

## Seasonality effects on lung and systemic markers following intratracheal instillation of pm10 and pm1

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### 1. Introduction

The World Health Organization (WHO) recently reported that over 800,000 deaths worldwide per year can be attributed to particulate matter (PM) air pollution. Recent epidemiological studies have linked short-term and long-term PM exposure to increased mortality. Inhalation of PM is known to cause inflammation in the lungs. The resulting pulmonary inflammation can lead to activation of the pulmonary endothelium and subsequent systemic inflammation (Cozzi et al., 2006). The major sources of ambient PM include automobile and power plant combustion processes, mechanical processes, and environmental dust.

Wilson and Suh (1997) provided a detailed description of the occurrence and composition of coarse (aerodynamic diameter  $<10\mu\text{m}$ ) and fine (aerodynamic diameter  $<1\mu\text{m}$ ) PM and concluded that they are separate classes of pollutants and should be measured separately in epidemiology and toxicology studies. Coarse PM typically derives from soil or abrasive mechanical processes in transportation or industry, and also can contain biogenic materials such as pollen, endotoxin, and mold spores known to have deleterious effects on human health, especially in those with pulmonary diseases such as asthma (Graff et al., 2009).

There is evidence to suggest differential toxicity of exposure to PM of varying size fractions. Because of the size of the particles, they can penetrate the deepest part of the lungs: PM10 can settle in the bronchi and in the proximal portion of the lungs (Kim and Hu, 1998); the smallest particles may be even more damaging to the cardiovascular system (Pope et al., 2002). Because lung deposition and chemical composition of fine and coarse PM are generally dissimilar, coarse PM may produce biologic activity and health effects distinct in nature and severity from those effects seen with exposure to fine PM. Therefore, we evaluated the different inflammatory and cytotoxic potential of PM10 collected in an urban area in Milano in two seasons (summer and winter) and of PM1 collected in the same area and in the same seasons. (Contract Grant Sponsor: Fondazione Cariplo).

## 2. Materials and methods

### 2.1 Animals

Male BALB/c mice (8 weeks old; Harlan) were used; food and water were administered *ad libitum*. The mice were housed in plastic cages under controlled environmental conditions (temperature 19-21°C, humidity 40-70%, lights on 7 a.m.- 7 p.m.). The established rules of animal care approved by Italian Ministry of Health (DL 116/92) were followed.

### 2.2 PM sources and endotoxin analysis

Atmospheric PM<sub>10</sub> and PM<sub>1</sub> were collected during summer (PM sum) or winter (PM win) in a Milano urban area as described (Gualtieri et al., 2010). Endotoxin content of PM was determined using a quantitative chromogenic Limulus Amebocyte Lysate (LAL) test (Pyrochrome-LAL test, Associates of Cape Cod, Inc.) following the manufacturer's instructions (Camatini et al., 2009).

### 2.3 Intratracheal PM instillation

Male BALB/c mice were briefly exposed to a mixture of 2.5% isoflurane (Flurane), 70% O<sub>2</sub> and 30% NO<sub>2</sub> anesthetic gas. Mice were intratracheally instilled by means of MicroSprayer® Aerosolizer system (MicroSprayer® Aerosolizer- Model IA-1C and FMJ-250 High Pressure Syringe, Penn Century, USA) with 100µl of NaCl 0.9% containing 100µg of PM<sub>10</sub> and euthanized at different times (3h, 24h); alternatively, mice were instilled with 100µl of a solution containing 100µg of PM<sub>1</sub> for three times, once every two days, and euthanized 24h after the last instillation. Control mice, running parallel to PM treated ones, were instilled with 100µl of saline solution. Treated and control mice were allowed to recover under visual control before placing them back in plastic cages. At different times, mice from each experimental group were euthanized, the trachea was exposed, cannulated, and three in-and-out washes with 0.6ml of isotonic saline solution were performed. The efficacy of BALF (BronchoAlveolar Lavage Fluid) collection ranged from 50 to 90% of the total solution injected. The BALF was centrifuged at 1500g for 15min at 4°C and pellets collected for cell counts. Supernatants were divided into aliquots and stored for subsequent biochemical analyses.

### 2.4 BALF analyses

#### *Cell counts*

After centrifugation, the BALF pellets were resuspended in 500µl of DMEM (10% FBS, 1% penicillin-streptomycin, 1% glutamine), and total cell counts performed with a Burker chamber, using the Trypan Blue exclusion method. A cell aliquot (240000 cells, 800 cells/µl) was smeared in duplicate onto slides using Cytofuge 2 (StatSpin, USA) 40g for 7 min at room temperature. The smears were stained with Diff Quik (Medion Diagnostic) for cell differential count, according to manufacturer instructions. Macrophages, polymorphonuclear leukocytes (PMNs) and lymphocytes were identified by their characteristic shapes.

#### *Biochemical analyses*

Total protein content was measured at 570 nm according to BCA method (Sigma Aldrich, USA). The commercially available kits for alkaline phosphatase (DALP-250

QuantiChrom Alkaline Phosphatase Assay Kit, Gentaur Molecular) and lactate dehydrogenase (DLDH-100 QuantiChrom Lactate Dehydrogenase Kit, Gentaur Molecular) were employed according to the manufacturers' instructions.

#### *Cytokine analyses*

The analyses of pro-inflammatory cytokine released in the BALF were performed by DuoSet ELISA kit for tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; R&D Systems) according to the manufacturer's protocols.

### 3. Results

#### 3.1 BALF analyses: PM10win and PM10sum

##### *Cellular inflammatory response (Table 1)*

BALF was analyzed for cellular indicators of inflammation and the percentage of different cell types was investigated as an inflammatory marker. Despite an extremely variety of results, total cells number never provide a clear indication of inflammatory status, as they didn't differ from that of control mice at any of the considered times. Otherwise, differential counts showed an increasing trend in PMNs percentage, that became statistically significant 24h after instillation of PM10sum and PM10win. At the same time, a decrease in AMs (alveolar macrophages) percentage was observed, and it became significant at 24h for both the PM10. No statistical difference in lymphocyte percentage was observed between Control and PM10 treated groups.

*Table 1 Cellular inflammatory response*

Total Cells (E+06)	mean $\pm$ SE	mean $\pm$ SE			
	3h	24h			
Co	2,76 $\pm$ 1,16	3,49 $\pm$ 0,89			
PM10win	1,55 $\pm$ 0,39	4,19 $\pm$ 1,07			
PM10sum	5,96 $\pm$ 1,84	2,28 $\pm$ 0,52			

  

AMs (%)	mean $\pm$ SE	mean $\pm$ SE	PMNs (%)	mean $\pm$ SE	mean $\pm$ SE
	3h	24h		3h	24h
Co	74,5 $\pm$ 6,21	68,1 $\pm$ 5,83	Co	15,83 $\pm$ 6,64	23,44 $\pm$ 4,81
PM10win	52,7 $\pm$ 14,82	29,4 $\pm$ 11,8	PM10win	45,64 $\pm$ 14,22	66,36 $\pm$ 12,79
PM10sum	64,6 $\pm$ 2,34	16 $\pm$ 4,94	PM10sum	30,4 $\pm$ 2,52	81 $\pm$ 4,97

##### *Cytotoxicity (Table 2)*

Concerning total proteins content, no statistically significant differences were observed 3h or 24h after PM10win and PM10sum instillation. LDH and ALP activities didn't show modifications between Control and treated mice, except a significant increase in LDH activity 24 after the instillation of PM10win. Moreover, it can be observed an ascending trend in total proteins in PM10sum treated mice at 3h, comparing to respective Control, and the same trend was also present in ALP activity at 3h and 24h for PM10sum treated mice.

*Table 2 Cytotoxicity*

Total Protein (mg/ml)	mean±SE	mean±SE	LDH (IU/L)	mean±SE	mean±SE
	3h	24h		3h	24h
Co	0,359±0,04	0,497±0,07	Co	20,23±2,67	13,75±2,38
PM10win	0,344±0,102	0,452±0,05	PM10win	17,36±0,3,29	23,81±2,7
PM10sum	0,56±0,178	0,336±0,05	PM10sum	16,9±206	17,06±0,1,78
ALP (IU/L)	mean±SE	mean±SE			
	3h	24h			
Co	0,137±0,019	0,143±0,043			
PM10win	0,16±0,055	0,102±0,027			
PM10sum	0,222±0,038	0,204±0,053			

*Cytokine release (Table 3)*

A significant increase in TNF- $\alpha$  was evident at 3h in BALF of both PM10sum and PM10win instilled mice. At 24h, TNF-a levels were notably lower.

*Table 3 Cytokine release*

TNF-a (pg/ml)	mean±SE	mean±SE
	3h	24h
Co	196,59±67,1	71,32±15,37
PM10win	1443,3±319,17	179,93±19,2
PM10sum	2292,4±239	205,24±32,68

**3.2 BALF analyses:PM1win and sum***Cellular inflammatory response (Table 4)*

Total cells number showed a significant increase 24h after the last instillation of PM1win, while no changes were obtained after PM1sum treated mice. Otherwise, differential counts showed an increasing trend in PMNs percentage only in PM1win treated mice. At the same time, a decrease in AMs percentage was observed, but these changes never became significant. No statistical difference in lymphocyte percentage was observed between Control and PM1 treated groups.

*Table 4 Cellular inflammatory response*

Total cells (E+06)	mean±SE		
Co	0,72±0,14		
PM1win	1,33±0,11		
PM1sum	1,09±0,29		
Macrophages (%)	mean±SE	PMNs (%)	mean±SE
Co	90,35±2,79	Co	9,42±2,75
PM1win	69,55±8,68	PM1win	29,35±8,76
PM1sum	84,72±5,86	PM1sum	14,27±5,51

*Cytotoxicity (Table 5)*

Concerning total protein content, no statistically significant differences were observed 24h after the last instillation of both summer or winter PM1. LDH activity was significantly higher both PM1win and PM1sum treated mice; on the contrary, ALP activity didn't show any statistically significant modification between Control and treated mice.

*Table 5 Cytotoxicity*

Total Protein (mg/ml)		LDH (IU/L)	
	mean±SE		mean±SE
Co	0,267±0,029	Co	19,41±1,5
PM1win	0,252±0,038	PM1win	30,02±1,05
PM1sum	0,312±0,052	PM1sum	25,6±1,33

  

ALP (IU/L)	
	mean±SE
Co	0,067±0,023
PM1win	0,154±0,026
PM1sum	0,160±0,033

*Cytokine release (Table 6)*

No significant increase in TNF-a was evident at 24h in BALF of both PM1 sum and PM10win instilled mice, comparing to Control group.

*Table 6 Cytokine release*

TNF-a (pg/mL)	mean±SE
Co	22,3±6,89
PM1win	27,13±12,83
PM1sum	14,57±2,74

**4. Discussion**

Particulate matter pollutant has been found to cause most serious effects on human health because of the broad range of diverse toxic substances that particles contain, and also depending on their different dimensions. Intratracheal instillation can provide appropriate and relevant information on the potential toxicity and lung injury caused by inhalable particulates.

The significant augment of PMNs percentage in PM10win and PM10sum treated mice confirm the pro-inflammatory potential of the coarse fraction of particulate matter; the highest mean was reached with PM10sum, showing an important contribute to inflammation due to the major endotoxin content. The presence of Gram- bacteria and endotoxins in the urban PM has been widely documented (Heinrich et al., 2006), especially in the coarse fraction (Monn and Becker, 1999); some studies suggest that the pathological effects of PM10 should be due to their endotoxin content (Monn and Becker, 1999) and that the endotoxin concentration is low during spring and winter, while it grows in summer (Heinrich et al., 2006). Consistent with this result is the concentration of TNF-a measured in BALF: the

highest levels were reached 3h after the instillation of PM10sum, even though also PM10win causes a significant increment comparing with control mice. Endotoxin content seems instead unable to induce cytotoxicity in intratracheally instilled mice, while PM10win cause an increment in LDH activity; probably, cytotoxicity is most strictly related to some chemical components present in higher concentration in PM10win than in PM10sum. While PM10 can settle in the bronchi and lungs, the smallest particles may be even more damaging to the cardiovascular system (Pope et al., 2002). Particles smaller than 100 nanometers can pass through cell membranes and migrate into other organs, including the brain (Kleinman et al., 2008). In our experiments, both PM1sum and PM1win show a low pro-inflammatory potential on lungs, but PM1win seems to be a little more effective in recruiting PMNs. Both these PM1 cause an increment in LDH activity, measured in BALF. So, consistently with the observations reported in other studies, the fine fraction of particulate matter demonstrates to affect and involve lungs less than the coarse fraction; in the next months, our research will direct on systemic effects correlated to PM1 instillation, i.e. inflammation, cytotoxicity and oxidative stress markers in heart and brain and coagulation markers in plasma of mice intratracheally instilled with PM1win and sum.

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