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Angiogenic activity *in vivo* of the particulate matter (PM10)

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ABSTRACT

Background: Particulate matter (PM) is the most efficient vehicle for the inhalation and absorption of toxic substances into the body.
Method: The present study was aimed at testing the hypothesis that PM10 samples collected on quartz filters exert an angiogenic activity *in vivo* in the chick embryo chorioallantoic membrane (CAM) assay.
Results: When the low, medium, and high PM10 concentrations filters were tested in the CAM assay, an increasing number of microvessels was detectable after 4 days of applications of the filters. Moreover, at histological level, numerous microvessels and a dense inflammatory infiltrate were recognizable in the CAM mesenchyme.
Conclusion: Our data show a clear dose-response relationship between the dose variable (PM10 and Bap) and the outcome variable. So far, the PM10 target value is determined on the basis of regulatory agreements and is not health-based. In addition, the mere gravimetric measure of PM10 cannot be considered a fully reliable surrogate of the overall toxicity of the mixture.

1. Introduction

The broad term "particulate matter" (PM) includes solid and liquid particles that can remain suspended in the atmosphere due to their small size. According to the aerodynamic diameter, particles can be classified as PM10 (< 10 μm diameter) and PM2.5 (< 2.5 μm). Respirable PM includes and adsorbs several organic and inorganic compounds; many of them are toxic and/or carcinogenic as the polycyclic aromatic hydrocarbons (PAHs) and heavy metals (Keukena et al., 2011; Azarmi et al., 2016).

Although PAHs contribute minimally to PM mass (less than 0.1%), they contribute predominantly to the toxicity of PM10 in urban areas. Some PAHs and some of their degradation products are known to have a high carcinogenic, mutagenic, teratogenic and allergenic potential, posing a threat to human health (Abdel-Shafya and Mansourb, 2016). PM is the most efficient vehicle for the inhalation and absorption of toxic substances into the body. Currently, air pollution from particulate matter is responsible for 3.2 million deaths per year (Lim et al., 2012).

Different potentially carcinogenic compounds mixture, including cigarette smoke (Wang et al., 2013), nicotine (Mousa et al., 2006), arsenic (Kao et al., 2003), dioxin (Ishimura et al., 2009), and trimethylchloride (Wang et al., 2013) have been suggested to induce pro-angiogenic effects, namely the ability to induce the formation of new vessels starting from pre-exisiting ones.

The present study was aimed at testing the hypothesis that PM10 samples collected on quartz filters exert an angiogenic activity *in vivo* in the chick embryo chorioallantoic membrane (CAM) assay.

2. Materials and methods

2.1. Sampling sites and collection

Taranto is the third most populated city in southern Italy. The most important seaport in southern Italy and one of the biggest steel plants in Europe are nearby the urban area. Moreover, a petrochemical center, a cement plant, a military and trade harbor, and a naval ship-repairing industry are sited close to the urban area. Daily PM10 samples were collected at "Cimitero" site, close to the industrial area. PM10 samples were collected on quartz filters (Whatmann, 47 mm diameter) by using a dichotomous low volume sampler with a flow rate of 2.3 $m^3 h^{-1}$

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(SWAM Dual Sampler, FAI Instruments s.r.l., Roma, Italy). The sampler was equipped with a sampling head for PM10 certified US EPA and UNI EN 12341:2014. In accordance with UNI EN 1234-1, the sampling head is able to select the particulate material of aerodynamic diameter lower or equal to 10 μm, with a sampling efficiency of 50%.

2.2. Determination of PM10

PM samples were conditioned by using a system supplied with a control system for the temperature and the humidity (20 ± 1 C° and 50 ± 5% RH) (Activa Climatic, Aquaria, Milano, Italy) and then weighted by the analytical balance (Sartorius series Genius, mod. SE2, Germany), with sensitivity of 0.0001 mg for mass determination.

2.3. Analysis of PAHs and N-PAHs

Polycyclic aromatic hydrocarbons (PAHs) and nitrated PAHs (N-PAHs) was obtained through a microwave assisted solvent extraction of an half of filter with acetone/hexane mixture (1:1) (Milestone s.r.l., model Ethos D, Sorisole (BG), Italy). Then, extracts were analyzed using a gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA USA) equipped with a programmable temperature vaporization injection system (PTV) and interfaced with a quadrupole mass spectrometer (Agilent MS-5973 N) to detect PAH concentration. The identification of each PAH was performed using Perylene-D12 (PrD, 264) as the internal standard (IS). The analytical performance of the whole procedure (extraction recovery, extraction linearity, analytical repeatability, LOD) was verified in our previous study (Bruno et al., 2007). N-PAH identification was conducted by using a Agilent 7000 A gas chromatograph (Agilent Technologies, Wilmington DE) equipped with a PTV and interfaced to a triple quadrupole mass spectrometer (QQQ) with an inert ion source (Agilent MS-5975). N-PAH quantitative determinations were performed in Multiple Reaction Monitoring (MRM) mode. The retention time (RT), the internal standard used (IS), precursor and product ions monitored for each NPAHs and deuterated (PAHs-d) are listed in Table 1.

2.4. Analysis of metals

A quarter of the sampled filter was used for element composition of PM. In detail, the filter was acid digested in 8 ml nitric acid and 2 ml hydrogen peroxide as reported by EN 14902:2005. The acid digested was performed in a microwave system (Milestone mod. Ethos D) that allowed to reach approximately 180 C° within 20 min (500 W), to increase slowly the temperature up to approximately 220 °C and then to hold the temperature for about 20 min (500 W). After the extract was transferred into labeled volumetric flasks (50 ml) containing ultrapure water (18.2 MΩ cm) and then analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES).

Table 1
Retention time (RT), precursor ions and product ions monitored for each NPAHs and for deuterated PAHs used as Internal standard (IS).

Compounds	Retention time	Precursor ion	Product ion
2 - Nitrofluorene	15.23	165	139
9 - Nitroanthracene	15.67	176	165
9 - Nitrophenanthrene	16.74	165	165
3 - Nitrophenanthrene	17.39	223	176
2 - Nitroanthracene	18.19	223	176
Benzo(a)anthracene-D12 (IS)	19.60	240	236
3 - Nitrofluoranthene	22.02	200	174
1 - Nitropyrene	23.15	201	176
2,7 - Dinitrofluorene	24.58	163	163
7 - Nitrobenz[a]anthracene	26.89	226	200
6 - Nitrochrysene	25.48	226	200
Perilene - D12 (IS)	26.85	264	260
1,3 - Dinitropyrene	31.46	200	174
1,8 - Dinitropyrene	28.68	200	174
1,6 - Dinitropyrene	31.11	200	174
6 - Nitrobenz[a]pyrene	34.70	267	239

2.5. Equivalent toxicity

The carcinogenic potency of total PAHs and N-PAHs (*i.e.*, total BaPeq) was calculated summing the benzo[*a*]pyrene equivalent concentrations (BaPeq) of each PAH and N-PAH. Calculation of the BaPeq concentration for a given compound uses its toxic equivalent factor (TEF), which represents the relative carcinogenic potency of the given PAH compound using benzo[*a*]pyrene as a reference compound, to adjust its original concentration. In this study the TEFs completed by Nisbet and LaGoy (1992) were employed.

2.6. In vivo angiogenesis assay

Fertilized White Leghorn chicken eggs (30 per group) were incubated at 37 °C at constant humidity. On day 3, a square window was opened in the shell, and 2–3 ml of albumen were removed to allow detachment of the developing CAM. The window was sealed with a glass, and the eggs were returned to the incubator. On day 10, a small piece of 10 quartz filters for each experimental group with low, medium, and high PM10 concentration was placed directly onto the CAM surface. Ten small pieces of blank quartz filters have been used as negative controls. A PM10 monitoring campaign lasting one month was carried out at the site of interest. Then, three PM10 samples to be tested were selected and identified as low (PM10 conc. equal to 35.4 $\mu g/m^3$), medium (PM10 conc. equal to 71.7 $\mu g/m^3$) and high (PM10 conc. equal to 103.2 $\mu g/m^3$).

CAMs were examined daily until day 14 and photographed *in ovo* under a stereomicroscope equipped with a camera and image analyzer system (Olympus Italia, Italy). CAMs were excised and then fixed with Karnovsky's fixative for 2 h at room temperature. Finally, CAMs were processed for light microscopy. CAM portions were removed and embedded in paraffin. Ten sections 10 μm thick were cut according to a plane parallel to the surface of the CAM for each blank quartz filters, low, medium, and, respectively, high PM10 concentration quartz filters, were stained with haematoxyl-eosin, and observed under a Leitz-Dialux 20 light microscope (Leitz, Wetzlar, Germany).

2.7. Image analysis

Microscopic images obtained from the stereomicroscope were converted in gray-scale and analyzed using the Aperio Positive Pixel Count algorithm embedded in the ImageScope v.11.2.0.780 (Leica Biosystems, Nussloch, Germany). All the images were analyzed with the exclusion of the quartz filter area using the negative pen tool to eliminate non-specific detection in this area. The algorithm input parameters were initially set to obtain the identification of pixels related to the blood vessels as strong positive and to the background as medium and weak positive and tuned to minimize non-specific pixel recognition as strong positive. The algorithm output is composed of the number of strong positive pixels (Nsp), the number of medium positive pixels (Np), the number of weak positive pixels (Nwp). A morphometric value is then defined and calculated by the algorithm as: $Number\ of\ strong\ positive\ pixels\,(\%) = \frac{Nsp}{Np + Nwp + Nsp} \times 100$

All the analysis were performed on images with equal area. Fold change data are reported as means ± SD. The Graph Pad Prim 5.0 statistical package (GraphPad Software, San Diego, CA, USA) was used for the analysis and $P < 0.05$ was considered as the limit for statistical significance.