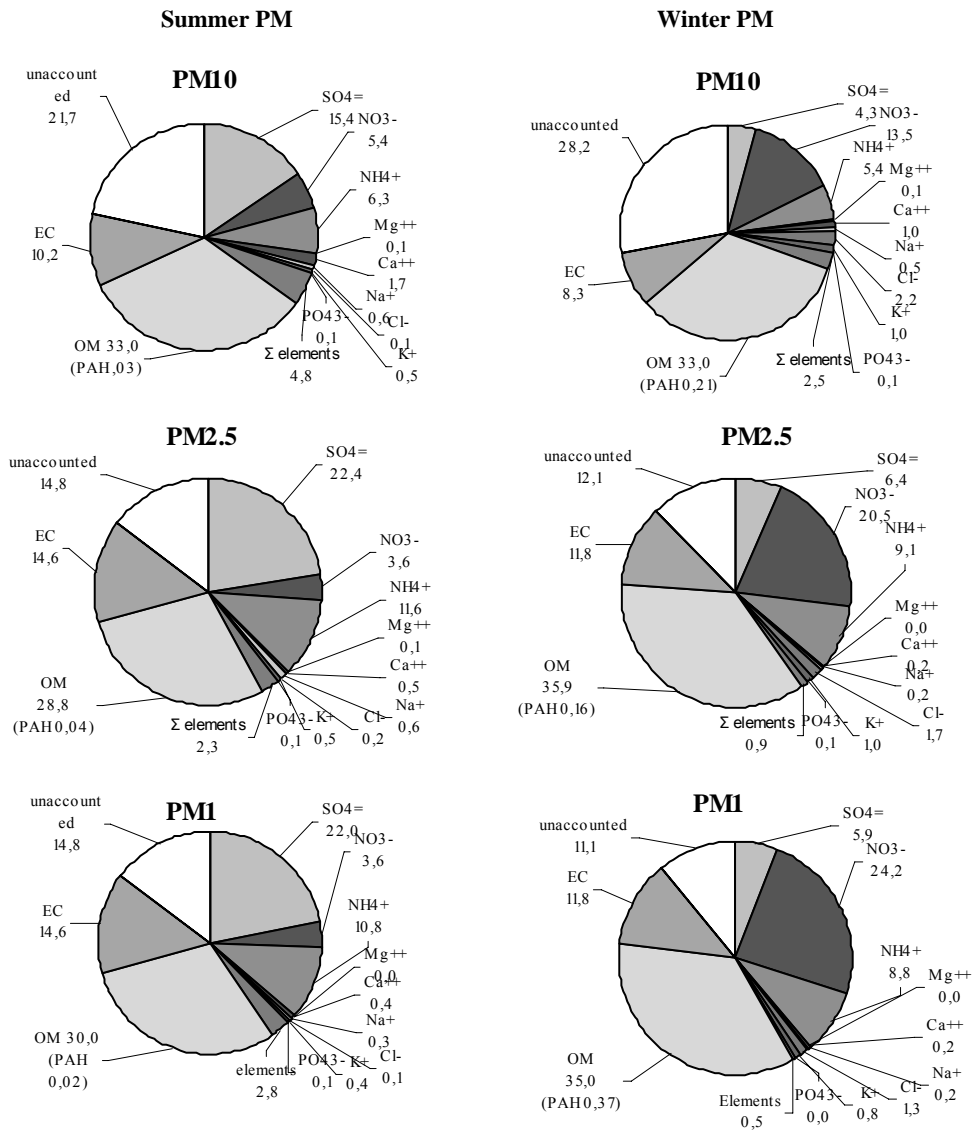


Fig 1. Chemical characterization of summer and winter PMs. Intra-season differences are low in term of total mass. The differences are much more evident comparing PM fractions of the different seasons. In summer sulphates are higher than in winter where the nitrates predominate. Although the total mass of organic matter (OM) is similar in summer and winter, nevertheless in winter the soot emission related compounds are predominant while in summer the contribution of biogenic OM are more consistent.

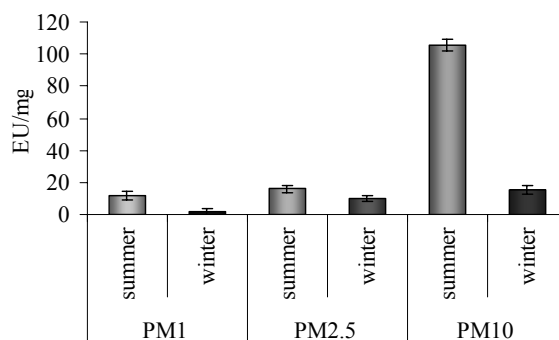


Fig 2. Endotoxin content in summer and winter PMs. Summer PM10 has the highest content of endotoxin as revealed by the limulus amoebocyte lysate (LAL) test, thus supporting the important role of biogenic OM in summer fractions.

2.2 In vitro results

In vitro experiments were performed on cells representative of the human lung epithelium. The doses used were not directly related to those of human exposure, but chosen to represent the mean PM quantity of air inhaled daily by a healthy human taking into account the clearance processes and the amount of deposition rates for the different dimensional fractions. The PM10 quantity supposed to reach the alveoli was $25\mu\text{g}/\text{day}$, and this quantity ($25\mu\text{g}/\text{cm}^2$) was chosen as the highest dose to be used in the *in vitro* experiments.

Cell viability is the first outcome analysed in defining the hazardous effect of a pollutant. PM chemical characterization evidenced its complexity and seasonal variability, suggesting potential differences in the biological effects produced. Cell viability was more affected by the summer PM fractions (Fig. 3) than the winter ones.

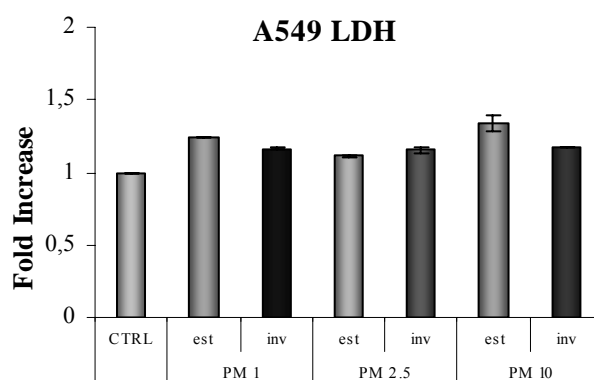


Fig 3. Cell viability of A549 cells exposed to summer and winter PMs.

The summer PMs reduction in cell viability was accompanied by a higher inflammatory effect, as demonstrated by the augmented level of pro-inflammatory proteins IL-1 β and IL-8, illustrated in Figs 4 and 5. Both IL-1 β and IL-8 are important mediator of the inflammatory response and may induce the release of other interleukins in different cell types. Summer PM10 induced a significant release of IL-1 β in THP-1-derived macrophages (Fig.4). Both the summer PMs induced an increase in the IL-8 release in THP-1 and A549 cells (Fig. 5), thus confirming the high pro-inflammatory potency of the summer fractions.

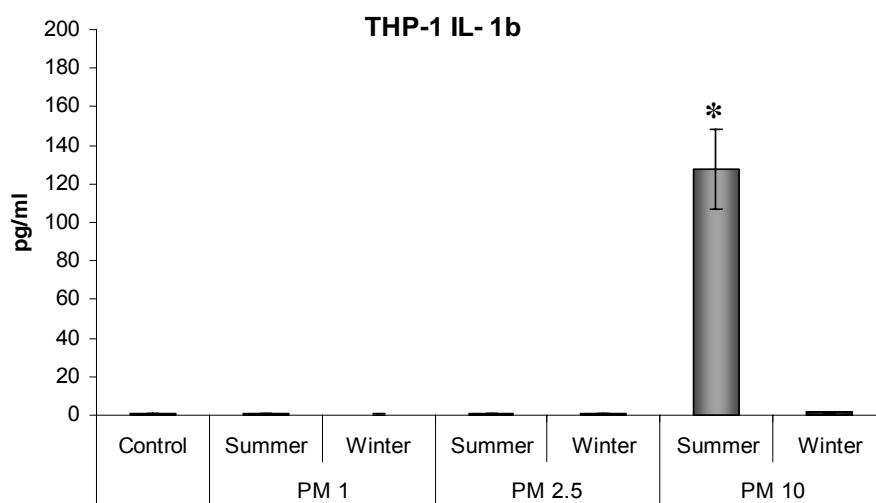


Fig 4. IL-1 β release in THP-1 derived macrophages exposed to summer and winter PMs

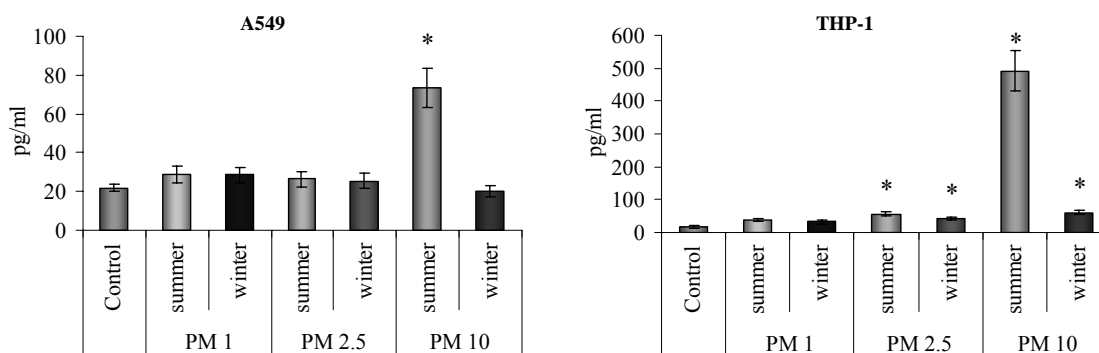


Fig 5. IL-8 release in THP-1 and A549 cells exposed to summer and winter PMs

Thus the inflammatory effects are significant in summer PM-exposed cells, while winter PM did not affect cytokine release. Beside these evidences, the winter fine fraction induced a significant increase in the expression of the enzymes belonging to the cytochrome P450 family, usually involved in the detoxification process of organic

compounds. In fact Cyp1B1 was over expressed in A549 exposed to fine winter PM (Fig. 6). Benzo[a]pyrene and carbon black (250nm) were used as positive control and reference material respectively .

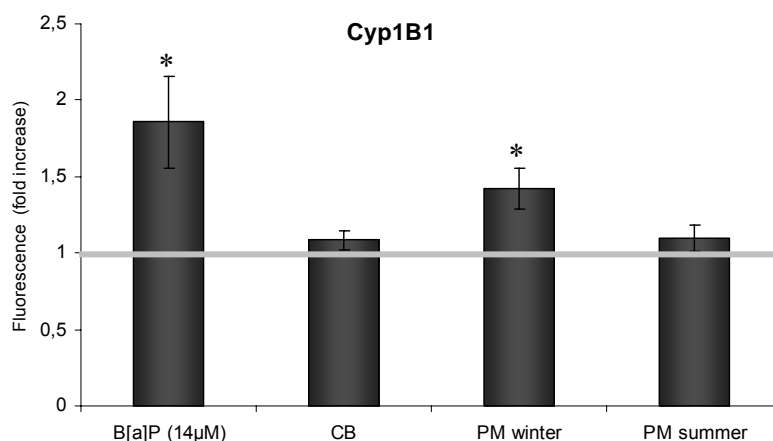


Fig 6. Cyp1B1 expression in A549 cells exposed to summer and winter fine PM.

2.3 In vivo results

In vivo experiments, generally performed on rodent laboratory mammals (mice or rats), are of primary importance for the understanding of the systemic effects of chemicals. Although the results obtained on *in vivo* systems are not strictly applicable to humans, they can improve the knowledge of the possible adverse effects obtained in *in vitro* experiments. The possibility to compare the results obtained by the use of the same material on *in vitro* and *in vivo* systems can furnish a reliable approach to understand the mechanisms of action and the biological effects produced by the different PM fractions. Mice were intratracheally instilled with summer PM10 and PM2.5 at the dose of 100 µg/mice and inflammatory, cytotoxic and oxidative stress markers in bronchoalveolar lavage fluid (BALF) were analysed at different times (from 3 h to 1 week). At 3 h a significant increase of the pro-inflammatory interleukin MIP-2, homolog of human IL-8, was evident (Fig. 7A) for both summer PM fractions, even the release was more relevant for the coarse fraction. The increment of TNF-α was consistent for summer PM10 (Fig. 7B) and TNF-α is the principal inflammatory mediator upstream of MIP-2 and it has been suggested to be a key-player in particles induced lung inflammation. A not significant decrease in AMs percentage was observed for PM2.5 after 3 h post instillation (Fig. 7D) however it became significant at 24h, while a sensible increase in PMNs percentage was induced just at 3 h (Fig. 7E).

Winter PMs induced a not significant release of inflammation mediators, while the expression of the enzymes involved in the detoxification processes was considerably increased. Figure 8 illustrates the expression of the cytochrome Cyp1B1 in the lung parenchyma of mice instilled with both winter PM fractions.

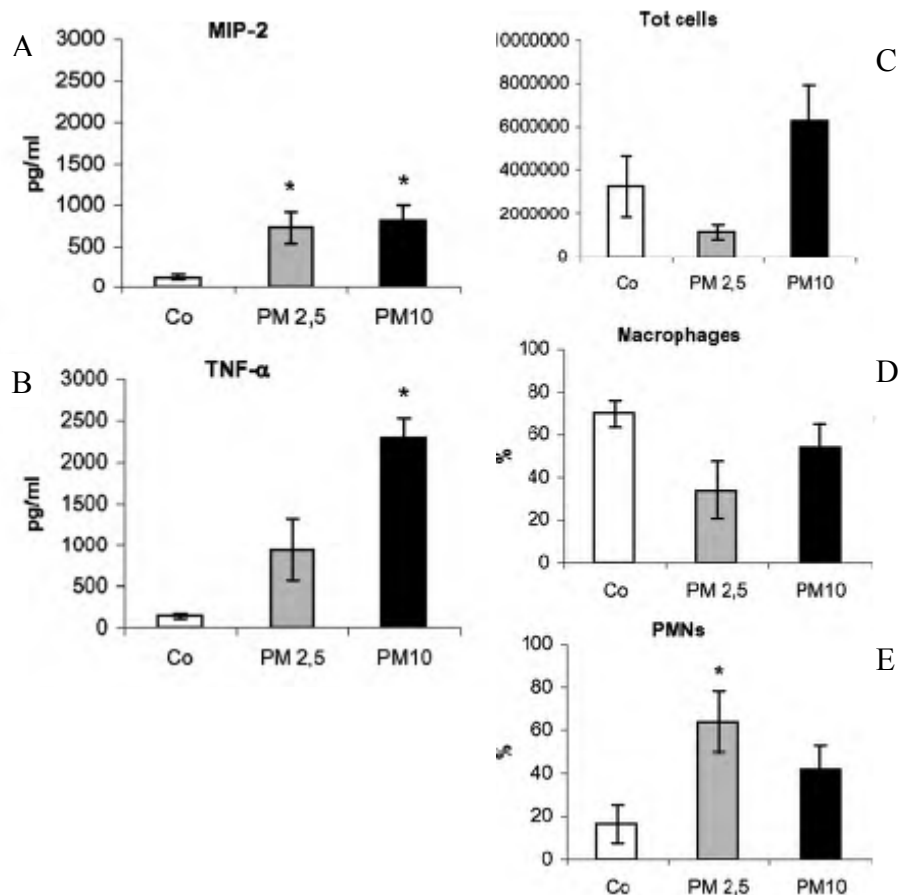


Fig 7. Cytokine concentration (A and B) and differential cell counts (C,D and E) in the BALFs collected at 3 h p.i. from PM exposed mice. Summer PM10 induced an increase of cytokine in the BALF while PM2.5 altered the relative percentage of cells.



Fig 8. Cytochrome Cyp1B1 in mice exposed for 24 h to winter PM10 and PM2.5. The fine fraction induced a significant increase of the enzyme.

3 Nanotoxicology of metal oxides on human lung cells

3.1 Background

Nanotoxicology is a recently coined term to designate the toxicology of particles with dimensions ranging in the nanoscale. As new discipline, it evolved from the experience and the last frontiers of the respiratory toxicology of particulate matter, with the specific advice of correctly investigate the potential health effects of natural and engineered materials with dimensions less than 100 nm. At this scale the physical and

chemical properties completely differ from those of bulk material and new unpredictable adverse biological effects may be elicited. This renders the investigation of the biological impact on nanoparticles (NPs) urgent, the first aim being the screening of the toxic potentials, the second the clarification of the mechanism of action of new nanotech material.

A quite huge literature is nowadays available on the toxic effects on *in vivo* and *in vitro* systems of different class of NPs, such as metal and metal oxide NPs, combustion derived carbon NPs, crystalline and amorphous silica NPs, single- and multi-walled carbon nanotubes. All these classes of nanomaterials are estimated to become serious environmental pollutants due to their high and constantly increasing production running parallel to the increasing global trend in the nanotech economy. Nanotechnology has been in fact projected to become a \$1 trillion market by 2015 (Nel et al., 2006), with an estimated production of about 60.000 tons nanoparticles (NPs) by 2020 (Lewinsky et al., 2008). A market with huge economical interests which implies the environment, and consequently humans, to be exposed to NPs. Due to their dimension, many of these nanomaterials are potentially constituents of the airborne particulate of outdoor (Donaldson et al., 2005b) and especially indoor environments, making the respiratory system the primary target of these new pollutants, whose respiratory toxicology profiles is the most urgent tools to address.

The major limitation to assess toxicity of NPs is the characterization of the experimental nanosystem prior, during and after exposure to living cells and organisms. This system is affected by many NP physicochemical characteristic, the most striking being size, surface chemistry, crystallinity, morphology, solubility, aggregation tendency and homogeneity of dispersion. All of these properties need to be assessed to correctly interpret their contribution to toxicity and to compare the results from different experiments and research groups.

The physicochemical characterization, the determination of appropriate exposure protocols and reliable methods for assessing NP internalization and their kinetics in living cells and organisms, represent aspects that claim for a comprehensive knowledge of the NP toxicity assessment to derive a potential threat to human health. In this scenario, multidisciplinary researches involving material scientists, physicians, chemists, toxicologists and microscopists should provide the adequate platform to identify their real hazard.

3.2 Cytotoxicity of metal oxide NPs

Basing on these assumptions and on the skills matured after the studies on the toxicity of atmospheric fine and ultrafine PM to the respiratory apparatus, our group focused new researches onto the *in vitro* biological effects of metal oxide NPs once administered to human alveolar epithelial cells in culture, which represent the main target of inhalable NPs. Metal oxide NPs are produced in large scale for nanotech industrial application and may pose serious hazards for human inhalation exposure. Commercial nanoTiO₂ and CuO (Sigma-Aldrich) were chosen as representative of two abundantly used NPs.

Particular attention has been devoted to the physicochemical characterization of these NPs and to the linkage between their cytotoxic effects and the interaction with cell structures at micro and nanoscale. Human alveolar epithelial cells, A549, were routinely maintained in lab and exposed to increasing concentration of NP suspensions. Cytotoxic effects and cell-particle interactions were investigate by biochemical and morphological techniques.

The TiO₂ and CuO suspensions revealed different behaviours. TEM analyses evidenced TiO₂ NPs regularly shaped in spheres with different diameters and a mean of 50.1nm, while CuO NPs appeared smaller, irregularly shaped, with a mean diameter of 34.4nm (Fig. 9). Both NPs had a tendency to aggregate in aqueous medium, and once dispersed in the cell culture medium, they produced aggregates larger for TiO₂ (mean diameter about 620nm) than CuO (mean diameter about 270nm) (Fig. 10). Surface charge was extremely different, since Z-potential of TiO₂ was -15.42mV, that of CuO was +15.45mV.

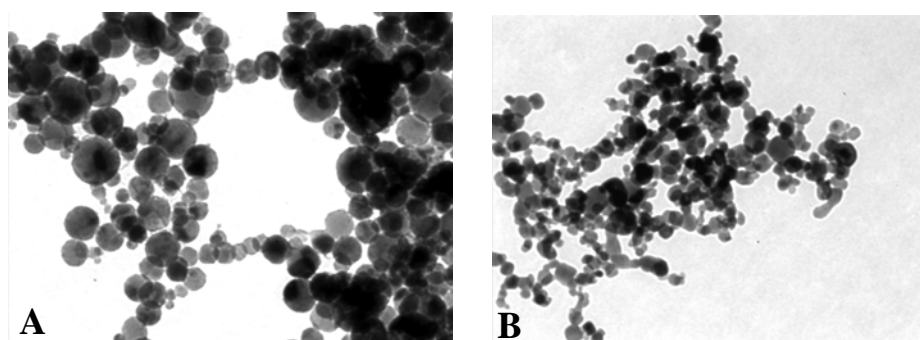


Fig. 9. TEM pictures of NP suspensions deposited and dried onto Formvar®-coated copper grids. A) TiO₂ aggregates ; B) CuO aggregates. Magnification x50000.

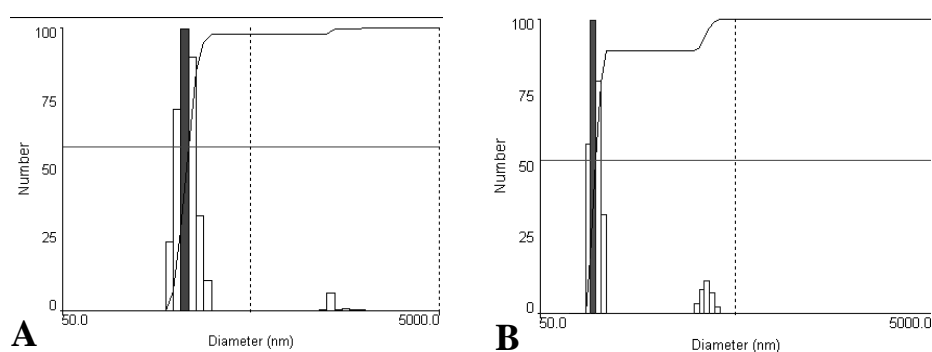


Fig. 10. Hydrodynamic behaviour of NPs as measured by DLS. Histograms represent the number of particles in function of their diameter. A) TiO₂; B) CuO.

The specific metallic composition of the NPs, coupled with these different hydrodynamic parameters, may be responsible of different biological responses. In fact A549 cells exposed to TiO₂ and CuO presented different reactions. The former ones did not induce decrease in cell viability, while the latter did it in a dose-dependent manner (Fig. 11). These data were obtained with MTT test, while LDH colorimetric assay failed to provide comparable results for an interference between the test reagent and the NPs. Similar problems were encountered when ROS species would be evidenced with the fluorescent probe DCFH to be quantitatively measured with cytometer.

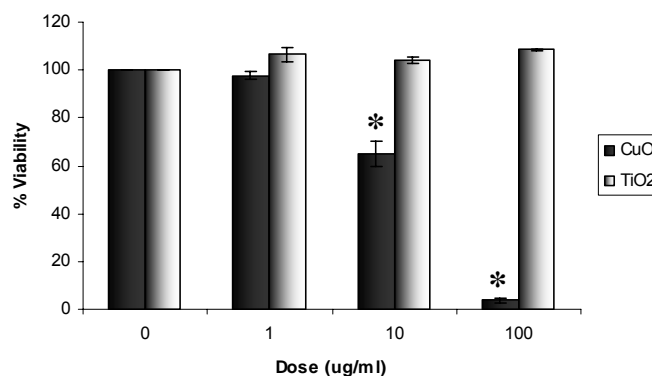


Fig. 11. MTT assay of A549 cells exposed to CuO and TiO₂NPs for 24 h

One of the open questions on NPs and especially on metallic NPs is if their effects are triggered by the release of metal ions from their surface area. In our experiments cells incubated in the medium, where CuO NPs were removed, were not affected, demonstrating that NPs reactivity was the main responsible for cell toxicity. Thus metal ions derived from the particles were apparently not involved in cell damage. This result is not in line with some researches, which attribute a significant role of dissolved copper ions on cells and organisms exposed to water NP suspensions (Kahru and Dubourguier, 2010).

The peculiar interaction and internalization processes of NPs into cells may also be responsible for the differential cytotoxic profile of CuO and TiO₂. Advanced microscopic techniques are helpful in understanding this aspect. RAMAN spectroscopy and AFM microscopy are nowadays available techniques to investigate the interaction of NPs and cell at molecular level. At the same time confocal and transmission electron microscopy techniques are extremely useful in the morphological characterization of the cell-particle interaction and structural modifications..

Nanobiotechnology and nanotoxicology development has determined a new life of TEM in the life sciences, since it is the most potent and efficient instrument able to visualize the interactions at nano level; moreover the detection and analysis of the NPs in cells and tissues often represent an essential step for nano-biologists. Traditional and energy filtering TEM (EFTEM) have been effectively adopted in our labs to map metal oxides NPs in the epithelial lung cells (Fig. 12).

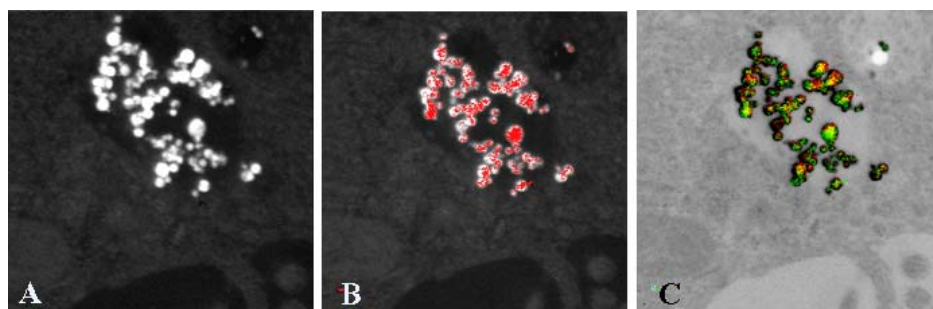


Fig. 12. TEM imaging of TiO₂ NPs internalized in A549 cells. A) dark field where white spots represents metallic particles; B) dark field where red colour corresponds to the energy loss of Titanium, evidenced by ESI technique; C) bright field where red colour corresponds to the energy loss of Titanium and green to that of Oxygen, evidenced by ESI.

TiO₂ was not cytotoxic, but was efficiently and abundantly internalized by cells, usually encapsulated as agglomerates in membrane bounded vacuoles (Fig 13 B). The cell-particle interaction determined a mild oxidative stress as revealed by 4-HNE immunohistochemistry, a marker of lipid peroxidation (Fig. 13 E).

CuO NPs were not easily detectable in the cells, since penetrated in form of very fine particle aggregates, but induced evident mitochondrial modifications and a pronounced oxidative stress as evidenced by the intense dark brown reaction of the 4-HNE immunochemistry (Fig. 13 C,F). Only at later stages, when cell membranes were probably very leaky, CuO NPs were found abundantly distributed throughout the cell (data not shown).

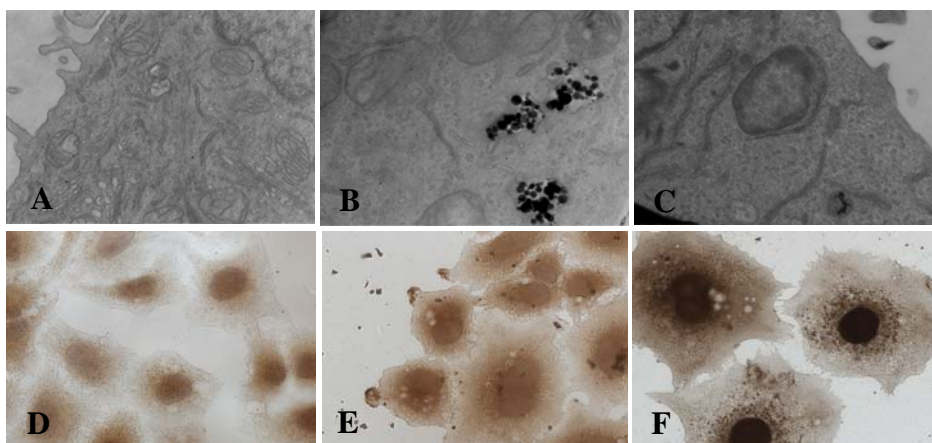


Fig. 13. Morphological effects of NPs on A549 cells, as revealed by TEM (A-C) and by 4-HNE immunocytochemistry, a marker of lipid peroxidation, evidenced by dark brown precipitate. A) Ultrastructure of a control cell, B) abundant TiO₂ NPs engulfed in cytoplasmic vacuoles; C) altered cell ultrastructure after CuO NPs exposure; D) light microscopy of control cells; E) TiO₂ exposed cells showing slight lipid peroxidation; F) CuO exposed cells showing strong lipid peroxidation.

4. Conclusions

PM

In conclusions the *in vitro* and *in vivo* experiments, performed with coarse and fine Milan PM collected during summer and winter, suggest there are no distinct qualitative differences in the biological effects produced. Summer fractions induced a more significant inflammatory process (Monn and Becker,1999) in comparison with the winter fractions and PM10 was more effective than summer PM2.5 in eliciting the expression of pro-inflammatory proteins (IL-1 β , IL-8, MIP-2, TNF- α). This effect is

probably linked to the presence of biogenic component. (Heinrich et al., 2006, Alfaro-Moreno et al., 2007; Camatini et al., 2010; Wegesser and Last, 2008). Winter PMs produced a not significant inflammatory process, while they were apparently involved in pro-carcinogenic events (Nebert and Dalton, 2006): in fact, PAHs content of winter fine PM induced an increased expression of Cyp1B1 suggesting that PM chemical composition plays a crucial role in eliciting its toxic effect. The role of particle size is an important issue too. The research is actually oriented to the identification of the part of PM most relevant to health, since PM is some kind of container comprising components which are closely related to health effects (Pope et al., 2002; Schwartz et al., 2008, Valberg,2004). There are good reasons to suppose that not all particles are equal with respect to health effects. It seems to be prudent not to neglect any fraction of inhalable particles. At present, the available data do not allow to absolve coarse particles and the role of ultrafine particles is only beginning to be assessed.

Nanoparticles

According to the here reported results, and to the suggestions of Jones and Grainger (2009), toxicologists have to pay particular attention to the characterization of the NP systems during the experiments, trying to minimize the interference with the detection systems and to prevent undesirable artefacts. Multiple internal controls and a correct set up of experimental panels are often needed to correctly interpret the hazards for human health. Metal oxide NPs likely represent a potential hazard for inhalation, since their reactivity seem to be associated to the bioreactivity of the metal constituting the particles, coupled with the particle small dimension and the consequent increase in reactive surface. These indications suggest the necessity to implement the research effort in the characterization of the toxicological profiles of the constantly increasing new nanomaterials, trying to bridge the enormous gap between nanomaterials development and the risk assessment for humans and environment.

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