ORIGINAL ARTICLE

Evaluation of DNA damage induction on human pulmonary cells exposed to PAHs from organic extract of PM10 collected in a coke-oven plant

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Abstract. Occupational exposure of coke oven workers, classified by IARC as human carcinogen, is characterized by the presence of PAHs emitted during pyrolysis of coal. We aimed to clarify the mechanism of action of complex mixtures of PAHs and to identify biomarkers of early biological effect, evaluating on lung epithelial cells (A549) genotoxic and oxidative damage of airborne particulate matter collected in a coke plant. Particulate matter was collected in the oven area on glass filter, extract and analysed by GC/MS. Direct/oxidative DNA damage induced by exposure to extract were evaluated by Fpg comet assay. The cells were exposed for 30 min, 2h and 4h to extract of half filter diluted at 0.004%, 0.008% and 0.02%. We evaluated comet percentage and analysed tail moment values of cells treated with Fpg enzyme (TMenz) and untreated (TM) that indicate respectively oxidative and direct DNA damage. Air sample contained 0.328 $\mu g/m^3$ of pyrene, 0.33 $\mu g/m^3$ of benzo(a)anthracene, 1.073 $\mu g/m^3$ of benzo(b)fluoranthene, 0.22 $\mu g/m^3$ of benzo(k)fluoranthene, 0.35 $\mu g/m^3$ of benzo(a)pyrene, 0.079 $\mu g/m^3$ of dibenzo(a,h)anthracene and 0.40 $\mu g/m^3$ of benzo(g,h,i)perylene. The dose-dependent increase of TM and TMenz in exposed cells was not significant, indicating only a slight direct and oxidative DNA damage in exposed cells. A small dose-time dependent increase of comet percentage was found. The study shows the high sensitivity of comet assay to measure early DNA damage also at low doses suggesting its use on lung epithelial cells to evaluate the effects of complex mixtures of genotoxic substances on target organ. (www.actabiomedica.it)

Key words: Polycyclic Aromatic Hydrocarbons, coke plant, Comet assay, lung epithelial cells, environmental monitoring

Introduction

Coke-oven workers are occupationally exposed to various polycyclic aromatic hydrocarbons (PAHs) emitted during pyrolysis of coal for coke production (1, 2). When coal burns, chemical and physical changes take place, and many toxic compounds are formed and emitted. Polycyclic aromatic hydrocarbons (PAHs) are among those compounds formed and are considered to pose potential health hazards because some PAHs are known carcinogens. PAHs are highly lipid soluble, and can be absorbed by the lungs, gut and skin of mammals because they are associated with fine particles from coal combustion (2). Coke production has been classified by the International Agency for Research on Cancer (IARC) as carcinogenic for humans (group 1) on the base of experimental studies and epidemiological reports indicating excess of risk principally for lung cancer (3). In particular, relatively to PAHs present in coking plant, the IARC classified benzo(a)pyrene as human carcinogen (group 1) (4), benzo(a)anthracene and dibenzo(a,h) antracene as probable carcinogens (group 2A); benzo(b)fluoranthene, benzo(k)fluoranthene and indeno

(1,2,3cd)perylene as possible carcinogens (group 2B) (5). Occupational exposure to coke-oven emission in coke production has been associated with an excess risk from cancer of respiratory system (particularly of lung), prostate and urinary tract (6-9). In particular in a recent study of Bosetti et al. 2007 an excess risk from lung/respiratory cancers, relative risk RR=1,58 (95% CI 1.47-1.69), was reported for coke production (7). Coke-oven workers could be chronically exposed both by inhalation and dermal contamination to polycyclic hydrocarbons (PAHs) during some processes such as the charging of coal into the oven.

Several studies on coke oven workers have evaluated cytogenetic endpoints such as chromosome aberrations, sister chromatide exchanges (SCE), micronuclei or DNA adducts but with contradictory results. In several studies biological effects like DNA adducts, micronuclei, SCE, DNA strand breaks and 8-hydroxy-2'-deoxyguanosine levels were shown to be increased due to PAHs exposure (10-16). In other studies no effects were observed (17-20). It is well known the capacity of PAHs to induce genotoxicity and carcinogenicity through direct interaction of PAH metabolites with DNA with consequent mutations in critic genes and carcinogenic process (21). Moreover, an increasing number of evidences demonstrate the involvement of Reactive Oxygen Species (ROS) in carcinogenesis by PAHs through the induction of oxidative DNA damage (22, 23).

In this study we evaluate the genotoxic and oxidative effects induced on lung epithelial cells (A549) from PAHs obtained by organic extract of airborne particulate matter (PM10) collected in the oven area of an Italian coking plant. The aim was to clarify the mechanisms of complex mixtures of PAHs at low doses, present in some occupational settings, and to identify biomarkers of early DNA damage useful as early indicators of biological effect.

Materials and Methods

Environmental monitoring

Airborne particulate matter (PM10) was collected in the oven area of an Italian coking plant. We used a GILAIR-5 air sampler (Gilian, USA) with a flow rate of 3 lt/min to collect PM10 on glass filter. The PAHs bound to respirable particulate matter on glass filter were extract with toluene by ultrasound. The obtained solutions were air dried, dissolved in 300 μl of toluene and analysed by GC/MS (GC System 6890 and HP Mass Selective Detector 5973) equipped with capillary column SE52 and mass detector. The analysis was performed by single ion technique using as standard the 16 PAHs listed by Environmental Protection Agency (EPA). The analysed PAHs were Pyrene, Benzo(a)anthracene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(a)pyrene, Benzo(g,h,i) perylene, Dibenzo(a,h)anthracene.

Cell culture and exposure conditions

A commercially available human lung epithelial cell line (A549) was used. The cells obtained from the American Type Culture Collection (ATCC) (Rockville, MD) were cultured in RPMI 1640 (EuroClone, United Kingdom) supplemented with 10% fetal calf serum, at 37°C in 5% CO_2.

The A549 cells were seeded into 15,6 mm diameter culture dishes (7x 10^4 cells/dish) and cultured for 24h before the exposure.

Semiconfluent cell cultures were exposed for 30 min, 2h and 4h to extract of half filter dissolved in DMSO and diluted at 0.004, 0.008 and 0.02%. Unexposed cells were used as negative control.

Fpg comet assay

We used comet assay modified with Fpg enzyme that recognizes and cuts the oxidized DNA bases indirectly allowing the detection of oxidative DNA damage. The experiments were performed at least in duplicate. The procedure of Collins et al. (1993) (24) was followed. Slides were examined at 200X magnification under a fluorescent microscope. Images of 50 randomly selected comets stained with ethidium bromide either from Fpg enzyme treated or untreated slides, were acquired and analyzed from each sample, with specific image analyzer software (Delta Sistemi, Rome, Italy). Measurements of Comet parameters: % DNA in the tail, tail length and tail moment (the

product of relative tail intensity and length, that provides a parameter of DNA damage), were obtained from the analysis. For each experimental point we calculated the mean tail moment of 50 comets from enzyme untreated cells (TM), which indicates the direct DNA damage, and the mean tail moment for Fpg enzyme treated cells (TMenz) (directly proportional to the number of oxidized DNA bases) indicating the oxidative DNA damage. The oxidative DNA damage was evaluated comparing TMenz value of exposed cells in respect to unexposed at each experimental point. The direct DNA damage was evaluated comparing TM of exposed in respect to unexposed cells. Moreover about 1000 cells from each slide were examined for presence of comets (cells with a detectable tail) by an experienced observer and the percentages of comets were calculated. The comet percentages values of exposed cells were compared with that found in unexposed cells at each experimental point. Treatment-related differences were evaluated using Student's t-test. A difference was considered significant at $p \leq 0.05$.

Results

Environmental monitoring

The monitored air samples contained 0.328 μg/m^3 of pyrene, 0.33 μg/m^3 of benzo(a)anthracene, 1.073 μg/m^3 of benzo(b)fluoranthene, 0.22 μg/m^3 of benzo(k)fluoranthene, 0.35 μg/m^3 of benzo(a)pyrene, 0.079 μg/m^3 of dibenzo(a,h)anthracene and 0.40 μg/m^3 of benzo(g,h,i)perylene.

Fpg comet assay

The organic extract of half filter, used for *in vitro* exposure, contained 425 ng of pyrene, 428 ng of benzo(a)anthracene, 1389 ng of benzo(b)fluoranthene, 289 ng of benzo(k)fluoranthene, 455 ng of benzo(a) pyrene, 103 ng of dibenzo(a,h)anthracene and 518 ng of benzo(g,h,i)perylene. Cells were exposed to 0,004, 0.008 and 0.02% dilutions of such extract. Comet results showed a slight dose-time dependent increase, although not significant, of comet percentage in cells exposed to extract in respect to unexposed (Fig. 1). Analysis of tail moment values (TM and TMenz) showed a slight dose-dependent increase at all exposure times indicating a small, not statistically significant, both direct and oxidative DNA damage induction (Fig. 2).

Discussion and Conclusion

In the present work we analyze the amount of some potential carcinogenic PAHs bound to respirable particulate matter collected in the oven area of an important Italian coke plant. Environmental monitoring showed low concentrations of PAHs including the carcinogen benzo(a)pyrene and the probable/possible carcinogens benzo(a)anthracene, dibenzo(a,h)anthracene, benzo(b)fluoranthene and benzo(k)fluoranthene.

The low levels of PAHs found in air correlate with comet results that did not show significant genotoxic and oxidative effects on lung epithelial cells exposed to the used dilutions of PM10 extract. However a slight dose-dependent increase of TM and TMenz was found in exposed in respect to unexposed cells. These findings indicate a small, not significant, induction of both direct and oxidative DNA damage on pulmonary cells (A549) exposed to complex PAHs mixtures.

The most available *in vitro* studies on genotoxic effects of PAHs mixtures from airborne PM10 concern organic extracts of urban air particulate matter (25-32) for which mutagenic effects, DNA strand break, DNA adducts and oxidative DNA damage induction were reported. Several studies are also available on genotoxic effects of complex PAHs mixtures contained in cigarette smoke extract for which DNA damage, oxidative stress, apoptosis, SCE and chromosomal aberrations were reported (33-36). This one represents the first *in vitro* study on genotoxic and oxidative effects of organic extract of PM10 collected in coke plant, on cell model reproducing target organ. Our study shows the high sensitivity of comet assay to measure early DNA damage also at low doses suggesting its use on lung epithelial cells to evaluate the effects of complex mixtures of genotoxic substances on target organ. Our findings contribute to assess the final effect of interaction of various PAHs contained in complex mixture that can induce synergistic, antagonistic and additive effects. It is particularly relevant

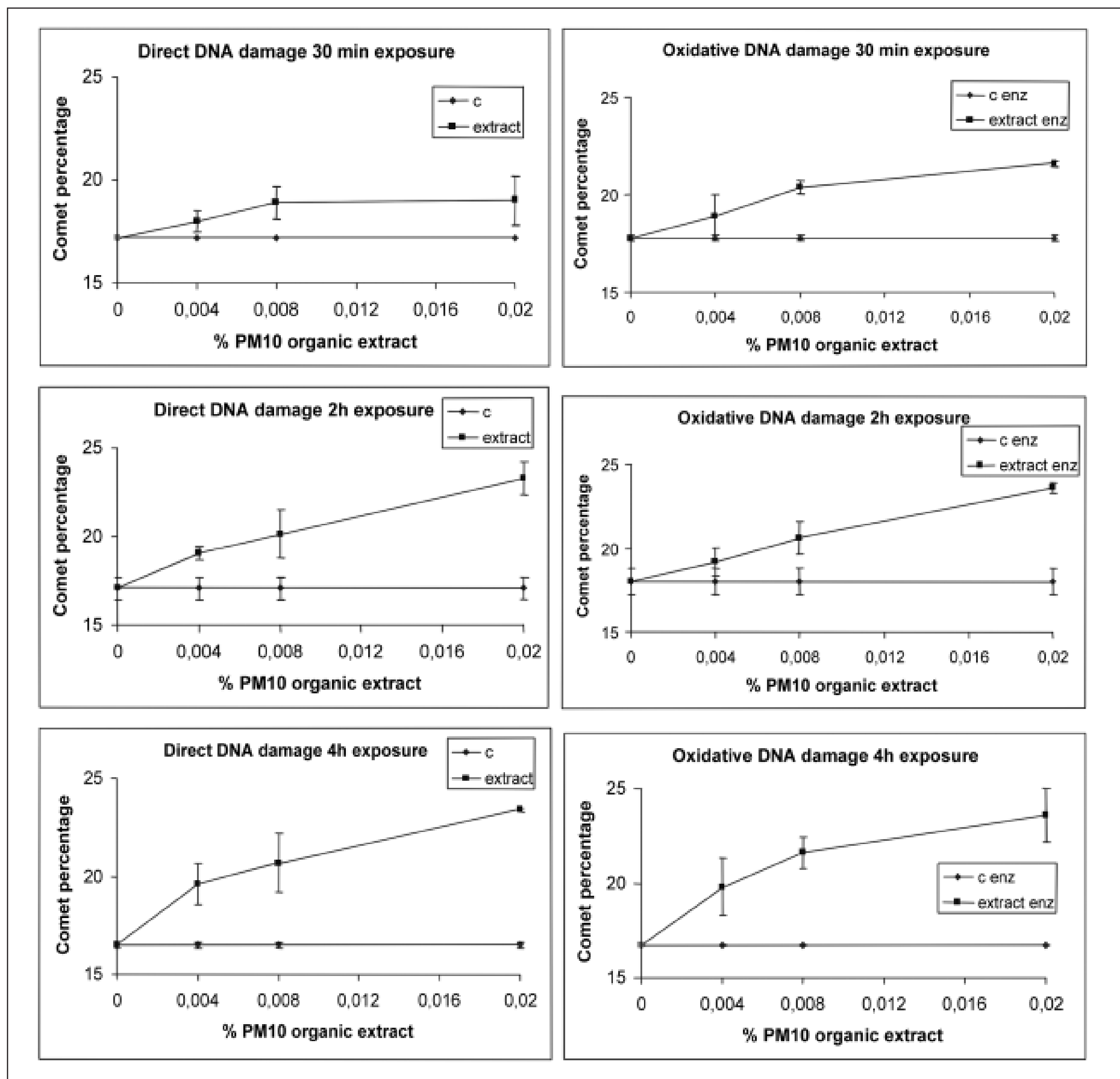

Figure 1. Comet percentages in A549 cells exposed for 30 min, 2 and 4 hours to PM10 extract evaluated by Fpg-Comet test. The left panels are related to comet percentage in cells untreated with Fpg enzyme, the right panels are related to comet percentage in Fpg enzyme treated cells. The experiments were performed in triplicate. DNA damage was evaluated comparing comet percentage value of exposed cells in respect to unexposed at each experimental point by Student's t test. Not statistically significant differences between exposed and unexposed cells were found

since complex mixtures of PAHs are present, also at very low doses, in several occupational settings such as coke plant, steel plant, airport, urban environment, asphalt paving, waste-incineration energy plant and in living environment.

Moreover our study furnishes useful information to identify biomarkers of early DNA damage useful as early indicators of biological effect.

The identification of early biomarkers of genotoxic and oxidative effects for occupational exposure to

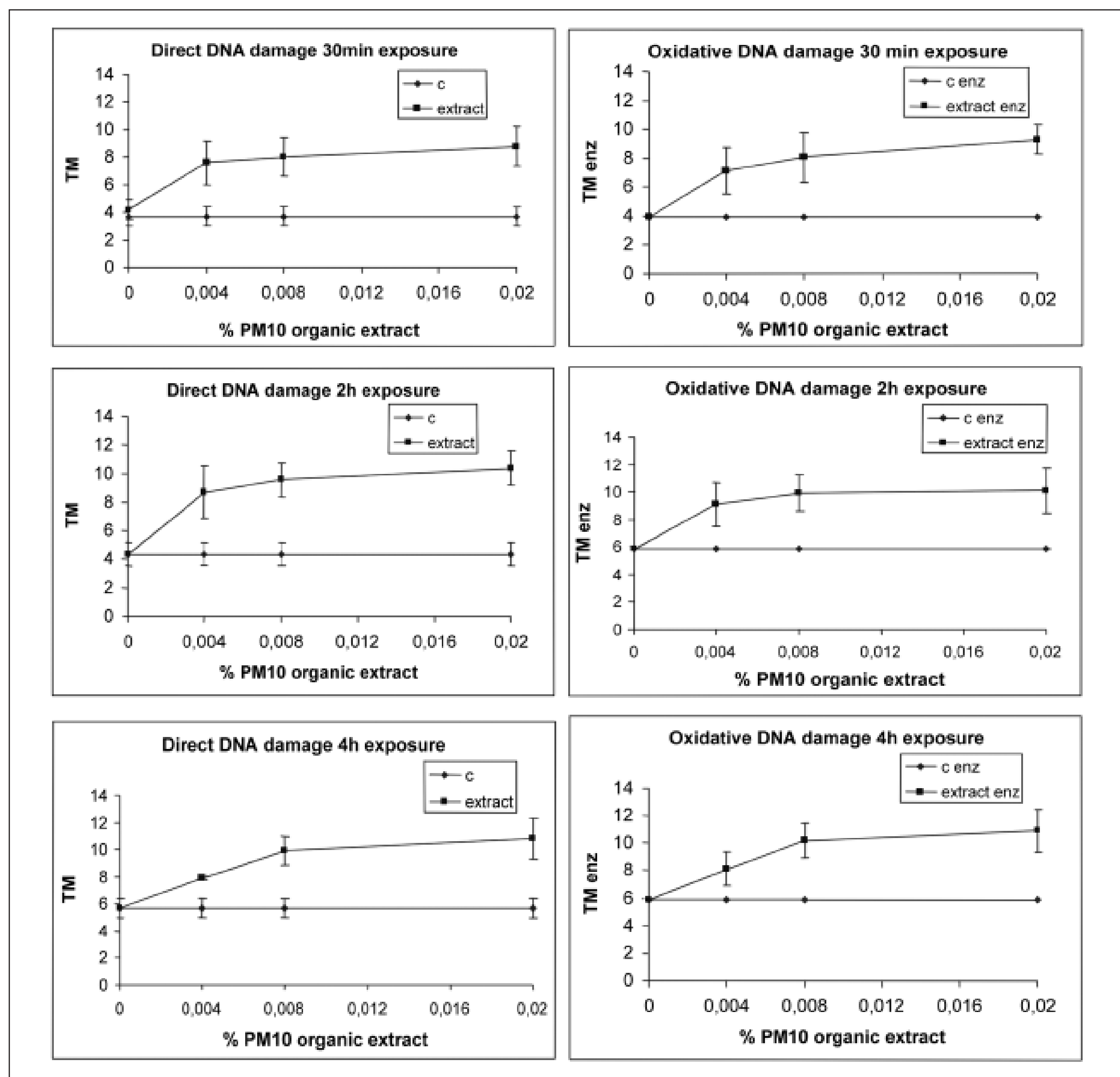

Figure 2. Direct and oxidative DNA damage induced in A549 cells by 30 min, 2 and 4 hours exposure to PM10 extract, evaluated by Fpg-Comet test. The left panels are related to Tail moment (TM) ± SD of cells untreated with Fpg enzyme, the right panels are related to Tail moment of Fpg enzyme treated cells (TMenz) ± SD. The experiments were performed in triplicate. At each experimental point Student's t test was used to compare TM value (indicative of direct DNA damage) and TMenz value (indicative of oxidative DNA damage) of exposed cells in respect to unexposed cells. Not statistically significant differences between exposed and unexposed cells were found

complex mixtures of PAHs in coke production, together with an accurate evaluation of PAHs exposure, may lead to a correct risk assessment and to the adoption of effective preventive measures to reduce exposure to coke oven emissions and related health effects. The consequences of the changes in such biomarkers, such as risk to develop cancer, warrant further investigations.

Acknowledgements

The research was supported by the Ministry of Health within the project "Strategie ed indicatori innovativi per la valutazione delle interazioni gene-ambiente nei tumori polmonari professionali" (PMS/026/2003).

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Accepted: May 15th 2008
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