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Genotoxicity induced by fine urban air particulate matter in the macrophages cell line RAW 264.7

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Abstract

Recent studies support a participation of fine airborne particulate matter (PM) with an aerodynamic diameter less than 2.5 μm in the effects of air pollutants on health. Particulate matter was collected in an urban area of L'Aquila during the winter 2004. Fine particulate samples were analyzed by X-ray photoelectron spectroscopy (XPS) to determine the chemical inventory of the aerosol particle surfaces and to evaluate the weight of characteristic functional groups of the most frequent carbon-containing organic pollutant compounds (C–C/C–H, C–O/C–N, C═O, COOH). The most important contributor to the mass of fine particulate matter was carbon. The overall purpose of this work was to determine the in vitro toxicity and genotoxicity of fine PM in cultured macrophages (RAW 264.7 cells) since the biological target of inhaled PM are the pulmonary epithelium and resident macrophages. In parallel in vitro toxicity assays were used including cell viability and apoptosis. Genotoxicity was evaluated by the micronucleus (MN) assay. The viability of macrophages was assessed by the MTT method; apoptosis by an ELISA test for programmed cell death (PCD) was determined after RAW 264.7 cells treatment. Concentration of 1, 3 and 10 μg/cm^2 of fine particles induced micronuclei in a dose-dependent manner. We also compared the effects of fine PM with those of fine carbon black particles (CB) in similar doses. Fine carbon black particles were consistently less genotoxic than the fine atmospheric particles, suggesting that the contaminants adsorbed on them (i.e. carbon-containing organic compounds in addition to metal oxides and metal salts) are involved in genotoxicity. Fine PM reduced cellular proliferation. Overall, the results presented here demonstrate the utility of in vitro tests in mouse cells for testing genotoxicity of urban air particulate matter.

Keywords: Fine airborne particulate matter; Genotoxicity; Micronuclei; Murine macrophages; RAW 264.7

1. Introduction

Urban air, particularly in high traffic areas, contains mutagenic and carcinogenic substances, including organic compounds such as benzo(*a*)pyrene and benzene, inorganic compounds such as nickel and chromium, and radionuclides (Cohen and Pope, 1995). Extracts of urban air particulates induce cancer in animals and are mutagenic in bacteria and mammalian cells (Crebelli et al., 1988). In recent years, the increase of PM_{10} and $PM_{2.5}$ particulate matter, particles with equivalent diameter less than 10 μm and 2.5 μm, respectively, has been associated with an increased number/frequency of cardiorespiratory hospital admissions, cardiorespiratory mortality, and respiratory-related emergency department visits (Schwela, 2000; Hruba et al., 2001; Biggeri et al., 2001). Correlations between mutagenicity data, airborne micropollutants, and meteorological parameters were established (Crebelli et al., 1995). Moreover, the ultrafine particle components of PM stimulate increases in proto-oncogene expression and proliferation in alveolar epithelial cells (Timblin et al., 2002).

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It is not known, however, which characteristics of urban air particulate matter (e.g., mass, size, shape, composition, or some combination of factors) contribute to the observed toxic effects. A recent theory is that transition metals, present as surface contaminants, can promote the production of free radicals which may cause the formation of reactive oxygen species, such as superoxide anions, and hydroxyl and hydroperoxy radicals (Wright et al., 1994). PM 2.5 as well PM10 (Li et al., 1997) act as vehicles for sulphuric acid, which initiates focal lung damage when it comes in contact with epithelial surfaces; these particles have therefore received recent attention (Kaiser, 2000). The research by Laskin et al. (2003) focused on hydrogen peroxide (H_2O_2) carried into the lower lung by fine particles; they demonstrated that biological effects of H_2O_2 are augmented by fine PM. Fine particles may exacerbate the symptoms of asthma and increase the morbidity and mortality from respiratory causes (Chalupa et al., 2004). Carbon particles inhaled at concentrations mimicking high episodic increases in urban air can exert extrapulmonary effects in old rats and they can change the systemic response to an inflammatory stimulus (Elder et al., 2004).

The monitoring of urban air for genotoxic and mutagenic micropollutants by conventional mutagenicity tests is receiving increasing consideration with the aim of evaluating health risks in urban areas (Monarca et al., 1999; Poma et al., 2002). However, few data are available on genotoxicity tests (such as micronucleus assays) applications in human and mouse macrophages. In previous works (i.e. D'Agostini et al., 1992) the occurrence of micronuclei in vivo was monitored in pulmonary alveolar macrophages; according to (Das et al., 1994), the genotoxic potential of inhalation of mosquito coil (MC) smoke was evaluated by using metaphase chromosome aberration and micronucleus assays in pulmonary alveolar macrophages (PAMs) of rats. Significantly higher frequencies of chromosome aberrations, including micronucleated PAMs in smoke-exposed animals, compared to controls, indicated a genotoxic capacity of MC smoke.

In the present study, we have evaluated the physical-chemical and genotoxic properties of fine particulate matter sampled in a site located in a slightly polluted residential area in the town of L'Aquila (winter 2004). Physical–chemical surface properties of PM 2.5 were determined by X-ray photoelectron spectroscopy (XPS). For the first time, the micronucleus (MN) assay was used in this work to evaluate the genotoxic effects of PM 2.5 particulate matter and carbon black in the murine macrophage RAW-264.7 cell line. Our observations provide the first evidence of the in vitro mutagenic effect of fine particulate in RAW-264.7 cells and indicate the possibility to detect chromosome mutations (micronuclei) in these cells. In addition, the relative cell toxicity (by determination of the mitochondrial-dependent reduction of MTT) and apoptosis (by an ELISA test) induced by the PM 2.5 fine and carbon black particles were determined.

2. Materials and methods

2.1. Airborne particulate matter: sample collection, extraction and chemical surface characterization

The particulate material was collected on a weekly basis between January 2004 and March 2004 by an eight-stages cascade impactor (Andersen particle fractionating sampler) with a pre-separator that eliminates particles with aerodynamic diameter above 10 μm. The average flow rate of the sampler was 28.317 l/min. The sampler separated the particulate matter into eight granulometric fractions within the 10–0.43 μm aerodynamic diameter range. The stage sampler cut-off diameters were 9 μm, 5.8 μm, 4.7 μm, 3.3 μm, 2.1 μm, 1.1 μm, 0.7 μm, 0.43 μm respectively for the stages 0, 1, 2, 3, 4, 5, 6, 7 of the impactor. The period of measurement was characterized by small changes of the meteorological average main parameters (temperature from −4 °C to 7 °C, humidity 82–88%).

In this work we studied the particles with aerodynamic diameter ranging from 2.1 μm to 0.43 μm collected by the last three stages of Andersen impactor.

For each stage, airborne particles were collected on a removable stainless steel disk with a 82.6 mm diameter. Half of the disk has been covered by an aluminum foil and the remaining part has been partially covered by a small portion (1 cm^2 area) of a Teflon filter (4.7 mm diameter, 0.4 μm pore size), coated with a thin gold film (5 nm).

In order to evaluate the particulate material concentration in air, the mass of the collected particles was determined by measuring the weight of the aluminum foil, before and after sample collection, using a Gibertini mod. E50S microbalance with 10 μg sensitivity. The aluminum foil was then put for 15 min in an ultra-sound bath with ethyl alcohol to remove the particles collected. The recovered particle suspension was put into the rotary evaporator to allow the ethanol evaporation. The residual particulate matter was used for biological tests.

The same procedure was applied to recover the particles collected by the uncovered stainless steel disk. The stainless disks of the cascade impactor were put in an ultra-sound bath with ethyl alcohol to remove the particles collected. In this case the suspension obtained was filtered on a Teflon membrane (PTFE) with 1 μm pore size, covered with thin gold film. The quantitative surface chemical composition of the fine particulate material collected on these filters was determined by X-ray photoelectron spectroscopy (XPS).

Both filters covered with gold were used to determine the chemical inventory of aerosol particles surface by means of X-ray photoemission spectroscopy (XPS).

Measurements have been performed using the ESCA PHI 1257 apparatus equipped with an Mg Kα photon source ($h\nu = 1253.6$ eV) and a concentric hemispherical analyser under ultra high vacuum conditions. The analysis chamber was maintained at a pressure lower than 10^{-9} torr. The gold $4f_{7/2}$ XPS peak located at 84.00 ± 0.01 eV was used to calibrate the spectra.

2.2. RAW 264.7 cell culture and exposure to particles

The murine macrophage cell line RAW 264.7 (a kind gift of Prof. M.G. Cifone, Department of Experimental Medicine, University of L'Aquila) was maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 20 mM Hepes, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The cultures were incubated in an atmosphere of 95% air and 5% CO_2 with of 80% humidity, and at a temperature of 37 °C. Cell detachment was carried out mechanically by scraping. The fine particulate samples for biological determinations were evaporated with a rotary evaporator and resuspended in culture medium. All particles were used at three different concentrations 1, 3 and 10 μg/cm^2 (2.2, 6.6 and 22 μg/ml).

2.3. Cell viability assay

RAW 264.7 cell viability was determined by trypan blue exclusion; cells were plated at a density of 25×10^4/well in 6-well plates and cultured for 48 h. 1, 3 and 10 μg/cm^2 of fine particles and carbon black (Degussa Huber NG90, diameter 200–250 nm) for 48 h were then added. After incubation, the cells were released with trypsin/EDTA (Life Technologies, Inc.) incubated with trypan blue, and counted using a hemocytometer.

Cell viability also was determined by the mitochondria-dependent reduction of MTT (3-(4,5-dimethyl-thiazol-2*y*)2,5-diphenyl-tetrazolium bromide, Sigma) to formazan. Cell cultures were established in microtiter plates and treated with 1, 3 and 10 μg/cm^2 of the fine particulate matter and carbon black for 48 h. The cells then were incubated with 100 μl of 0.2 mg/ml MTT for 2 h at 37 °C, followed by a 15 min incubation at 37 °C with 100 μl DMSO (dimethyl sulfoxide, Sigma). Microtiter plates were read at 595 nm in an ELISA plate reader. The results are expressed as absolute optical density (O.D.) readings.

2.4. Micronucleus assay

RAW-264.7 cells were incubated with complete medium supplemented with 1, 3 and 10 μg/cm^2 (2.2, 6.6 and 22 μg/ml) of fine particulate matter samples or carbon black. Negative controls were incubated with the same volume of medium. Cells were then assayed for micronucleus production according to the procedure of Tawn and Holdsworth (Tawn and Holdsworth, 1992). After 44 h of incubation, Cytochalasin B (Cyt B; Sigma) was added at a final concentration of 6 μg/ml in complete medium containing the particles samples, carbon black and/or positive controls. After further 28 h of incubation, the cells were harvested. Three parallel cultures were used for each experiment and three independent determinations were carried out for each particulate matter and carbon black concentration. After treatment, RAW-264.7 cells were collected by low speed centrifugation (350 g for 10 min), washed twice in Dulbecco's phosphate-buffered saline, and finally resuspended in 1 ml of 3:1 methanol:acetic acid fixative; the fixed cells were smeared onto clean glass slides, air dried, and stained with Giemsa for 3 min. To detect micronucleus formation, 3000 binucleated cells per treatment group (1000 from each culture) were scored at 1000× magnification by light microscopy. Student's *t*-test was applied to quantitatively determine the difference between treated and control MN frequencies.

2.5. Evaluation of apoptosis

Apoptosis was evaluated using an ELISA Plus kit (Roche) which employs a quantitative sandwich enzyme immunoassay with monoclonal antibodies directed against histone-associated DNA fragments. Cells were treated in microtiter plates at a concentration of 1×10^4/well with particulate matter and carbon black (1, 3, 10 μg/cm^2, 5 h and 24 h); the supernatants had to be discarded and then cells lysed with lysis buffer (according to the instructions of the Roche ELISA Plus kit). Twenty microliters of lysate (cytoplasmic fraction) was transferred into wells of the streptavidin-coated microtiter plate. Subsequently, a mixture of anti-histone-biotin anti-DNA-peroxidase conjugated antibody was added. After the removal of unbound antibody by washing, the amount of nucleosomes was quantified by the peroxidase retained in the immunocomplex. Samples were read in triplicate in a spectrophotometer at 405 nm. Fold increase (EF, enrichment factor) in the levels of mono and oligonucleosomes in the cytoplasmic fraction was determined by comparing these results with the levels of the untreated controls.

3. Results

3.1. X-ray photoelectron spectroscopy (XPS) analysis

Fig. 1 shows a typical survey spectrum of particulate material acquired in the range 0–1100 eV. The XPS peaks were recorded in the following sequence of increasing binding energy Au4f, Si_{2p}, S_{2p}, C_{1s}, N_{1s}, O_{1s}, and Na_{1s}. The resolution was about 0.7 eV per step and pass energy of 80 eV. High resolution spectra were collected for the unresolved composite C_{1s} peak at a resolution of 0.0 eV per step and a pass energy of 20 eV in the range 279–292 eV. Once the Shirley background has been removed from these XPS spectra, the carbon peaks were decomposed (Hutton and Wiliams, 2000) in three components attributed to carbon singly bound to carbon and hydrogen (C–C/C–H at 284.6 eV, FWHM = 1.4 eV), carbon singly bound to oxygen or nitrogen (C–O/C–N at 286.3 eV, FWHM = 1.4 eV) and carbon making a double bond with oxygen (C═O/COO^- at 288.6 eV FWHM = 1.4 eV) in order to evaluate the relative contributions of the characteristic functional groups of the most frequent carbon-containing organic pollutant. (C–C/C–H, C–O/C–N, C═O, COOH).

A typical spectrum of a sample obtained according to the previous procedure is reported in Fig. 1A(a) (black

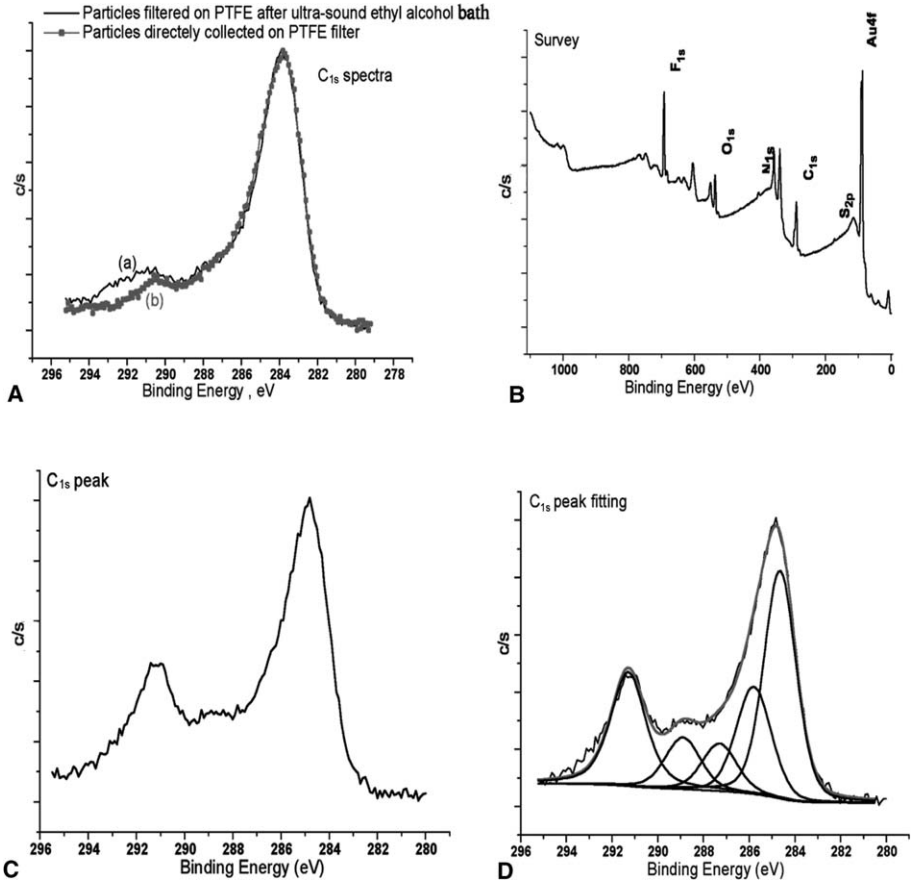

Fig. 1. (A) Overlapping C_{1s} spectra. (a) (black solid line): Particles filtered on PTFE after ultra-sound ethyl alcohol bath; (b) (grey dashed line): Particles directly collected on PTFE filter. (B) XPS survey spectra. (C) High resolution C_{1s} spectrum. (D) Fitting of high resolution C_{1s} spectra.

solid line). For the same sample the particles collected on the small piece of Teflon filter was directly analysed with the XPS technique. Fig. 1A(b) (grey dashed line) shows the obtained XPS spectrum. Within the experimental error limit (about 5%), the two spectra coincide in the energy range of interest (279–292 eV) where the characteristic functional groups of PTFE filter appear. This comparison shows that the ultra-sound ethyl alcohol bath does not produce variations of the organic composition of particulate matter.

Fig. 1B and C shows the survey and high resolution C_{1s} spectra. The survey spectrum (Fig. 1B) shows the presence of C, O, N, S and F and the important contribution to the mass of the fine particulate matter of carbon and carbon compounds. The chemical inventory of samples collected is given in Table 1: C shows the highest percentage with respect to O, N and S during all the sampling periods. The high resolution C_{1s} XPS spectrum (Fig. 1C), extended over the energy range 280–295 eV, indicates the overlapping peaks as coming from a mixture of organic carbon compounds. A fitting procedure has been performed to quantify the percentage of functional groups of carbon. Five Gaussian curves were used to fit the carbon 1s peak. Fig. 1D shows a typical result of this procedure. The most intense peak at 284.7 eV is due to C–C and C–H bonds. The remaining three low intense peaks at energy 285.8 eV, 287.3 eV and 288.9 eV are due to C–O/C–N, C═O, COOH respectively. The peak at 291.3 eV originates from the C–F bonds of PTFE filter. In order to obtain a quantitative result, a region of interest for each functional groups is defined and simply integrated. The percentages of functional groups of each sample are given in Table 2, where C–C/C–

Table 1
Chemical inventory of aerosol particles collected in different periods

	January 2004 (%)	February 2004 (%)	March 2004 (%)
C	73 ± 4	73 ± 4	72 ± 4
O	20 ± 5	21 ± 5	23 ± 5
N	5.9 ± 0.3	5.4 ± 0.3	5.2 ± 0.3
S	1.3 ± 0.7	1.3 ± 0.7	0.8 ± 0.7

Table 2
Characteristic functional groups of the most frequent organic pollutants compounds containing carbon

	January (%)	February (%)	March (%)
CC/CH	54 ± 4	53 ± 4	54 ± 4
CO/CN	23 ± 2	22 ± 2	21 ± 2
C═O	13 ± 3	12 ± 3	12 ± 3
COOH	10.8 ± 1.3	12.4 ± 1.3	10.8 ± 1.3

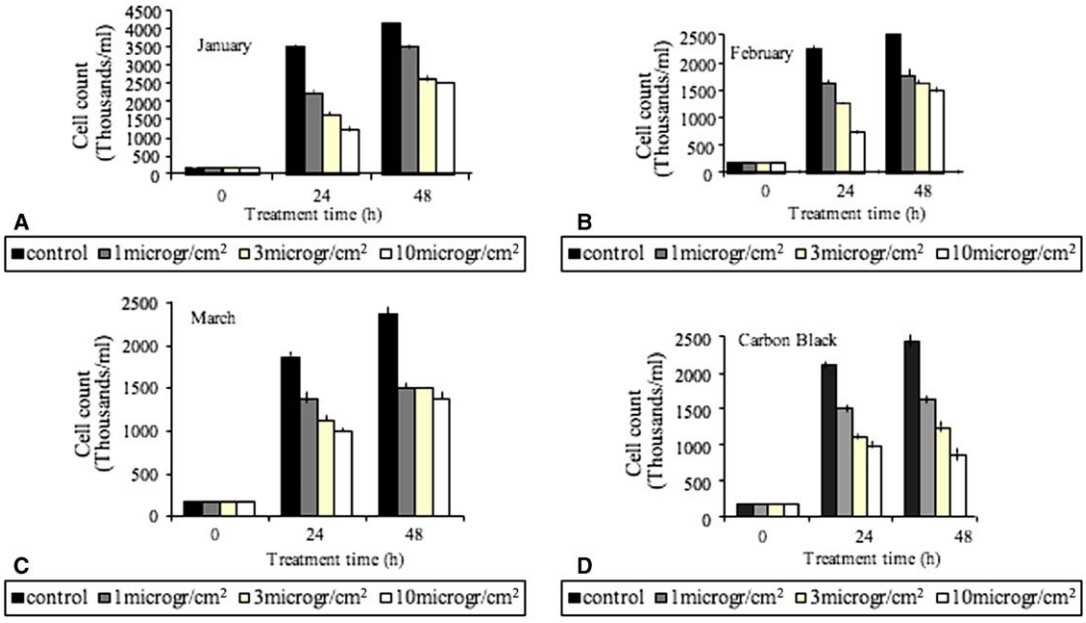

Fig. 2. Cell proliferation of fine-treated RAW-264.7 viable cells as measured by counting cells after trypan blue exclusion. (A–C): cell treatments with fine particulate collected in January, February, March, respectively; (D) growth after cell treatment with carbon black. Values are averages (SD) of three independent experiments assayed in duplicate.

Table 3
Effects of fine airborne particulate matter and carbon black on the viability of RAW 264.7 macrophage line evaluated by MTT reduction assay (the results are expressed as absolute optical density readings at 595 nm)

Time (h)	μg/cm²	January	February	March	Carbon black
24	1	0.11 ± 0.03	0.12 ± 0.01[a]	0.11 ± 0.01	0.18 ± 0.01
	3	0.09 ± 0.02[a]	0.07 ± 0.01	0.09 ± 0.01[a]	0.19 ± 0.01
	10	0.07 ± 0.01[a]	0.04 ± 0.01	0.07 ± 0.01[a]	0.20 ± 0.16[a]
	C	0.21 ± 0.03	0.21 ± 0.14	0.22 ± 0.02	0.21 ± 0.03
48	1	0.44 ± 0.01[a]	0.42 ± 0.01	0.41 ± 0.01	0.44 ± 0.03
	3	0.38 ± 0.01[a]	0.33 ± 0.01	0.33 ± 0.01[a]	0.36 ± 0.2[a]
	10	0.25 ± 0.01[a]	0.24 ± 0.02	0.22 ± 0.01	0.26 ± 0.01[a]
	C	0.56 ± 0.01	0.55 ± 0.09	0.50 ± 0.01	0.55 ± 0.02

[a] Mean value significantly different, Student's t-test $p < 0.05$, for values compared with the control C.

H shows the highest percentage during all the sampling periods according to the most intense peak in Fig. 1D.

3.2. Viability and MN assays in cells exposed to particulate

The cytotoxic and genotoxic effects of fine particulate matter were assayed in murine RAW-264.7 cells. The effect of fine particulate matter and carbon black on the proliferation of viable cells was determined by cell counts over 48 h using the trypan blue dye exclusion. Both the fine particulate and carbon black reduced the proliferation of RAW-264.7 cells in a dose-dependent manner (Fig. 2A–D). Moreover, fine particulate matter and carbon black reduced the cellular metabolism of the macrophages when assessed by the MTT method as shown in Table 3, where any decrease from the control would indicate toxicity.

In order to examine the effect of fine particles on the stability of chromosomal DNA, we examined MN induction in RAW-264.7 cells. Fine particulate matter from all sampling periods induced micronuclei in a dose-dependent manner (Table 4). The frequency of micronuclei produced by the fine-treated samples in RAW-264.7 cells was higher than that produced by equal weights of carbon black. The appearance of micronuclei in the cytoplasm of binucleated and treated cells could be correlated also to other nuclear damages (apoptotic) as evaluated by the following ELISA test.

3.3. Effects of fine particulate matter on apoptosis in RAW- 264.7 cells evaluated by ELISA test

The effect of fine particulate matter on the induction of apoptosis was evaluated by the ELISA^{PLUS} assay, which measures internucleosomal degradation of genomic DNA occurring during PCD. Both carbon black and the fine particulate matter induced significant amounts of apoptosis in RAW-264.7 cells (Fig. 3): a strong fold increase (EF, enrichment factor) was followed by a EF steady state/slow

Table 4
Frequency of micronuclei (referred to the total number of cells with MN micronuclei in 1000 cells) in RAW 264.7 cultured cells 48 h exposed to fine particulate matter different concentrations

μg/cm^2	January	February	March	Carbon black
	MN/1000 cells[a,b]	MN/1000 cells[a,b]	MN/1000 cells[a,b]	MN/1000 cells[a,b]
1	45.0 ± 0.7[c]	41.5 ± 0.7[c]	31 ± 3[c]	22.0 ± 1.4[c]
3	51.0 ± 1.4[c]	74.0 ± 0.7[c]	66 ± 3[c]	36.5 ± 0.7[c]
10	68 ± 1[c]	81 ± 2[c]	81 ± 2[c]	50 ± 2[c]
Control	13 ± 1	16 ± 1	15.2 ± 1.1	14 ± 11
Positive control 200 ng/ml lead acetate	90 ± 1[c]	95.2 ± 1.1[c]	100.0 ± 1.7[c]	100 ± 2[c]

[a] Range of micronuclei per binucleated cell:1.
[b] Values are means ± S.E.M. from three independent samples.
[c] Mean value significantly different, Student's *t*-test; ($^c p < 0.01$), for values compared with the control C.

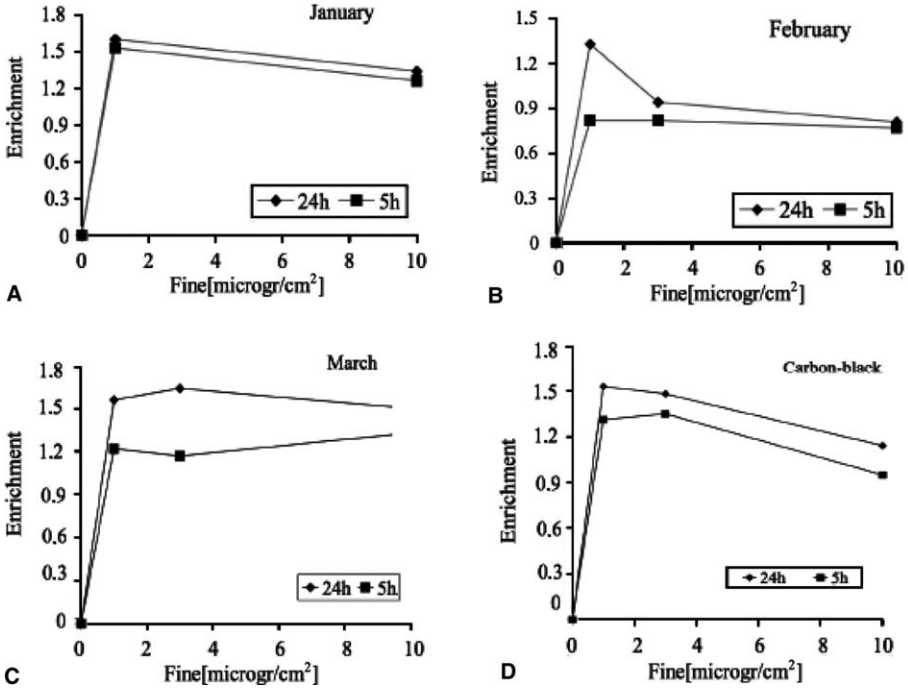

Fig. 3. Effects of fine particulate on the induction of apoptosis in RAW-264.7 cells as evaluated by the ELISA Plus assay; fold increase (EF, enrichment factor) in the levels of mono and oligonucleosomes in the cytoplasmic fraction, was determined by comparing these responses in treated cells with the levels of the untreated controls. (A–C) EF after cell treatments with fine particulate (1, 3, 10 μg/cm^2, 5 h and 24 h) collected in January, February, March respectively; (D) EF after cells treatment with carbon black (1, 3, 10 μg/cm^2).

decrease, indicating that at 3 and 10 μg/cm^2 treatments there was a trend versus necrosis, whereas apoptosis was the main cytotoxic effect induced by 1 μg/cm^2 treatments.

4. Discussion

In the present study we have characterized the chemical state of each compositional elements by XPS. The quantitation of functional groups containing carbon have been obtained using a fitting procedure of a C_{1s} spectrum. The results indicate that the fine particulate matter has a high percentage of C-rich particles. The C-rich particles are derived from vehicle traffic and are the most common type within the fine granulometric fractions. Polycyclic aromatic hydrocarbons (PAH) in an urban atmosphere are associated mainly with the fine particles and these are also found in intrapulmonary depositions (Stocks, 1960; Crebelli et al., 1988; Crebelli et al., 1995; Wright et al., 1994; Li et al., 1997); the physical characteristics of the particles (number, sizes, shape, aggregation properties) and compositional elements also contribute to ill health.

The genotoxic activity of fine PM (as assessed by micronuclei and apoptosis tests) presented in this study is in good agreement with other reports (Hornberg et al., 1998). The characteristic functional groups of the most frequent organic pollutants containing carbon are shown in this work. In order to evaluate the role of adsorbed contaminants by PM on induced genotoxic responses, we have compared the effects of PM 2.5 fractions with carbon black, characterized by significantly less substances adsorbed on its surface (Nikula et al., 1995), which was less genotoxic than PM 2.5. These findings suggest that contaminants

adsorbed onto the particles (as determined by XPS analysis) are involved in the genotoxic and apoptotic effects observed in RAW-264.7 cells. Further studies evaluating the contributions of metal oxides and metal salts implicated in PM cyto- and genotoxicity (Molinelli et al., 2002), may also help in demonstrating the action of fine particles in RAW-264. Particle surface chemistry represents an important factor for biological and cyto/genotoxic effects on cells and in particular macrophages. Moreover, the results presented here demonstrate the utility of an urban particle genotoxicity in vitro test in animal cells as an alternative to microrganisms. The murine macrophage cell line RAW-264.7 is a reliable in vitro model for this purpose that is also useful for the study of inflammatory mediators release induced by coarse and fine urban air particles, as previously reported (Diociaiuti et al., 2001; Pozzi et al., 2003). We conclude that the genotoxic test using murine macrophages combined with XPS analysis is useful for determining the genotoxic load and risk of the environmental fine air particulate matter.

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