Chemical characterisation and cytotoxic effects in A549 cells of urban-air PM10 collected in Torino, Italy.

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Abstract: Human type II alveolar cell line (A549) was exposed to aqueous and organic solvent PM10 extracts in order to evaluate cell proliferation, proinflammatory cytokines and cytotoxicity (lactate dehydrogenase , LDH). PM10 samples, collected in Torino (north-west Italy), were analyzed for inorganic chemical species (bioavailable iron and secondary particulates) and endotoxins, potentially inflammatory promoters to human airways.
Meanly, in the considered period, PM10 concentration was 55.4 ± 39.1 µg/m3, iron concentration was 0.078 ± 0.095 and secondary particles constituted 42 ± 9 % of the PM10 total mass. PM10 inhibits cell proliferation and induces both IL-6 and LDH release in a dose and time dependent manner, with a seasonal trend. The different roles of aqueous and organic solvent PM10 extracts demonstrate the importance of particle composition for the biological effects. Nevertheless few significant correlations were found between the biological effects and PM10 components evaluated in this methods development study.

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*** Cover Letter**

Dear Editor,

We are sending the manuscript "*Cytotoxic effects induced on A549 cells and chemical characterization of urban-air PM10 collected in Torino (Italy)*" that we submit for possible publication on *Environmental Toxicology and Pharmacology.*

PM10 samples were collected in an urban site of Torino, an industrial north-western Italian city, with high levels of particulate matter among European cities. PM10 samples were analyzed for the amount of inorganic chemical species (bioavailable iron and secondary particulates: sulfates and nitrates) and endotoxins, described as potentially inflammatory promoters to human airways. In order to better understand how ambient particulate matter affects lung health, A549 cells were exposed to aqueous and organic solvent PM10 extracts. Effects on cell proliferation, on the release of proinflammatory cytokines and cytotoxicity, measuring lactate dehydrogenase (LDH) release in the extracellular medium, were evaluated.

In general PM10 inhibits cell proliferation and induces both IL-6 and LDH release in a dose and time dependent manner and these biological effects have a seasonal trend. Few significant correlations were found between the biological effects and PM10 components evaluated in this methods development study. Nevertheless the different roles of aqueous and organic solvent PM10 extracts demonstrate the importance of particle composition for the biological effects.

Best regards

Tiziana Schilirò

CYTOTOXIC EFFECTS INDUCED ON A549 CELLS AND CHEMICAL CHARACTERIZATION OF URBAN-AIR PM10 COLLECTED IN TORINO (ITALY).

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Running head: Biological effects of PM10 induced on A549 cells.

Abstract

Human type II alveolar cell line (A549) was exposed to aqueous and organic solvent PM10 extracts in order to evaluate cell proliferation, proinflammatory cytokines and cytotoxicity (lactate dehydrogenase , LDH). PM10 samples, collected in Torino (north-west Italy), were analyzed for inorganic chemical species (bioavailable iron and secondary particulates) and endotoxins, potentially inflammatory promoters to human airways.

Meanly, in the considered period, PM10 concentration was 55.4 ± 39.1 μg/m^3, iron concentration was 0.078 ± 0.095 and secondary particles constituted 42 ± 9 % of the PM10 total mass. PM10 inhibits cell proliferation and induces both IL-6 and LDH release in a dose and time dependent manner, with a seasonal trend. The different roles of aqueous and organic solvent PM10 extracts demonstrate the importance of particle composition for the biological effects. Nevertheless few significant correlations were found between the biological effects and PM10 components evaluated in this methods development study.

Keywords: Particulate Matter, Cell Proliferation, Cytotoxicity, LDH, Cytokine.

1. Introduction

The health burden due to particulate matter (PM) air pollution is one of the biggest environmental health concerns in the World Health Organization (WHO) European Region and all around the world. PM is a complex and heterogeneous mixture, whose composition (particle size distribution and chemical characteristics) changes in time, space and depends on emissions from various sources, atmospheric chemistry and weather conditions (WHO, 2007).

The breathable fraction of ambient PM is often referred to PM10, defined as particles with a median aerodynamic diameter less than 10 μm, that is a quality air indicator widely measured (WHO, 2006). PM10 may further be divided into two main size fractions, a “coarse” (2.5–10 μm) and a “fine” (0.1–2.5 μm). The c*oarse* fraction is dominated by natural sources (geological material: fugitive and resuspended dust; biological material: pollen, endotoxin) and its composition changes depending on the geology of the site considered. The *fine* fraction is dominated by anthropogenic emissions: mixture of carbon particles from combustion processes and secondary particles produced by photochemical reactions in the atmosphere (sulfates and nitrates). The carbonaceous fraction consists of aggregates of organic and inorganic carbon on which are adsorbed transition metals (Pb, Cd, V, Ni, Cu, Zn, Mn, Fe), organic compounds and biological constituents (US EPA, 1996).

Epidemiological and experimental studies have shown that particulate air pollution may induce and aggravate respiratory and cardiovascular diseases. Significant associations between exposure to ambient air particles and increased morbidity and mortality have been demonstrated (Englert 2004; Katsouyanni et al. 2001; Krewski and Rainham 2007; Pope 2007). Most of the available studies do not attribute the observed health effects to a particular characteristic of PM (WHO, 2007). Moreover the exact physiochemical mechanism by which PM produces adverse effects is still poorly

known; one of the hypotheses considered on PM's mechanisms of action is the oxidative potential of the particles or specific components.

Several *in vitro* studies with collected particulate matter fractions indicate that particles with a higher oxidative potential have a greater ability to deplete antioxidant defences and to induce airway inflammation (Ghio et al. 2002; Moller et al. 2008; Schins et al. 2004). In this process transition metals such as Fe (Carter et al. 1997; Hutchison et al. 2005; Schins et al. 2004), organics such as polycyclic aromatic hydrocarbons (PAHs) (Billet et al. 2008) and endotoxins (Becker et al. 2003; Oberdorster et al. 2000) in PM seem to be involved (Donaldson et al. 2003; Schwarze et al. 2006; Sorensen et al. 2003). Moreover, although secondary inorganic aerosols have less toxic activity when tested under controlled laboratory conditions, epidemiological studies show significant associations between sulfates and nitrates and various health outcomes. In ambient air, this fraction may act as a carrier for other components or as a surrogate for PM emitted from the combustion of sulfur-containing fuels (Schwarze et al. 2006).

Recent studies have emphatised the ability of particles to induce cellular responses in different types of lung cells, as well as inflammation and toxic effects in the whole lung (Calcabrini et al. 2004; Churg and Brauer 2000; de Kok et al. 2006; Gualtieri et al. 2008; Hetland et al. 2004; Monn and Becker 1999; Shi et al. 2006). *In vitro* studies on the toxicity of PM10 and its fractions were made by exposing cultures of macrophages and pulmonary epithelial cells to suspension of particles (Becker et al. 2005; Churg and Brauer 2000). The results of these studies show that exposure to environmental particles induces cytotoxicity, increase in transcription factors expression (NFκB) and release in the culture medium of cytokines (such as IL-1, IL- 6, IL-8, MIP-2, GM CSF, TNF) and reactive oxygen species (Becker et al. 2005; Huang et al. 2002; Monn and Becker 1999; Ning et al. 2000). Other *in vitro* studies show that particles can induce damage in plasma membrane and release of cytosolic enzyme lactate dehydrogenase

(LDH) (Billet et al. 2007; Garcon et al. 2006; Geng et al. 2006; Molinelli et al. 2002); LDH is considered a marker for acute cytotoxicity (Seagrave and Nikula 2000).

The aim of the present study was to investigate the biological *in vitro* effect of urban-air PM10 collected in Torino, a north-western Italian city, during two years (2005-2006). PM10 samples were analyzed for the amount of inorganic species (bioavailable iron and secondary particulates: sulfates and nitrates) and endotoxins, described as potentially toxic to human airways. Adherent A549 cells were exposed to PM10 aqueous and organic solvent extracts at different concentrations. A549 cells constituted a useful *in vitro* model for studying human respiratory epithelial cell biology as they present characteristics similar to human alveolar type II cells (Hatch 1992; Li et al. 2003; Shi et al. 2006; Veronesi et al. 2002) . This study investigated whether different PM10 extracts could 1) affect the cell proliferation, 2) increase the release of proinflammatory cytokines e.g. IL-6, and 3) induce cytotoxicity by measuring LDH release in culture medium. The association of the chemical and *in vitro* toxicological characterization of urban-air PM10 in one of the most industrialized areas of Italy, with high levels of particulate matter among European cities (Hazenkamp-Von Arx et al. 2004; Marcazzan et al. 2003) may provide scientific evidences for environmental and sanitary purposes.

2. Materials and Methods

2.1 PM10 sampling

PM10 (PM passing through a size-selective inlet with a 50% efficiency cut-off at 10 µm aerodynamic diameter) was sampled on glass microfiber filters (Type A/E, 8'' x 10'', Gelman Sciences, Michigan, USA), with a Sierra Andersen High Volume Sampler 1200/VFC (Andersen Samplers, Atlanta, Georgia, USA) using a flow of 1160 L/min. Sample duration was controlled by a timer accurate to ± 15 min over a 24 hr sample period. The exact flow was calculated daily, corrected for variation in atmospheric

pressure and actual differential pressure across the filter. The filters were pre- and post- conditioned by moving them to a dry and dark environment for 48h, and they were weighed in a room with controlled temperature and humidity. Procedures were conducted according to the European Committee for Standardization (CEN, 1998). The PM10 concentration, C ($\mu g/m^3$), in the air volume sampled, V (L), was calculated as follows:

$$C = [(W2-W1)-(B2-B1)]*10^3/V$$

where W_1 is the mean of three tare weights of the same filter before sampling (mg), W_2 is mean of three post-sampling weights of the same sample-containing filter (mg), B_1 is mean tare weight of blank filters (mg). B_2 is mean post-sampling weight of blank filters (mg) and V is volume as sampled at the nominal flow rate.

Samplings were carried out from January 2005 to December 2006 (2 filters every month, one sampled at the half and one at the end of every month, without considering week-ends) in a meteorological-chemical background station located in the urban centre of Torino, an industrial north-western Italian city (Gilli et al. 2007b).

2.2 Particles extractions

Each PM10 filter (typically 20.32 cm by 25.40 cm) was treated individually: two different 6.35 cm by 20.32 cm wide strips were cut from the same filters and each portion has been used with different extraction medium: acetone and the Dulbecco's Modified Eagle's Medium (DMEM) culture medium without fetal calf serum (FCS). Acetone was chosen as solvent to extract organic-extractable compounds (Claxton et al. 2004). DMEM without FCS was chosen to extract water-soluble components, hypothetically comparable to the extraction at the lung cells (Calcabrini et al. 2004; Hetland et al. 2004). Each portion of the filter has been cut into little strips and placed in 50 mL polypropylene sterile tube with 15 mL of the extraction medium. The tubes were placed in an ultrasonic water bath for 10 min followed by 1 min of vortexing. This procedure was repeated 3 times. The samples were centrifugated at 5000 rpm for 10 min to

remove the filter material and the supernatant was collected. Acetone extracts were evaporated with a rotary evaporator and re-suspended in culture medium. Aqueous extracts were directly assayed. It was assumed that particles were totally removed from the filters through extraction procedures (Hutchison et al. 2005). Unless specified otherwise, all chemicals were purchased from Sigma, USA.

2.3 Iron and bioavailable iron determinations

Iron determination was performed according to the procedure of Gilli et al. (2007b). Briefly, the metals were extracted from filter strips (3.18 cm by 20.32 cm) by a nitric acid solution. After cooling, the sample was mixed and centrifuged and the trace element concentrations in each sample were determined by atomic absorption spectrometry, Varian GTA-96.

The bioavailable iron from urban particulates was determined as previously described (Lund and Aust 1990), with some modifications as reported by (Smith and Aust 1997) and Gilli et al. (2007b). Briefly, filter strips (3.18 cm by 20.32 cm) were suspended in NaCl 50nM, mixed and pH adjusted to 7.5. Citrate was then added to samples to obtain a final concentration of 1 mM and all samples were placed on a wrist-action shaker in the dark for 24 h. One mL samples were withdrawn and centrifuged to remove the particulates. The amount of iron mobilized as the citrate: Fe complex in the supernatant was determined using a spectrophotometric total non-heme iron assay (Brumby and Massey, 1967). The concentration of iron mobilized by citrate was expressed as µg of Fe/m^3 and as ng of Fe/µg of particulate.

2.4 Sulfates and nitrates determination

Sulfates and nitrates determination was performed according to the procedure of Gilli et al. (2007a, 2007b). Briefly each filter strip (3.18 cm by 20.32 cm) was extracted in 15 mL in distilled deionized waters, via 30 min sonication, 30 min agitation and an overnight refrigeration at 4°C. Prior to the analysis, the samples were centrifuged to remove particles. Ion chromatography (IC) was used to determine the soluble ion

content sulfates ($SO_4^{=}$) and nitrates (NO_3^-), applying a Dionex DX-100 ion chromatograph with $NaHCO_3$ 0.3 mM and Na_2CO_3 2.7 mM for eluent and IonPac analytical column S12A for anions. Both the utilized standards, sodium sulfate and sodium nitrate (71959 and 71759 FLUKA respectively) ranged from 0.1 µg/mL to 100 µg/mL. The ions were identified by their elution/retention times (about 8.75 min and 12.5 min for nitrates and sulfates respectively) and quantified by the conductivity peak area or peak height (300A method, US EPA, 1996).

2.5 Endotoxins determination

Endotoxin was assayed with an end-point chromogenic Limulus amebocyte lysate (LAL) method (QLC-1000 n° 50-648U, Cambrex, Walkersville, MD, USA) at 37°C with an automated micro-plate reader (ELX 800 UV, Bio-Tek Instruments, Inc) following manufacturer's instructions. *Escherichia coli* 0111:B4 endotoxin was used as standard endotoxin. Sample concentration were reported as endotoxin units (EU) per millilitre of eluant, EU per milligram of PM10, and EU per cubic meter of air collected. The limit of detection (LOD) was 0.01 EU/mL. To control for the potential interference of samples substances with the LAL assay, all samples were spiked with a known amount of endotoxin (0.4 EU/mL). The spiked solution was assayed along with the unspiked samples and their respective endotoxin concentrations are determined. The difference between these two calculated endotoxin values should equal the known concentration of the spike ± 25% to accept the results of the determinations.

2.6 Cell culture

The human lung epithelial cell line A549 (non-small cell lung cancer) from Interlab Cell Line Collection (Genova, IT) was used as a model for human epithelial lung cells. Cells were grown as a monolayer, maintained and treated in DMEM supplemented with 10% FCS, 2% L-glutamine 200mM, 2% HEPES 1M, 1% sodium pyruvate 100 mM and 1% penicillin/streptomycin 10mg/mL, at 37°C in an humidified atmosphere containing 5% CO_2.

2.7 Cell proliferation

Cell proliferation was evaluated using crystal violet method (Kueng et al. 1989). Cells were seeded in 24-well plates at a density of $4*10^4$ cell/well and exposed to particle concentration of 50 and 100 µg/mL (30 and 60 µg/cm^2), (Alfaro-Moreno et al. 2002; Calcabrini et al. 2004; Veronesi et al. 2002). Cell proliferation was determined at 24h, 48h and 72h of exposure measuring the residual cell number by crystal violet by determining the absorbance at 595 nm with a micro-plate reader (ELX 800 UV, Bio-Tek Instruments, Inc). At the same time blank filters were treated in the same way. All experiments were performed in triplicate. Percentage cell proliferation was calculated comparing the absorbance of exposed cultures with the absorbance of non-exposed cultures.

2.8 Proinflammatory cytochine in cell supernatant

IL-6 is a multifactorial cytokine protein that plays a major role in the mediation of airways inflammation associated with infection or toxic insult. A549 cells were exposed to particle concentration of 50 and 100 µg/mL (30 and 60 µg/cm^2) and after 24, 48 and 72h of treatment, supernatant aliquots were removed for the cytochine assay. IL-6 was measured in the culture supernatant by enzime-lynked immunoabsorbent assay (ELISA) kits (PeliPair reagent set, Sanquin, Netherlands) with an automated micro-plate reader (ELX 800 UV, Bio-Tek Instruments, Inc) according to the manufacturer's recommendations. Results were calculated by expressing samples IL-6 increment as a percentage of IL-6 expression from non exposed cells.

2.9 LDH assay

To evaluate PM10 cytotoxicity, released lactate dehydrogenase (LDH) activities, from damaged cells, were measured in cell-free culture supernatants. Enzymatic activities, measured spectrophotometrically as absorbance variation at 340 nm (37°C), were expressed as nmol NADH oxidized/min in the conversion of pyruvate to lactate (Golladay et al. 1997; Kinnula et al. 1994; Riganti et al. 2002). A549 cells were seeded

in 6 well plates at a density of $1*10^6$ cell/well and exposed to PM10 extracts containing 50, 100 and 200 µg/mL of particles (30, 60 and 110 µg/cm^2), (Billet et al. 2007; Geng et al. 2006; Hetland et al. 2000). At the same time blank filters were treated in the same manner. At 24h, 48h and 72h LDH activity was measured in supernatant and cell lysate. LDH activity was calculated as ratio between the extracellular LDH (measured in the supernatant) and total LDH (expressed as sum of LDH measured in supernatant and cell lysate). To obtain cell lysate, cells were washed with PBS, detached with trypsin-EDTA, resuspended with 1 mL of TRAP (82.3 mM triethanolamine hydrochloride, pH 7.6) and sonicated for 10''. Then LDH was measured by adding 250 µl of mix containing NADH 0.25 mM and pyruvate 0.5 mM and consumption of NADH was measured as absorbance in a microplate reader (Benchmark Plus Microplate Reader, Biorad) at 340 nm. All experiments were performed in triplicate. Results were calculated by expressing samples LDH activity as a percentage of LDH activity from non exposed cells.

2.10 Statistical analysis

Statistical analyses were performed using the SPSS Package, version 14.0 for Windows. T-test analysis was used to compare means and Spearman correlation coefficient was used to assess relationship between variables. The mean difference and correlation were considered significant at $p<0.05$.

3. Results

3.1 *PM10 concentration*

A total of 46 PM10 filters were analyzed during 2005 and 2006 without considering week-ends. The mean PM10 concentration during the whole sampling period was 55.4 ± 39.1 µg/m^3. The highest value was observed in winter (198.5 µg/m^3), while the lowest was observed in summer (12.4 µg/m^3); the T-test analysis show significant ($p < 0.01$)

difference for the seasonal (autumn/winter *vs* spring/summer) PM10 concentrations (Table 1).

3.2 *Iron and bioavailable iron concentrations*

The total iron mean concentration was 1.035 ± 0.811 μg/m^3 and, in relation to PM10, 19.43 ± 11.64 ng/μg particles. The highest value was observed in winter (3.751 μg/m^3), while the lowest was observed in summer (0.099 μg/m^3). A statistically significant difference was found for the seasonal concentrations (autumn/winter *vs* spring/summer) expressed as μg/m^3 (t-test, $p<0.01$) but not as ng/μg particles (Table 2). A significant positive correlation was found with PM10 concentrations (Spearman correlation: $r=0.800$, $p<0.01$).

Table 2 shows also the mean concentrations of bioavailable iron, 6.9 ± 4.5% of total iron was bioavailable. No statistically significant differences were found for the mean seasonal concentrations expressed both as μg/m^3 (t-test, $p<0.01$) and as ng/μg particles. A positive significant correlation was found with PM10 concentrations (Spearman correlation: $r=0.670$, $p<0.01$).

3.3 *Sulfates and nitrates concentrations*

The sulfates mean concentration was 10.5 ± 5.7 μg/m^3 and, in relation to PM10, 216.7 ± 72.7 ng/μg particles, such that sulfates represented 21.2 ± 6.9 % of total PM10 mass. The nitrates mean concentration was 11.7 ± 9.2 μg/m^3 and, in relation to PM10, 216.3 ± 80.5 ng/μg particles, such that nitrates represented 20.6 ± 6.1% of total PM10 mass. Considering the sum of sulfates and nitrates as principal components of secondary particulate, it was found that they meanly constituted 42 ± 9 % of the PM10 total mass (Table 1). A statistically significant difference was found for the seasonal concentrations for both sulfates and nitrates expressed as μg/m^3 (t-test, $p < 0.05$ and $p < 0.01$, respectively). Considering the concentrations of the two species in relation to PM10 (percentage), it was found that in the hot season the sulphate amount (23.6 ± 5.6 %) was more elevated than in the cold season (19.2 ± 7.3 %) and this trend was

significant (t-test, $p < 0.01$), while for nitrates the amount was similar in the two seasons. A significant positive correlation was found with PM10 concentrations both for sulfates (Spearman correlation: $r=0.740$, $p<0.01$) and for nitrates (Spearman correlation: $r=0.900$, $p<0.01$) and also a significant positive correlation between sulfates and bioavailable iron (Spearman correlation: $r=0.490$, $p<0.01$).

3.4 *Endotoxins concentrations*

Endotoxin mean concentration during the whole sampling period was 0.19 ± 0.07 EU/m^3 and in relation to PM10 3.70 ± 0.95 EU/mg. A statistically significant difference was found for the seasonal concentrations (autumn/winter *vs* spring/summer) expressed as EU/m^3 (0.24 ± 0.07 *vs* 0.14 ± 0.01 respectively) (t-test, $p<0.05$) but not as EU/mg particles. Endotoxins, EU/mg, were inversely correlated with PM10 concentrations (Spearman correlation: $r = - 0.710$, $p < 0.05$).

3.5 *A549 cell proliferation*

Mean absorbance values at 595 nm of the negative control at 24, 48 and 72 h were respectively 0.120 ± 0.010, 0.225 ± 0.010 and 0.410 ± 0.015. Extracts from blank filters had no significant effect on cell proliferation. Figure 1 shows effects of organic solvent (1a) and aqueous (1b) PM10 extracts on cell proliferation. PM10 inhibits cell proliferation in a dose and time dependent manner. Aqueous extracts seem to have stronger effects than organic solvent extracts on cell proliferation, this trend was confirmed by T-tests for inhibition at 48 and 72 h ($p < 0.05$) but not at 24 h, for both the tested concentrations. The maximum inhibition of cell proliferation, 25.3 ± 5.8 %, was achieved at 72h by the 100 µg/mL aqueous extract.

Proliferation inhibition of A549 cells due to organic solvent extract, in some cases (50 µg/mL at 72h, 100 µg/mL at 48 and 72h) was correlated with PM10 concentrations (Spearman correlations: $r=0.860$, $p<0.01$; $r=0.810$, $p<0.01$ and $r=0.620$, $p<0.05$

respectively), no correlations were found with any other parameters or for the aqueous extracts.

Figure 3(a) shows different seasonal (autumn/winter *vs* spring/summer) proliferation inhibition of A549 cells at 72h after their incubation in the continuous presence of two concentrations of particle matter (50 and 100 µg/mL). Despite PM10 organic solvent extracts and PM10 aqueous extracts always had higher effect in the cold months these differences were not significant (T-tests $p > 0.05$).

3.6 *IL-6 release in A549 cells*

In order to investigate the proinflammatory potential of the particulate, the production of IL-6 in the presence of two different extracts of PM10 was analyzed. Mean IL-6 release in the negative control at 24, 48 and 72h range from 0.2 to 1.0 pg/mL. PM10 extracts induce IL-6 release in a dose and time dependent manner. As shown in figure 2a, the organic solvent extract induce significant release of IL-6 in relation to the control at 48h and 72h of exposure (T-test $p < 0.05$) for both the concentrations, the lowest concentration at 24h of exposure has no significant increase of IL-6 release in relation to the control ($6.2 \pm 3.5\%$).The two tested concentrations exhibited similar expression of cytokine without significant difference (t-test $p > 0.05$).

In figure 2b, the aqueous extract induce significant release of IL-6 in relation to the control at 24h, 48h and 72h of exposure (T-test $p < 0.05$) for cells exposed to 100 µg/mL while for cells exposed to 50 µg/ml only at 72h.

Despite the aqueous extract exhibit higher mean levels of IL-6 expression the difference between the production of IL-6 in the presence of organic solvent extract or aqueous extract was not statistically significant (T-test $p>0.05$) for any concentrations or times. The maximum increment of IL-6 release, 108.3 ± 39.1 %, was obtained at 72h by the 100 µg/mL aqueous extract.

IL-6 release by A549 due to organic solvent extracts (50 and 100 µg/mL at 72h) was correlated with PM10 concentrations (Spearman correlations: r=0.680, p<0.05 and r=0.730, p<0.05 respectively). No correlations were found with any other parameters. IL-6 release by A549 due to aqueous extracts was always correlated with PM10, sulfate, nitrate and Fe concentrations for both the extract concentrations and at each time of exposure in a range of Spearman correlation between r = 0.720 to r = 0.920, p<0.05.

Figure 3(b) shows different seasonal (autumn/winter *vs* spring/summer) IL-6 release at 72h after their incubation in the continuous presence of two concentrations of particle matter (50 and 100 µg/mL). PM10 organic solvent extracts and PM10 aqueous extracts always had higher effect in the cold months and these differences were statistically significant (t-test, $p < 0.01$) except for one case (50µg/mL organic solvent extract).

3.7 *LDH release in A549 cells*

An increase of extracellular LDH enzyme activity reflects an increase in the amount of membrane-damage cells (Lobner 2000). The release of the cytoplasmatic enzyme LDH into the culture supernatant was used to measure cell cytotoxicity. Extracts from blank filters had no significant effect on LDH release. Incubation of A549 cells with particles at a concentration of 50 µg/mL had no effect on LDH release as compared to control cells (data not shown). Results are shown in table 3: for organic solvent extract and for both the concentrations the highest and significant release of LDH was obtained at 72h of exposure (T-tests, $p < 0.05$), while at 24h and 48h no significant effects were observed in relation to the control (T-tests, $p > 0.05$). For aqueous extracts exposure to 100 µg/mL of particles increase LDH release by approximately 30-35% for all incubation time. Statistically significant increase of extracellular LDH activities were observed at 24h, 48h and 72h after cell exposure at 200 µg/mL of particles concentration (T-tests, $p < 0.05$). The maximum increment of LDH release, 87.6 ± 41.9

% and 83.7 ± 44.1 %, was respectively obtained at 48h by the 200 µg/mL aqueous extract and at 72h by the 200 µg/mL organic solvent extract .

LDH increase by A549 due to both organic solvent extracts and aqueous extracts was never correlated with PM10 concentrations or with any other parameters, with the exception of the aqueous 200 µg/mL extract at 72h *vs* PM10 concentrations.

Figure 3(c) shows different seasonal (autumn/winter *vs* spring/summer) release of LDH activity from A549 cells at 72h after their incubation in the continuous presence of two concentrations of particle matter (100 and 200 µg/mL). PM10 organic solvent extracts had similar effect in the two seasons while PM10 aqueous extracts always had higher effect in the cold months, this difference was significant only for the lowest extract concentration.

4. Discussion and Conclusions

The PM10 monitoring in Torino has showed concentrations of PM10 often over the daily (50 µg/m^3) and yearly (40 µg/m^3) quality targets (Air Quality Directive, 2008/50/CE). High pollution episodes are frequent during winter time in Northern Italy. This is due to the regional unique climate and topography which hamper particles dispersion. Human alveolar epithelial cells were exposed to concentration of particles comparable to 100 µg/m^3 such as those found in winter season in Torino, indeed the averages were 93.5 ± 54.1 µg/m^3 for 2005 and 78.4 ± 53.1 µg/m^3 for 2006 (Oberdörster and Yu 1999).

In urban sites like that utilized in this study, significant seasonal differences in total Fe concentrations occur probably due to minor presence of anthropogenic sources (for example domestic and industrial heating) in the warm season (Gilli et al. 2007b; Ziemacki et al. 2003). A small amount of iron is presumably bioavailable (about 6% of total Fe) and this form is capable of generating ROS and induces inflammation in

cellular systems (Aust et al. 2002). In this study this bioavailable Fe has not a significant seasonal trend and it is less correlated with PM10 concentration than total Fe.

In relation to secondary PM10 components, the seasonal trend of the two species is normally due to the photochemical reactions that, in warm season occur more frequently. It was found that the secondary airborne particulate matter (as sum of sulfates and nitrates) mass concentration was about 42% of the total PM10, and this value was confirmed by other studies conducted in the Po valley of Northern Italy (Gilli et al. 2007b; Marcazzan et al. 2003).

Among relevant air pollution components in causing toxicity there are also endotoxins; in this study the mean endotoxins concentration was low and quite comparable with those present in literature (Heinrich et al. 2003; Morgenstern et al. 2005; Mueller-Anneling et al. 2004; Solomon et al. 2006).

Many studies have investigated the toxicity and mechanism of PM in the airway epithelial cells (Scapellato and Lotti 2007); PM induces different biologic effects depending on sampling site (Rosas-Pérez et al., 2007), size fraction (Alfaro-Moreno et al. 2002; Hetland et al. 2004; Osornio-Vargas et al. 2003), sampling time (Frampton et al. 1999) and contaminants adsorbed on the particles (Baulig et al. 2003; Billet et al. 2007; Calcabrini et al. 2004; Muller et al. 2006). Moreover all in vitro studies with PM10 usually used high concentration of particles so that a potential confounding effect of high doses need to be taken into account when extrapolating to *in vivo* conditions (Oberdorster and Yu 1999).

In this study PM10 inhibits cell proliferation and induces both IL-6 and LDH release in a dose and time dependent manner according to other studies on epithelial cells (Alfaro-Moreno et al. 2002; Osornio-Vargas et al. 2003). These biological effects have a seasonal trend as well as other biological effects such as mutagenicity evaluated in a study conducted in the Torino city (Gilli et al. 2007a).

In general the maximum biological effect on A549 cells was obtained in term of IL-6 release (108.3 ± 39.1 %), then LDH production (87.6 ± 41.9 %) and in the end inhibition of cell proliferation (25.3 ± 5.8 %). Indeed other studies have shown that PM10 induce - first the production of reactive oxygen species and inflammatory mediators such as interleukin IL-6 - than acute citotoxicity as release of LDH and in the end - an alteration of the cellular function highlighted by inhibition of the cellular proliferation (Jalava et al. 2006). It is also important to consider that these outcomes might occur independently or in association with each others (Seagrave and Nikula 2000).

In our investigation aqueous extracts seem to induce a greater IL-6 release and a greater proliferation inhibition than organic solvent (acetone) ones, while, on the contrary, organic solvent extract induce a greater release of LDH. Probably ,although both the two PM10 extracts produce effects on cells, water-soluble components, such as transition metals, could rapidly cross the plasma membrane and induce the inflammatory response, while polar compounds, from organic solvent extracts, cross the plasma membrane more slowly and are able to directly interact with DNA and induce mutations (Gilli et al. 2007a; Gilli et al. 2007b).

In general the biological effects are frequently attributed to the intracellular generation of ROS, usually correlated with aqueous PM extracts, that may damage protein structure, plasmatic membrane associated lipids and other cellular compartments (Knaapen et al. 2004; Sevanian and Ursini 2000; Sorensen et al. 2003). Soukup and Becker (Soukup and Becker 2001) found that expression of cytokines by human alveolar macrophages incubated with ambient insoluble PM10 was more than 50 times higher than that caused by the water-soluble fraction. Another study, conducted by Javala et al. (2008), is focused on the stronger cytotoxic effects of insoluble (organic) than water-soluble fraction of PM. In another investigation, mRNA levels of IL-1α and IL-8 were significantly more expressed by human bronchial epithelial cells when

incubated with the water-soluble fraction of ambient PM10 than with the corresponding suspensions (10–500 μg/mL, up to 24 h) (Fujii et al. 2001). To our knowledge, few studies in literature compare the different effects of urban PM10 extracts.

Few significant correlations were found between the biological effects and PM10 components (bioavailable iron, secondary particulates and endotoxins) evaluated in this methods development study and it is important to note that the correlations have become significant only after some days of exposure (72h). These results confirmed other recent studies in which, for *in vitro* lung cells exposure, there were inconsistent associations between both cell proliferation and IL-6 release and secondary particulates or metals (Happo et al. 2008). One possible explanation for the presence of few correlations could be that the biological end-points and the PM10 components were quantified in chemically separate fractions. Furthermore the aqueous extracts did not reflected the activity of any particular PM10 chemicals components but only highlighted the activity of physiologic-soluble components.

Nevertheless the significant difference in biological effects induced by the two different extracts (aqueous and organic solvent) and the observed seasonal trend, support the need of further studies, in order to determine particle components responsible for biological effects associated both to PM10 and fine particulate matter.

The identification of PM components carrying the relevant burden in causing inflammation remains unsettled, and it was said to represent one of the biggest gaps in our knowledge (Pope and Dockery 2006).

In the future, better understanding of the relative toxicity and health effects of particles from various sources could facilitate targeted abatement policies and more effective control measures to reduce the burden of disease due to air pollution.

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Figure Captions

Figure 1. Proliferation inhibition of A549 cells after their incubation in the continuous presence of increasing concentrations of particulate matter (50 and 100 µg/mL) from PM10 organic solvent extracts (a) and PM10 aqueous extracts (b). Inhibition of cell proliferation was calculated comparing the absorbance of exposed cultures with the absorbance of non-exposed cultures. Asterisks indicate statistically significant differences from the control , $p < 0.05$ (T-test).

Figure 2. IL-6 release by A549 cells after their incubation in the continuous presence of increasing concentrations of particulate matter (50 and 100 µg/mL) from PM10 organic solvent extracts (a) and PM10 aqueous extracts (b). Results were calculated by expressing samples IL-6 relies as a percentage of IL-6 release from non exposed cells. Asterisks indicate statistically significant differences from the control , $p < 0.05$ (T-test).

Figure 3. Different seasonal (autumn/winter *vs* spring/summer) proliferation inhibitions 3(a), IL-6 releases 3(b) and LDH releases 3(c) from A549 cells at 72h after their incubation in the continuous presence of PM10 organic solvent extracts and PM10 aqueous extracts (50, 100 µg/mL or 200 µg/mL). Asterisks indicates statistically significant differences for the two seasons, $p<0.05$ (T-test).

*** Conflict of Interest Statement**

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Conflict of Interest Policy

Manuscript number (if applicable): Article Title: Cytotoxic effects induced on a549 cells and chemical characterization of urban-air PM10 collected in Torino (Italy).	Author name: Tiziana Schilirò, Luca Alessandria, Raffaella Degan, Deborah Traversi, Giorgio Gilli

Declarations

Environmental Toxicology and Pharmacology requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

Please state any competing interests

No conflict of Interest

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

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Print name

Tiziana Schilirò,
Luca Alessandria
Raffaella Degan,
Deborah Traversi
Giorgio Gilli

Table 1. Means and standard deviations of PM10, sulfates and nitrates concentrations (μg/m^3) of the whole sampling period (2005/2006) and divided by seasons.

	PM10 (μg/m^3)	**$SO_4^=$ (μg/m^3)**	**NO_3^- (μg/m^3)**	**$SO_4^=$ (% of PM10 mass)**	**NO_3^- (% of PM10 mass)**
Whole period	55.4 ± 39.1	10.5 ± 5.7	11.7 ± 9.2	21.2 ± 6.9	20.6 ± 6.1
Winter/Autumn	71.3 ± 43.1**	12.4 ± 6.4*	14.9 ± 10.1**	19.2 ± 7.3*	20.8 ± 6.0
Summer/Spring	37.4 ± 24.1**	8.3 ± 5.7*	8.0 ± 6.3**	23.6 ± 5.6*	20.3 ± 6.3

** statistically significant differences, (autumn/winter *vs* spring/summer) $p < 0.01$ (T-test).

* statistically significant differences, (autumn/winter *vs* spring/summer) $p < 0.05$ (T-test).

Table 2. Means and standard deviations of Fe concentrations (μg/m^3) of the whole sampling period (2005/2006) and divided by seasons.

	Fe total (μg/m^3)	**Fe bio (μg/m^3)**	**% Fe bio (of total)**
Whole sampling period	1.035 ± 0.811	0.078 ± 0.095	6.9 ± 4.5
Winter/Autumn	1.355 ± 0.919**	0.097 ± 0.089	7.5 ± 4.8
Summer/Spring	0.672 ± 0.463**	0.056 ± 0.099	6.2 ± 4.1

** statistically significant differences, (autumn/winter *vs* spring/summer) $p < 0.01$ (T-test).

Table 3. Released lactate dehydrogenase (LDH) activity from A549 cell 24h, 48h and 72h after their incubation in the continuous presence of increasing concentrations of particle matter (100 and 200 µg/mL) from PM10 organic solvent extracts and from PM10 aqueous extracts. Results were calculated by expressing samples LDH activity as a percentage of LDH activity from non exposed cells.

LDH increase %		24 h	48 h	72 h
PM10 organic solvent extracts	100 µg/mL	-2.7 ± 7.9	3.9 ± 7.1	64.0 ± 22.5*
	200 µg/mL	-7.3 ± 3.1	2.9 ± 5.5	83.7 ± 44.1*
PM10 aqueous extracts	100 µg/mL	30.2 ± 24.3	33.5 ± 23.0*	35.6 ± 18.3*
	200 µg/mL	78.1 ± 34.1*	87.6 ± 41.9*	53.6 ± 24.6*

* statistically significant differences in relation to the control, $p < 0.05$ (T-test).

Figure 1a

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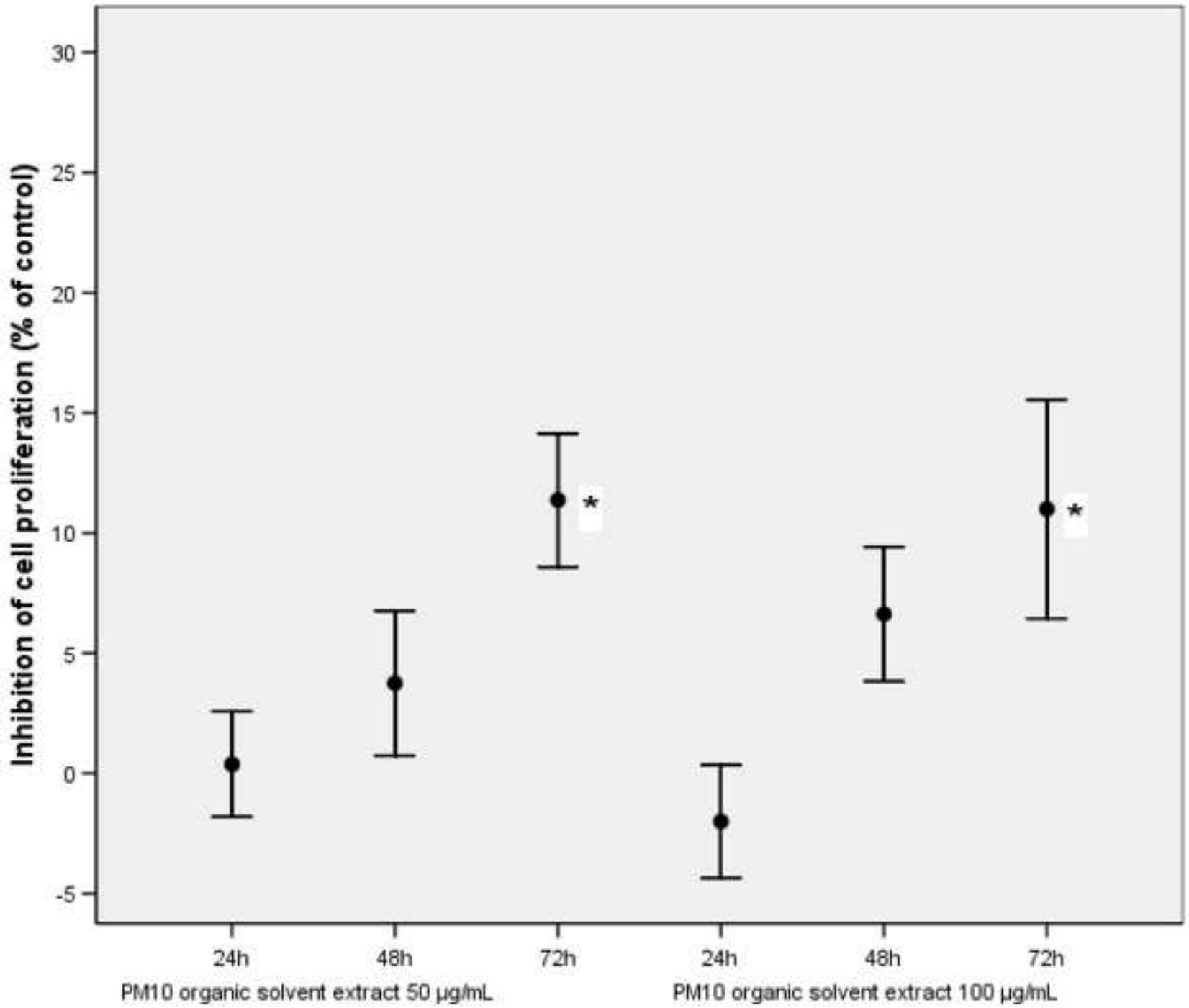

Figure 1b

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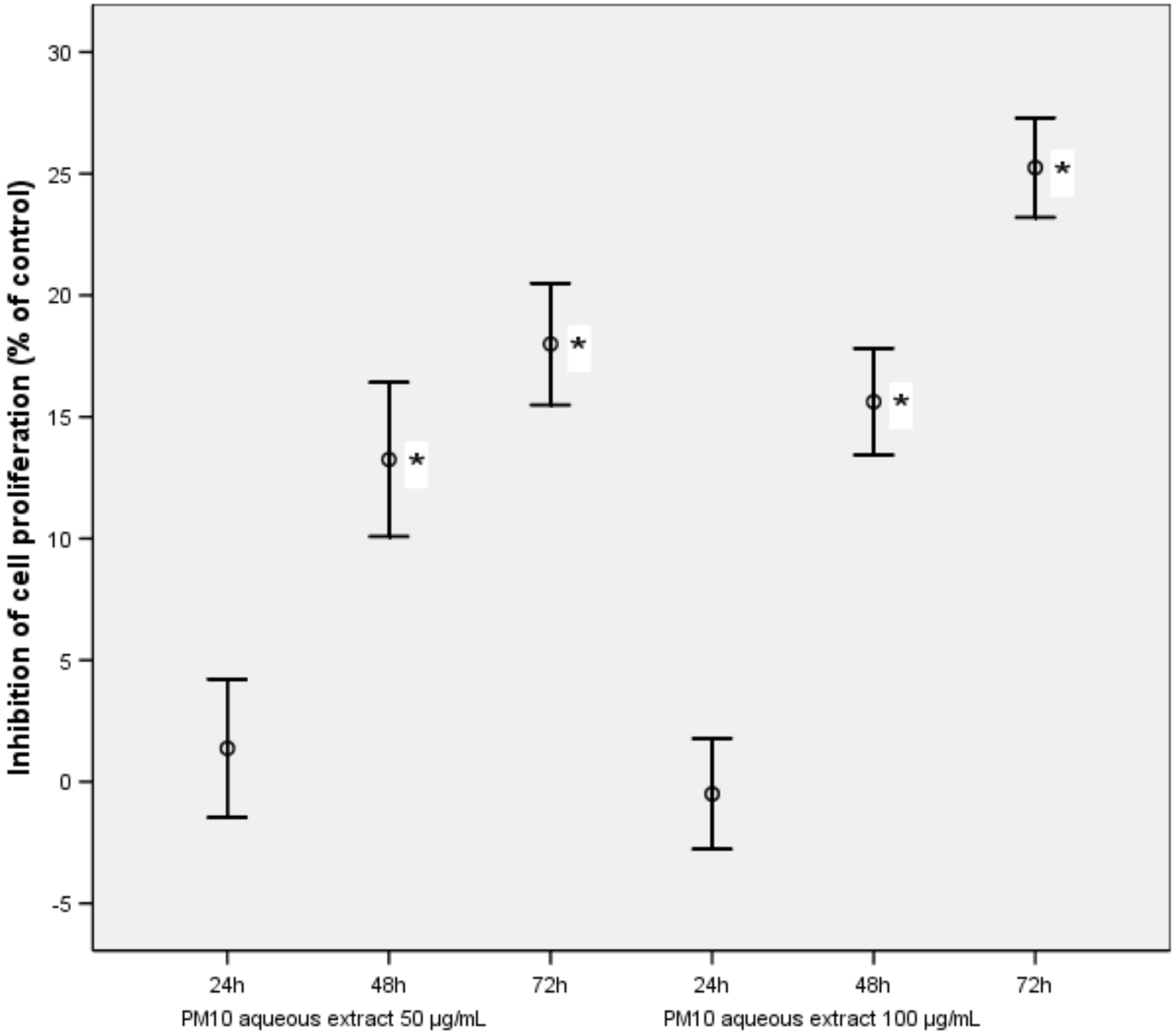

Figure 2a

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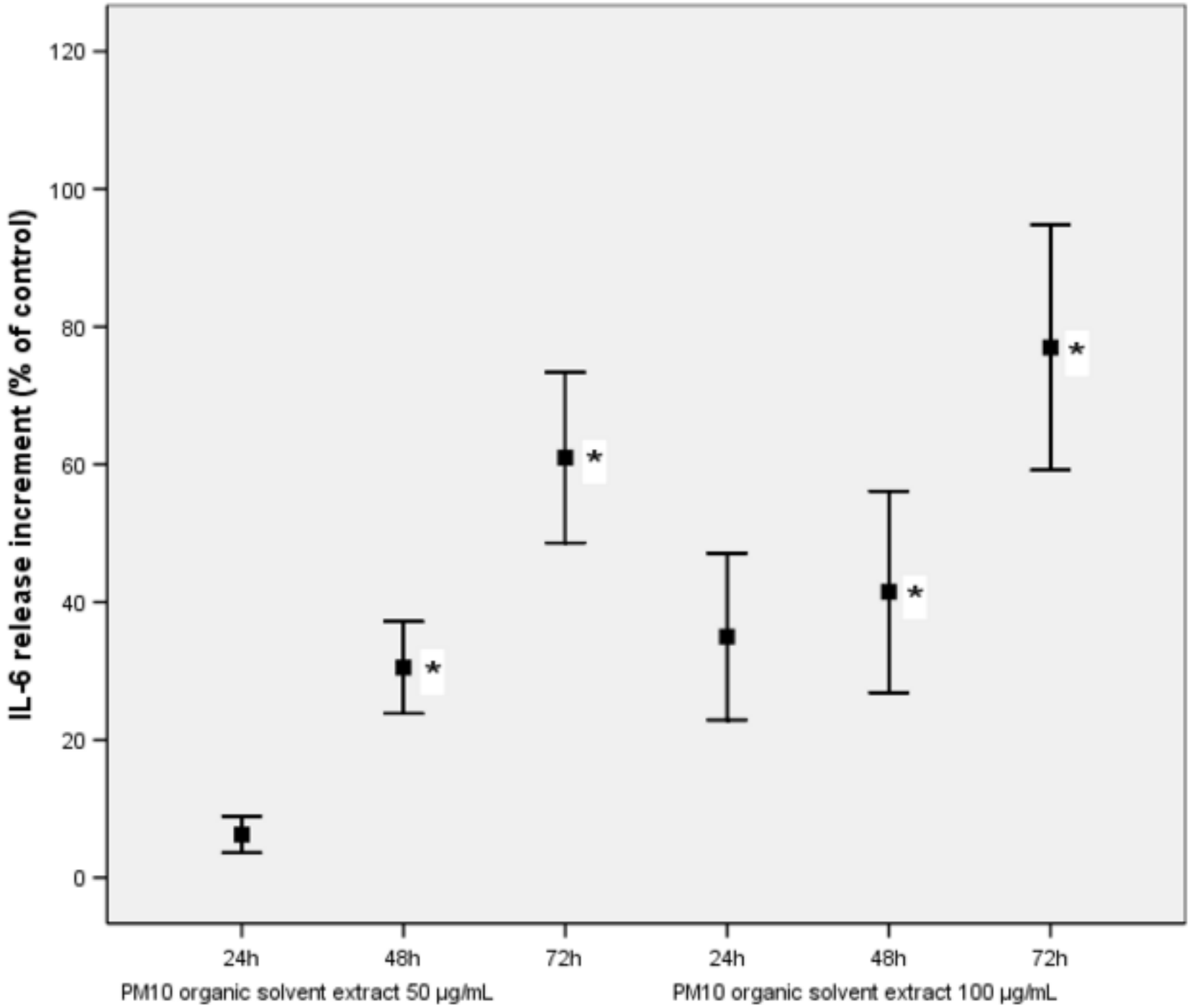

Figure 2b

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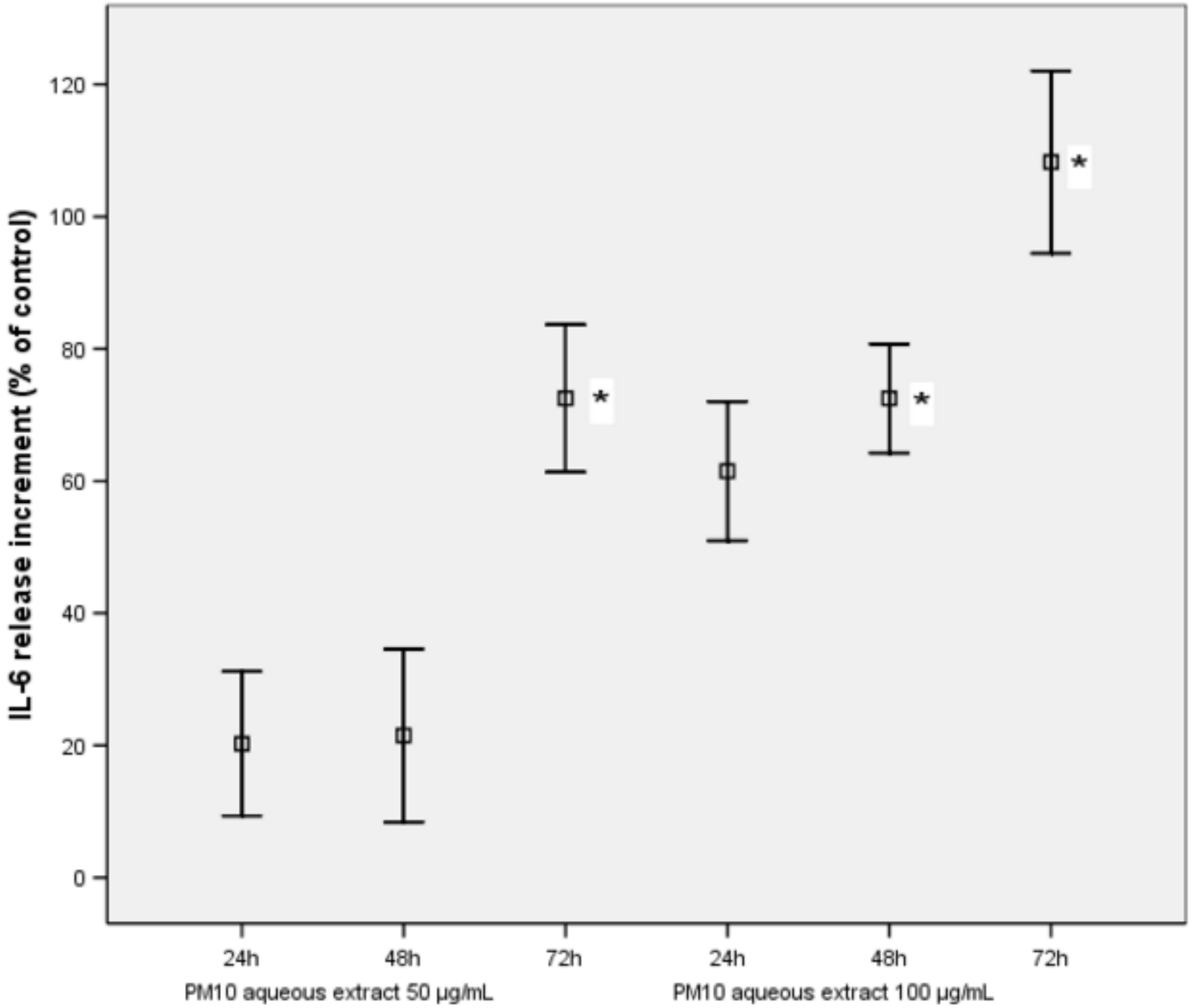

Figure 3

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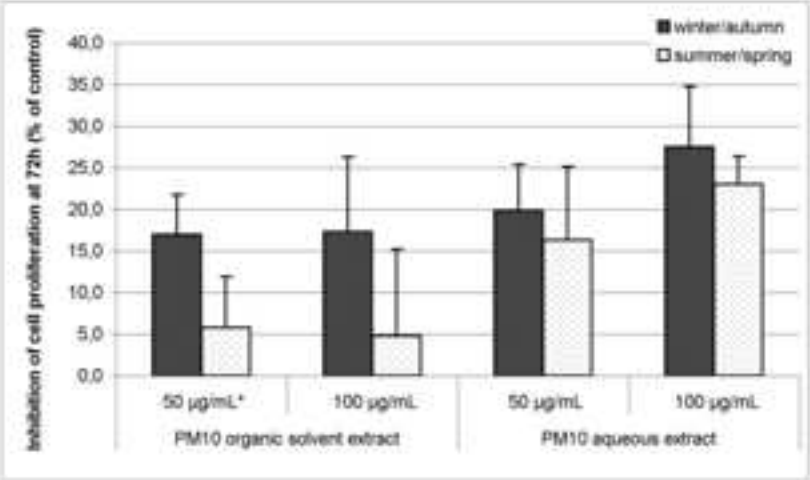

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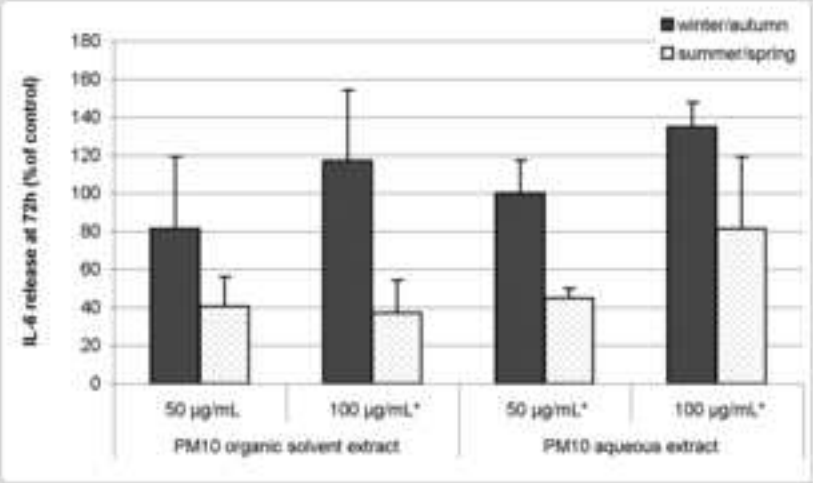

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