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Combustion-Derived Particles from Different Fuels Induce Different Cytotoxic Effects on A549 cells

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The biological effects induced in human alveolar cells by biomass combustion-derived particles (PM10), collected from the emission of heating systems operating with different fuels, have been analysed. Particles emitted from pellet, charcoal and wood combustion, were chemically characterized and used for the exposure of monocultures of human A549 alveolar cell line. Cell viability, pro-inflammatory cytokine expressions, oxidative stress and DNA damage were analysed. Pellet-derived particles seem to have higher toxic properties in comparison with charcoal and wood ones, suggesting a correlation between their chemical properties and toxicological profile. These data demonstrate that biomass combustion-derived particles may activate different toxicological pathways, suggesting that the type of fuel and its quality may have an important role in the strategies to prevent respiratory diseases.

1. Introduction

Airborne pollution, which has been classified by the International Agency for Research on Cancer (IARC), as carcinogenic to humans (Group 1), is generally recognized as a significant environmental and health hazard.
Several epidemiological studies have associated particulate exposure with adverse health effects including respiratory and cardiovascular diseases and with increased morbidity and mortality (Anderson *et al.*, 2012). Generally, the adverse effects of PM on human health are determined by its size, surface area and chemical composition. PM dimensional class is an important parameter, depending on which particles can be classified as PM10 (particles with an aerodynamic diameter less than 10 μm), PM2.5 (Ø < 2.5 μm) and PM1 (Ø < 1 μm) (Camatini *et al.*, 2010). The size and the surface area determine how deeper the particles can deposit within the respiratory system and induce cytotoxic effects, like inflammatory injury or oxidative damage (Longhin *et al.*, 2013). Furthermore, PM is a mixture of chemical and biological elements, including metals, elemental and organic carbon, polycyclic aromatic hydrocarbons (PAHs), and endotoxins, whose physico-chemical characteristics depend on the emission sources (Billet *et al.*, 2007). Regarding the sources, PM can be classified as natural or anthropogenic. The first one includes volcanoes, fires and dust storms; the other, includes particles emitted from mechanical and industrial combustion processes, vehicle emissions and tobacco smoke. Most of the studies related to air quality and their health effects have been performed in urban area, where air pollution is dominated by anthropogenic sources such as diesel engines exhaust and biomass combustion-derived particles (Anderson *et al.*, 2012).
Although air pollutant emissions are dominated by outdoor sources, PM human exposure is strictly related also to the indoor pollution. Biomass combustion for cooking and heating is the main source of indoor pollution, especially in developing country, where it is burned in rudimentary stoves or open fires and represents the primary source of energy (IARC 2010). IARC in 2010 has classified household biomass fuel combustion as Category 2A, probably carcinogenic to humans. Different kind of fuels can be classified under the name of biomass, such as wood, animal dung, agricultural residues, coals or logs (Capistrano *et al.*, 2016).

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The adverse health effects induced by biomass-combustion change according to their composition, strongly influenced by the specific fuel (Sussan *et al.*, 2014), the combustion conditions and technology used (Jalava *et al.*, 2012) and the time of exposure (Akunne *et al.*, 2006). Moreover, particles toxicity is strongly influenced by the compounds of the core or their surface area. PAHs and some heavy metals are the principal elements (Buonanno *et al.*, 2015).
Recently, several studies performed to evaluate the biological effect of combustion-derived particles emitted by different fuels, have shown a high variability of results.
For instance, Jalava and coworkers have evaluated the toxicological effects of particulate emissions from seven different residential wood combusting furnaces, evidencing that combustion technology largely affects the particulate emissions and their toxic potential (Jalava *et al.*, 2012). Furthermore, other authors have found that pellet stoves generated less active particles, in terms of pro-inflammatory activity, compared to the ones generate from wood stoves. However, even if pellet stoves have shown reduced *in vitro* effects when compared to wood stoves, they still can occasionally generate biologically active particulate (Corsini *et al.*, 2017).
The different toxic responses are supposed to be linked to the biomass properties. In this work, the toxicity of biomass combustion-derived particles from different fuels (pellet, charcoal and wood) was investigated with the intent to outline possible differences in the cytotoxicity able to provide further information on the effects produced. Biomass particles were chemical characterized and then tested on A549 cell line to evaluate their effects. The mechanisms activated by the interaction between particles and cells are still unclear and PM-induction of oxidative damage has been evaluated.

2. Methods

2.1 PM sampling and processing

PM10 samples were collected in an indoor environment equipped with an open fireplace and processed for cell culture experiments. Particles were obtained by extraction in pure sterile water with an ultrasound bath, dried in a desiccator, weighted and stored at -20 °C until use. For biological investigations, PM were suspended in pure sterile water (R=18.2 MΩcm; TOC=3 ppb) to obtain aliquots at a final concentration of 2 µg/µL and sonicated for 30 seconds with a sonicator equipped with a probe (SONOPULS Bandelin, 0,105 kJ, A 10%, 001.0 s) just prior to cell exposure.

2.2 Chemical characterization

PM samples were chemically characterized for elements (ng/µgPM) and PAHs (ng/mg_{PM}) with a gas chromatograph coupled with a TSQ mass spectrometer (Thermo Fischer Scientific, St Peters, MO, USA) to perform gas chromatography-mass spectrometry (GC/MS). For the separation was used the column Meta-XLB (60 m length, 0.25 mm internal diameter, 0.25 µm film thickness; Teknokroma, Barcelona, Spain), while He (99.9995% purity) was used as carrier gas. The set temperatures were 260 °C, 280 °C and 250 °C for the injector, transfer line and ion source respectively. The extraction and purification recovery was assessed by adding 5 ng of recovery standard (L429-RS Recovery Standard D-IPA Stock Solution, Wellington Laboratories) before injection into the GC/MS. The calculation of the recovery was obtained as the percentage ratio between the internal standard (added before the extraction) and the recovery standard (added before the injection in GC/MS).

2.3 Cell culture and treatment

A549 cells (ATCC CCL-185, American Type Culture Collection, Manassas, USA) were routinely maintained in OptiMEM medium (Gibco, Life Technologies, Monza, Italy) supplemented with 10% inactivated fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (100 X, Euroclone, Pero, Italy). For *in vitro* experiments, cells were cultured in OptiMEM medium with 1% FBS. The concentration of 5 µg/cm^2 was selected as the lowest tested dose able to induce significant effects in the *in vitro* system here described after a single acute exposure. Cells were exposed after 24 h from seeding to 5 µg/cm^2 of pellet, charcoal and wood particles, respectively.

2.4 Cell viability, inflammatory response, oxidative stress and DNA damage

At the end of the exposure, the media were collected and the cellular responses analyzed. Cell viability was determined by lactate dehydrogenase (LDH) detection. The assay, that provides information on cell membrane integrity, was performed according to manufacturer's guidelines (Pierce, Thermo Fischer Scientific). The cytokine release as marker of pro-inflammatory response in cells was evaluated. The levels of TNFα were determined in culture medium by ELISA assay following the manufacturers' instructions and the absorbance measured by a multiplate spectrophotometer reader (Tecan, Männedorf, Switzerland) at 450 nm

and 630 nm. Standard curves were used to determine the concentration of proteins in pg/mL. Oxidative stress response was assessed by flow cytometry using the Carboxy-DCFDA probe, which detects the reactive oxygen species (ROS) production. A549 cells were incubated with the Carboxy-DCFDA probe for 20 minutes in Hank's Balanced Salt Solution (Thermo Fisher Scientific; Monza, Italy) and successively, exposed to biomass for 2 h. At the end of the incubation, cells were detached, re-suspended in PBS and analysed at the flow cytometer (CytoFLEX 13/3, Beckman Coulter, USA). Fluorescence of 10,000 events was detected using 525 nm band pass filter (FITC). Data were analysed as mean percentage of positive cells for staining. The activated form of Ataxia-Telangiectasia mutated ATM (p-ATM) and histone H2AX phosphorylation (γH2AX) were used as markers of DNA damage and analysed with immunocytochemical technique. Cells were grown on cover-slips, exposed for 24 h to PM and etoposide (1,65 μM), used as positive control, and finally, fixed with 4% paraformaldehyde. Later, cells were incubated for 1 h with blocking-permeabilizing solution (0,1% Tween20, 0,1% Triton X-100 and 1% BSA in PBS) and stained O/N with p-ATM (1:100, Santa Cruz Biotechnology, Dallas, Texas, USA) and γH2AX Alexafluor-488 conjugated antibody (1:100, Cell Signaling). The day after, cells were incubated with the secondary antibody Alexa Fluor 594 goat anti-mouse IgG. Finally, cell DNA was counterstained with DAPI. Images were taken under a fluorescence microscope (AxioObserver, Zeiss, Germany) equipped with a digital camera. Digital images were taken with the dedicated software (AxioVision4, Carl Zeiss Vision).

2.5 Statistical analysis

The data are reported as mean values of independent experiments ± SEM. Statistical differences between samples were tested with unpaired t-test or two-way ANOVA and post hoc comparisons performed with Dunnett's method, by GraphPad Prism 6 software. Statistical differences were considered significant at the 95% level ($p < 0.05$).

3. Results

3.1 Biomass characterization

Chemical characterization revealed specific differences between biomasses. PM composition indeed, seems to be strongly affected by the specific type of fuels used. PAHs were the major chemical fraction with a concentration of 8 and 11 times higher in charcoal and wood particles in comparison with pellet. Phenanthrene and Pyrene were the most abundant PAHs in all the particles collected. Pellet-derived PM was enriched in metals, with the higher concentration of Zinc in pellet increased 1.5 and 3 times respect to wood and charcoal particles (data not shown).

3.2 *Biological investigations*

In vitro experiments were performed on A549. Cell viability, measured with LDH assay, was the first outcome analysed to define the biomass hazardous effect. Cell viability at 24 h of exposure to PM 5 μg/cm^2 presented a significant decrease (Figure 1 A). As shown, pellet and wood-derived PM had the major response.

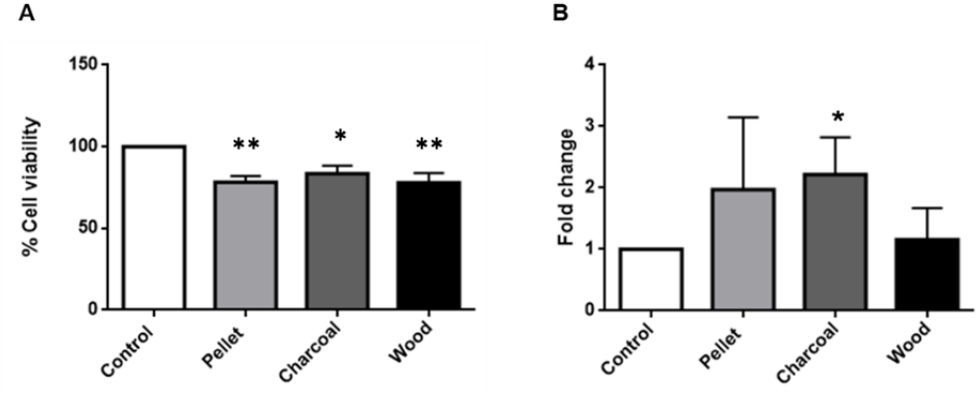

*Figure 1: A) Cell viability measured with LDH release. Data are expressed as percentage of viable cells with respect to control. Statistical analysis was performed by Two-way ANOVA with Dunnett's multiple comparison test. B) Intracellular ROS production. Statistical analysis was performed by unpaired t test. **p<0.01 and *p<0.05 vs control cells.*

The reduction in cell viability was not accompanied by a higher inflammatory effect. The levels of the pro-inflammatory cytokine TNFα, were not affected by biomass exposure (data not shown).
The oxidative stress response after exposure to biomasses was also investigated. The cytometric analysis confirms the statistically significant ROS production only after A549 exposure to charcoal (Figure 1B). A slight, but not significant increase in the response was observed also after pellet exposure.
The oxidative DNA damage was assessed by evaluating the phosphorylation of two DNA markers: the histone 2AX (γH2AX), marker of DNA double-strand breaks (DSBs) and DNA repair, and ATM, involved in the genotoxic stress response. Fluorescence microscopy evidenced the presence of characteristic distinct foci of p-ATM and γH2AX in chromatin of cells treated with pellet-derived PM (Figure 2).

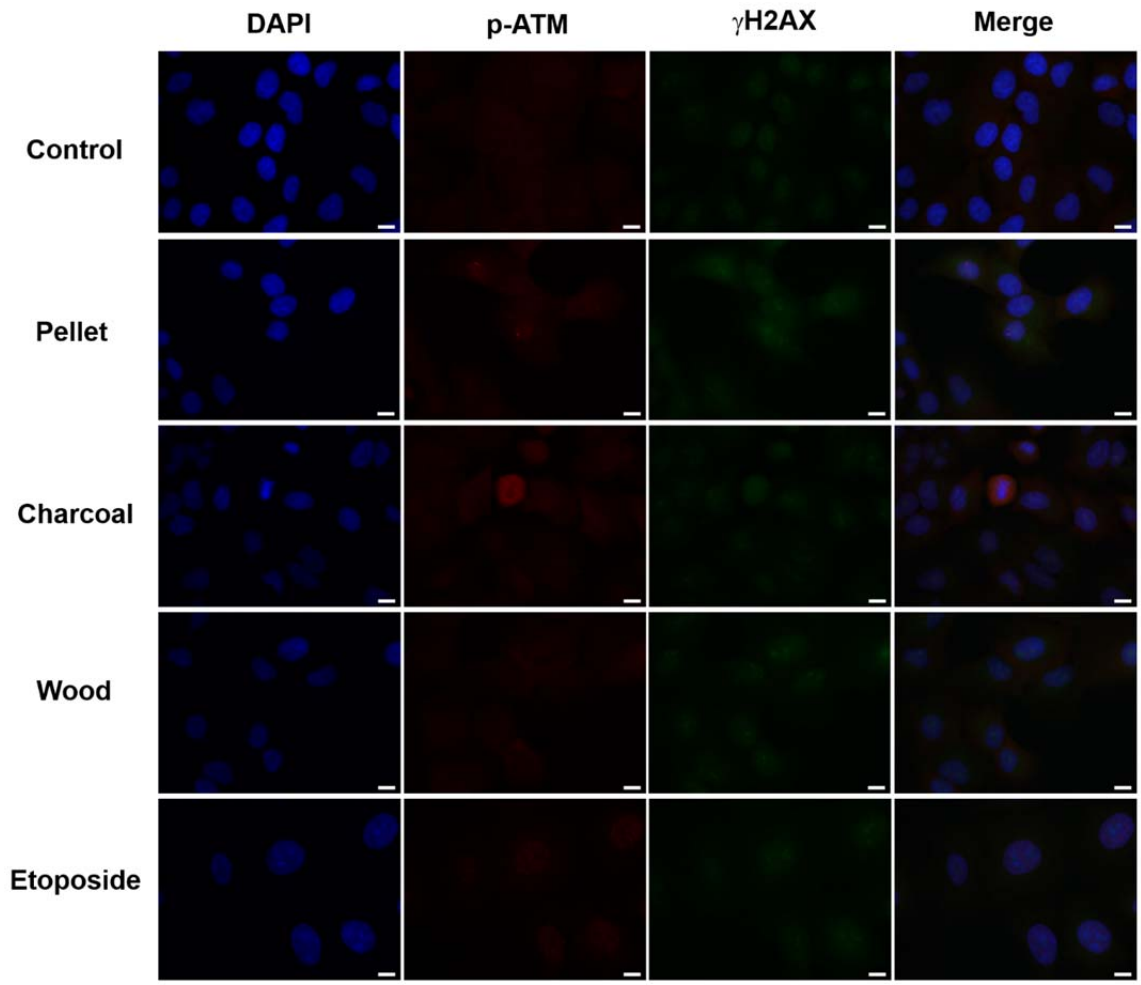

Figure 2: Fluorescence microscopy images showing DNA damage. Scale bar= 10 μm.

4. Discussion

Biomass particles showed a deep difference in chemical composition. These findings can explain the different toxic effects observed. Pellet-derived PM revealed a higher capacity of triggering cytotoxic and genotoxic effects on A549 cells, since it induces cell death, ROS production and DNA damage. In order to investigate the biological effect of biomasses derived from different emission sources on human A549 cells, the ability of particles to affect cellular metabolic activity and viability at 24 h of exposure was examined at first. As expected from literature data (Marabini *et al.*, 2017), biomasses were able to damage the cell plasma membranes, suggesting the activation of mechanisms of cell death.
Important mechanisms related to PM toxicity and involved in health effects from biomass exposure, are oxidative stress and inflammation (Longhin *et al.*, 2013). The modulation of the inflammatory response after PM exposure was investigated by measuring the release of the cytokine TNFα. Not surprisingly, A549 cells

showed a low inflammatory response, in agreement with previous observations reporting that wood smoke particles are not able to induce a high cytokines release respect to other PM sources in the *in vitro* systems (Longhin *et al.*, 2016). The oxidative stress in response to biomass was also investigated and the cytometric analysis confirms the statistically significant ROS production only after A549 exposure to charcoal. PM-induced ROS formation has been widely reported and linked to metals and PAHs (Yang *et al.*, 2016). Despite a higher presence of metals in pellet-derived PM, this fraction resulted to be less effective on ROS formation in comparison with particles collected from charcoal and wood. The exposure to biomass can also lead to genotoxic effects, such as alterations of DNA integrity and function, analyzed with DSBs.
The ROS increase could be related to the soluble metals as well as to the presence of PAHs and quinones. One of the most severe consequence of ROS damage is its interaction with DNA. DNA damage, if not repaired, can lead to genetic instability and can play an important role in lung cancer development. There are evidences indicating that the exposure to traffic-related air pollution is linked to DNA oxidative damage and this might be associated with an increased risk of cancer (Møller *et al.*, 2014).
In order to evaluate PM-induced genotoxic effect, the presence of DNA lesions by the expression of p-ATM and γH2AX was investigated. ATM is a protein involved in genotoxic stress and when activated it triggers a cascade of events leading to the phosphorylation of different substrates, including H2AX, which are able to mediate the effects of ATM on DNA repair. These findings indicate an increased expression of p-ATM and of γH2AX only after pellet exposure, suggesting the induction of DNA damage and the subsequent activation of the DNA repair machine. This effect has been previously linked to the presence of PAHs (Longhin *et al.*, 2013). However, a PM10 high content of metals too may be one of the main factors producing the biological responses, including DNA oxidative damage (Van De Huevel *et al.*, 2016).

5. Conclusions

In the last years, great attention has been devoted to the indoor PM fraction, since several studies have reported its involvement in the onset of pulmonary and cardiovascular diseases. The results of this study evidence that pellet-derived PM, which has the higher concentration of metals, activates death mechanisms and oxidative responses in alveolar cells, with consequent oxidative lesions at DNA level. Wood-derived PM is unable to affect the biological endpoints in this research, except for cell viability. The lower toxic properties observed after wood exposure could be explained by the activation of different molecular pathways, still unknown. Charcoal combustion particles induced oxidative stress on A549 cells but not genotoxic damage, suggesting a role of another pathway, probably related to the xenobiotics metabolism. From the obtained results it may be deduced that diverse biomasses may activate lung different toxicological pathways which may depend to their chemical composition. The role of combustion-derived particles on human health is still under investigation and further studies are needed to understand the molecular mechanisms responsible for the lung toxic effects.

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