Toxicology in Vitro 44 (2017) 164–171

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

# Particulate emissions from modern and old technology wood combustion induce distinct time-dependent patterns of toxicological responses *in vitro*

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ARTICLE INFO

*Keywords:*
Time course
Macrophages
Combustion emissions
Particulate matter
Inflammation
Cytotoxicity

ABSTRACT

Toxicological characterisation of combustion emissions *in vitro* are often conducted with macrophage cell lines, and the majority of these experiments are based on responses measured at 24 h after the exposure. The aim of this study was to investigate how significant role time course plays on toxicological endpoints that are commonly measured *in vitro*. The RAW264.7 macrophage cell line was exposed to $PM_1$ samples (150 μg/ml) from biomass combustion devices representing old and modern combustion technologies for 2, 4, 8, 12, 24 and 32 h. After the exposure, cellular metabolic activity, cell membrane integrity, cellular DNA content, DNA damage and production of inflammatory markers were assessed. The present study revealed major differences in the time courses of the responses, statistical differences between the studied samples mostly limiting to differences between modern and old technology samples. Early stage responses consisted of disturbances in metabolic activity and cell membrane integrity. Middle time points revealed increases in chemokine production, whereas late-phase responses exhibited mostly increased DNA-damage, decreased membrane integrity and apoptotic activity. Altogether, these results implicate that the time point of measurement has to be considered carefully, when the toxicity of emission particles is characterised in *in vitro* study set-ups.

## 1. Introduction

Particulate air pollution is one of the most important factors that is linked to reduced life quality and adverse health effects. It is known to reduce life expectancy through exacerbation of chronic cardiovascular and respiratory diseases, but also inducing new disease cases (World Health Organization (WHO), 2003, Pope and Dockery, 2006). One of the main sources for increased air pollution worldwide is biomass combustion (Silva et al., 2013). Above all, the adverse health effects of biomass combustion are remarkable in countries with a high prevalence of indoor cooking with solid fuels. However, their effects on health are also noticeable in the countries with generally low particulate air pollution levels (Sigsgaard et al., 2015).

In numerous toxicological studies, biomass combustion particles have caused a large variety of toxicological outcomes. These effects include genotoxicity, cytotoxicity and inflammatory activity as well as oxidative stress *in vitro* (Kasurinen et al., 2016; Danielsen et al., 2011; Kocbach et al., 2008a; Samuelsen et al., 2008; Leonard et al., 2000); inflammation, tissue damage and genotoxicity in mice (Happo et al., 2013; Uski et al., 2012); and oxidative stress, reduced heart rate variability and increased arterial stiffness in studies with human volunteers (Unosson et al., 2013; Barregård et al., 2006). It has been shown that the combustion technology, as well as the quality of the fuel and user-related practices have significant effects on particle concentrations and the chemical composition of wood combustion emissions (Kelz et al., 2012; Kelz et al., 2010a; Kelz et al., 2010b; Lamberg et al., 2011; Tissari et al., 2008; Fine et al., 2002). Therefore, the overall particulate emissions from wood combustion can be greatly reduced by improving both combustion technology and optimising the furnaces. However, particles emitted from different biomass burning units and combustion qualities act differently in biological systems due to the variation in their chemical composition. Usually, in cell experiments, decreased combustion quality has been associated with more cytotoxic and genotoxic effects of particulate emissions (Tapanainen et al., 2012, 2011; Jalava et al., 2012, Jalava et al., 2010). However, in recent studies it has been observed that particulate matter (PM) from modern

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http://dx.doi.org/10.1016/j.tiv.2017.07.005
Received 10 January 2017; Received in revised form 12 June 2017; Accepted 7 July 2017
Available online 12 July 2017
0887-2333/ 

technology combustion can cause stronger toxicological responses in animal lung (Happo et al., 2013; Uski et al., 2012) than PM from old technology appliances, which has been detected also in cell cultures (Uski et al., 2014).

A large share of the *in vitro* toxicological studies with collected airborne particles is conducted with macrophage cell lines. Moreover, mouse macrophages are also largely used, because they have a high comparability to results gained from animal experiments. In airways, macrophages mediate inflammatory and innate immune responses immediately after exposure to inhaled particles, which is followed by removal of deposited particles from the respiratory tract by phagocytosis. A large share of the published *in vitro* studies with particulate combustion emissions are conducted using dose-dependent approach with only one time point for the analysis of the induced responses, and only rarely the studies have focused on the toxicity of emissions from modern combustion units even at single time point of measurement (*e.g.* Karlsson et al., 2006; Jalava et al., 2012; Kasurinen et al., 2016). Therefore, it is important to study, how PM from different combustion devices affects the time course of the evoked responses. It is known that not all the toxicological effects happen simultaneously but rather as a cascades (Li et al., 2002), thus, the toxicity caused by the particles is usually a complex pathway of events. Some of the first-phase reactions may be lost or the down regulation of some pathways may already diminish the results if only one time-point is used in the experiments. Though, the combustion emission induced time course of the toxicological responses in macrophages has remained unresolved.

Therefore, we investigated the time courses of toxicological responses induced by $PM_1$ emission samples from wood combustion from selected small-scale combustion sources representing old and modern combustion technologies. In the present study, we measured several markers of cytotoxicity (DNA content, metabolic activity and cell membrane permeability), inflammation (tumor necrosis factor alpha [TNFα] and macrophage inflammatory protein [MIP-2] production) and DNA damage (comet assay) in RAW264.7 macrophages at six different time points (2 h, 4 h, 8 h, 12 h, 24 h and 32 h) after the exposure. The time course of the toxicological responses was compared to the measured chemical composition of the PM samples to find possible factors affecting the provoked responses in macrophages.

## 2. Materials and methods

### 2.1. Small-scale biomass combustion appliances

The particulate emissions for this study were produced using five different commonly used wood combustion appliances that represent prevailing European equipment. Units representing modern combustion technology were a fully automated modern pellet boiler (PB) and a modern masonry heater (MMH). A conventional masonry heater (CMH), an old technology log wood boiler (LWB) and sauna stove (SST) represented old technology systems.

The modern masonry heater consisted of an improved combustion concept including secondary combustion air compared to the conventional masonry heater where the combustion air was mainly derived through the grate. The sauna stove was a light, metal stove designed for rapid combustion and heat release. The masonry heaters and the sauna stove were loaded manually with dry birch logs and the log wood boiler with beech logs. Commercial softwood pellets were used as fuel in the pellet boiler. Detailed descriptions of the furnaces and fuels as well as OC and EC concentrations of the emissions are presented in Supplementary Table 1 and in previous publications (Tapanainen et al., 2012, 2011, Jalava et al., 2012; Kelz et al., 2012, Kelz et al., 2010a).

### 2.2. Particle sample collection

All $PM_1$ samples were collected from diluted flue gas of the biomass combustion appliances on polytetrafluoroethylene (PTFE) filters with a particle sampling system for toxicological and chemical characterisation, which has been previously validated by Ruusunen et al. (2011). The sampling system consisted of a pre-cyclone with 10 μm cut-off, a porous tube diluter, a Dekati® gravimetric impactor (DGI, Dekati Ltd., Tampere, Finland) and a pump. The DGI includes a backup-filter for PM $<$ 0.2 μm and four impaction stages with aerodynamic cut-off sizes of 0.2, 0.5, 1.0 and 2.5 μm. The PM $<$ 0.2 μm collected on the backup-filter and the two lowest impaction stages ($PM_{0.2–0.5}$ and $PM_{0.5–1}$) of the DGI were used to form a $PM_1$ sample for each combustion appliance, since most of the particles from good quality biomass combustion falls in that size range. Blank control filters were collected from all sampling campaigns separately and treated exactly as the other filters.

### 2.3. Preparation of $PM_1$ samples to analyses

The $PM_1$ samples were prepared for chemical and toxicological analyses according to previously validated procedures (Tapanainen et al., 2011). Briefly, before their usage the filters were washed with methanol and weighed with an analytical balance. The effects of the surrounding temperature, barometric pressure and relative humidity were corrected by including control filters and appropriate conditioning time in the weighing procedure. The collected particle mass was determined by repeating the weighing of the filters before and after the sample collection and extraction procedure to gain the extraction efficiency. The particulate material was extracted twice from the filters with methanol in a water-bath sonicator for 30 min, temperature not exceeding + 35 °C. Three stages ($PM_{1.0–0.5}$, $PM_{0.5–0.2}$ and $PM_{0.2}$) were pooled to form $PM_1$ sample representing each biomass combustion sample and concentrated using a rotary evaporator at + 35 °C and 150 mbar. Finally, the particle suspension was dispensed into glass tubes on a mass basis and the samples were dried under nitrogen flow on a heating block (+ 35 °C). The same procedures were followed when the blank filters were prepared. All $PM_1$ samples were stored at − 20 °C for subsequent toxicological and chemical analyses.

For cell exposures, dry $PM_1$ samples and blanks were thawed and stabilised to room conditions for 30 min. Thereafter, dimethyl sulfoxide (DMSO, final concentration of 0.3%) and pathogen-free water were added to a concentration of 5 mg/ml, and sonicated in an ultrasonic water-bath for 30 min at + 20 °C. The blank filter sample was prepared in the same way as the $PM_1$ samples. Thereafter, the particle suspension was diluted in cell culture medium to obtain the PM concentration of 150 μg/ml that was used in the cell exposures.

### 2.4. Chemical characterisation of emission particles

Chemical characteristics of the $PM_1$ samples including ions and elements are presented in our earlier publications (Tapanainen et al., 2011, 2012; Jalava et al., 2012) and summarised in Table 1. Anions ($Cl^-$, $SO_4^{2-}$, $NO_3^-$) and cations ($Na^+$, $NH_4^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$) were analysed using ion chromatography systems as described in detail by Teinilä et al. (2000).

Two different methods were used to analyse elements (Al, As, Cd, Co, Cr; Cu, Ni, V, Fe, Mn, Pb, Zn) in the PM samples. All samples, except for those collected from the old technology log wood boiler, were analysed using an inductively coupled plasma mass spectrometer (ICP-MS) as described by Pakkanen et al. (2001). Ash forming elements from the old logwood boiler samples, excluding Cl, as well as S were determined by a multi-step pressurised digestion with $HNO_3$(65%)/HF (40%)/$H_3BO_3$ followed by a measurement using inductively coupled plasma optical emission spectroscopy (ICP-OES) or ICP-MS, depending on detection limits. Cl was determined according to EN 15289 as described in Kelz et al., 2010a. A total of 30 PAH compounds were analysed using a gas-chromatograph mass-spectrometer with a single ion monitoring technique (GC–MS-SIM) as described by Lamberg et al. (2011). Genotoxic PAHs were determined based on the WHO-IPCS criteria (World Health Organization, 1998). Elemental carbon (EC) and