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Particulate emissions from modern and old technology wood combustion induce distinct time-dependent patterns of toxicological responses \textit{in vitro}

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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 hours 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 hours. 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.

Keywords: time course, macrophages, combustion emissions, particulate matter, inflammation, cytotoxicity
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 (WHO 2009, 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 \textit{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, 2010a, 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, 2010). However, in recent studies it has been observed that particulate matter (PM) from modern 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, 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 minutes, 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$_3$BO$_3$ 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 (WHO 1998). Elemental carbon (EC) and organic carbon (OC) were detected from parallel low volume particulate samples as described by Frey et al. (2009). The determination of the contents of OC and EC in the aerosol samples from the old technology log wood boiler was performed with a carbon/ hydrogen analyser as described in Kelz et al. 2010a.

2.5. Experimental design

The RAW264.7 macrophage cell line was obtained from American Type Culture Collection (ATTC, Rockville, MD, USA). The cells were maintained at +37 °C in 5% CO₂ atmosphere in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Paisley, USA) supplemented with 10 % of heat inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), 2 mM L-glutamine and 100 U/ml penicillin-streptomycin (Gibco, Paisley, UK). The cell suspension (5 x 10⁵ cells/ml) was dispensed into 6-well plates (Costar, Corning Inc., NY, USA), 2 ml per well, and the cells were allowed to adhere for 24 h. Fresh complete medium was changed 1 h before the exposures.

Macrophages were exposed for 2, 4, 8, 12, 24 or 32 hours to 150 µg/ml of PM₁ samples or the corresponding blank sample at 150 µg/ml. All cell exposures were conducted in duplicate in three independent experiments. The used PM dose was selected based on our previous dose response studies on small-scale wood combustion particles (Jalava et al. 2012, 2010, Tapanainen et al. 2011). After exposure at each time point, the macrophages were resuspended into cell culture medium by gently scraping the bottom of the wells with a cell lifter and mixing with a pipette. Immediately after the resuspension, cell metabolic activity (CMA) was determined from each well using the MTT-test. The remaining cell suspension was centrifuged (5 min, 6.082 x g, +4°C and the supernatant was stored at -80°C for analysis of the proinflammatory cytokine TNFα and the chemokine MIP-2. The cells were suspended into phosphate buffered saline (PBS) and a part of them was used for analysis of the DNA content and propidium iodide (PI)-staining of the fresh cells. Prior to the analysis of the DNA
content, the cells were fixed with ethanol (70% v/v) and stored at +4 °C. The other duplicate portion of the cells in PBS was used without a delay in the comet assay to measure DNA damage.

2.6. Analysis of cytotoxicity

**MTT test.** The proportion of cells with decreased mitochondrial activity was estimated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) test, which detects the metabolic activity of mitochondria and endoplasmic reticulum in cells. The optical density at wavelength 570 nm was measured with a microplate reader (Victor3™, PerkinElmer Corp., Waltham, MA, USA) and analysed using WorkOut 2.0 software (Dazdaq Ltd., Brighton, UK). The observed absorbance of the exposed cells was compared to control cells.

**PI-exclusion test.** Cells with a lowered cell membrane integrity were measured by flow cytometry. Briefly, the cells were separated from the culture medium by centrifugation (5 min, 370 x g) and washed once with 1 ml of PBS. Thereafter, the cells were resuspended into 0.5 ml of PBS and propidium iodide (PI) was added to reach 1 µg/ml concentration and mixed with the suspension. The samples were incubated for 15 min at room temperature in dark. After the incubation, a total of 10,000 cells per sample were analysed immediately by flow cytometry (CyAn ADP, Beckman Coulter, USA) and Summit software version 4.3 (Beckman Coulter).

**Analysis of cellular DNA content.** DNA content and thus the cell cycle phase of non-apoptotic mouse macrophages was analysed with PI staining of fixed permeabilised cells. In this analysis, cells at different cell cycle phases are recognised and apoptotic cells are identified as cells containing fragmented DNA (Ranalli et al. 2003; Darzynkiewicz et al. 1992; Nicoletti et al. 1991). The ethanol-fixed cells were centrifuged (10 min, 400 x g), and thereafter resuspended into PBS. Cells were treated for one hour with 0.15 mg/ml ribonuclease A at +50 °C and then stained for two hours with 8 µg/ml PI at +37 °C in the dark. A total of 10,000 cells per sample were analysed at an emission wavelength
of 613±20 nm (FL3) by flow cytometry (CyAn ADP, Beckman Coulter). Possible interference of the particles in the analysis was checked by running fixed particles suspension only with the flow cytometry and gated to exclude most of the PM from the analysis.

**Analysis of inflammatory responses.** The concentrations of the proinflammatory cytokine TNFα and the chemokine MIP-2 were analysed from the cell culture medium using commercially available kits (R&D Systems, Minneapolis, MN, USA) following the instructions of the manufacturer. The absorbance was measured at 450 nm using a microplate reader (Victor3™, PerkinElmer). Concentrations of the proinflammatory mediators were calculated by interpolation from the standard curve using WorkOut 2.0 software (Dazdaq).

**Analysis of genotoxicity.** DNA damage in mouse macrophages was detected by the comet assay. The alkaline version of the comet assay was performed according to the procedure described by Singh et al. (1998). The comet analysis was performed in ethidium bromide-stained slides analysing 100 cells/nuclei per test sample in each experiment using the comet assay IV (Perceptive Instruments Ltd., UK) image analysing system. Three replicate experiments were analysed. The comet response parameter used in the statistical analysis was the Olive Tail Moment (OTM) [(tail mean – head mean) x tail%DNA / 100].

**Statistical analysis.** All the measured values were analysed with Levene’s test for equality of variances before analysing the data with the analysis of variance (ANOVA) and Dunnett’s test. In cases where Levene’s test gave values < .05, the Kruskal-Wallis test was applied. The differences in data were regarded as statistically significant at \( p < .05 \). Differences between time points were tested by Tukey’s HSD or Dunnett’s C test. All data was analysed using the IBM SPSS Statistics 21.0 (IBM ®, New York, NY, USA).

10
3. RESULTS

3.1 Chemical composition of PM samples

The concentrations of Ca, Cd, Cl, K, Mg, Mn, Na, Pb, Zn and PAH compounds of the studied PM samples are summarised in Table 1 and individual PAH content in the samples in Supplementary Table 2. The chemical composition of the particulate samples from the different combustion sources were described in detail by Tapanainen et al. (2012 and 2011) and Jalava et al. (2012). Briefly, the modern masonry heater and pellet boiler PM had higher concentrations of Cl, K and Mn than appliances representing conventional combustion technologies. Ca, Cd, Mg, Na, Pb and Zn concentrations had more variation between all combustion sources. Regarding PAH compounds, the old combustion systems showed significantly higher PAH emissions than modern systems.

3.2. Cytotoxicity

Mitochondrial activity. CMA, measured with the MTT test was significantly affected by most of the samples compared to the level of activity in control cells (Figure 1). The blank filter sample slightly decreased the metabolic level of the cells at 2-hour exposure and had small variation to control level at rest of the time-points. Pellet boiler (PB) samples consistently decreased the metabolic activity at exposure durations from 2 to 12 hours, with a slightly lesser effect at 24 and 32 hours. Similar effect was seen with modern masonry heater (MMH) samples, especially at 8 and 12 hours. None of the exposure durations induced a significant effect with conventional masonry heater. The PM\textsubscript{1} sample from poor combustion quality in the log wood boiler first induced significantly elevated CMA, but after 8 hours of the exposure, CMA started to decline, being down to 23 ± 1 % of control at 32 h. A similar, but not as drastic effect was seen with the sauna stove emission sample, where the smallest share of metabolically active cells, 46 ± 3 %, was detected at 24 hours after the exposure. However, at 32-hour exposure duration, the response was not as large anymore. The statistical differences
between the samples were mostly limited to differences between the modern (PB, MMH) and old technology (LWB, SST) samples.

*Cell membrane integrity.* In the PI-exclusion test, PM$_1$ samples had variable responses in different exposure durations depending on the combustion quality (Figure 2). Only minor changes in the cell membrane integrity were seen with water control and blank samples. The cell membrane permeability did not increase with the pellet boiler and conventional masonry heater derived PM$_1$ samples compared to controls. PM emissions from modern masonry heater increased the cell membrane permeability at 2h to 41±12% and at 4h to 27±3% of the cell population. This response however vanished at the later time points. With the two samples from the furnaces with poor combustion quality, cell membrane integrity was compromised only after 24 and 32 hours, but the response levels were large. The old technology log wood boiler sample caused more extreme results, 55 ± 15% at 24 hours and 85 ± 6% at 32 hours, compared to 18±5% and 50±21%, respectively, with the sauna stove. Of all of the studied samples and exposure duration combinations, only 2 and 4 hours for modern masonry heater, 24 and 32 hours for log wood boiler and 32 hours for sauna stove had significantly increased cell membrane permeability compared to control. In the inter-sample comparison, only MMH at early time points and LWB at later time-points had statistically different response compared to other samples.

*DNA content analysis.* The DNA-content of the cells was analysed by flow cytometric analysis of PI stained permeabilised cells (Figure 3). The method reveals different phases of the cell cycle and apoptotic cells as the subG$_1$ phase of the analysis, which is shown here. Water and blank control exposed cells did not differ from each other and also the pellet boiler sample was on a similar level with control with only slightly elevated response. In all of these cases, the amount of cells in subG$_1$ phase was higher at the early time-points, but then decreased until 12-hour time-point, with a slight
increase again towards 32-hour time-point. MMH sample induced the highest number of cells in subG₁ phase at 2-hours, with a significant response until 12-hours. With this sample the response was diminished at 24 and 32 hours. CMH sample did not induce any clear time-response. With LWB sample, the response consistently increased towards the longer exposure durations compared to control. However, only the 32-hour time point had significant response. With sauna stove sample, the apoptotic response, indicated as SubG₁ cells increased drastically from 12-hours to 24 and 32-hour time-points. These particles also restricted the progression of normal cell cycle in S-G₂/M – phase at 8 and 12 h (data not shown). The inter-sample differences were again limited to early time-points of MMH and late time-points of LWB and SST, compared to the other samples respectively.

3.3. Inflammatory activity

Chemokine production. Only LWB and SST PM₁ samples induced large increases in MIP-2 levels that started to rise 4 hours after the exposure (Figure 4). LWB sample evoked the highest measured MIP-2 concentration (1670±374 pg/ml) 12 hours after the exposure that was also the highest measured during the study. However, at the 24 hours and 32-hour time points, the chemokine level started to decrease from the highest measured concentrations both with the LWB and SST samples. PM samples from conventional and modern masonry heaters and PB had only minor effects on MIP-2 concentration, especially when chemokine concentration at control level was taken into account.

Cytokine production. The obtained cytokine responses by the PM₁ samples from different biomass combustion sources remained at a low to moderate level at all time points (Figure 5). The highest increases in TNFα production were induced by the LWB, SST and MMH PM₁ samples. Similar to the chemokine responses, the increase of cytokine concentration started 4 hours after the exposure, including negative control sample and extracted blank filter sample. The response by LWB sample reached its highest level (180 ± 24 pg/ml) at the 12 hours time point. Modern and conventional
masonry heater samples evoked their highest TNFα production 24 hours after the exposure. Both SST and LWB activated cytokine productions started to decrease and were near the control level at 24 hours, but only with the sauna stove sample TNFα started to increase again at the latest 32 hours time point. Response to the PM₁ sample from MMH started to decrease from its highest at the latest measured time point of 32 hours. Meanwhile, response to the pellet boiler sample remained near the negative control level, even though at the 32 hours it was increased significantly from the control level.

3.4. Genotoxicity

DNA damage. LWB sample evoked clear genotoxic response after exposure to PM₁ emission samples (Figure 6). DNA damage induced by the LBW sample started to increase 8 hours after the exposure, and was its highest at the 24 hours time point, but started to decrease again 32 hours after the exposure. SST sample revealed its highest, but not statistically significant response also 24 hours after the exposure. CMH sample produced low, but significant increases in the measured DNA damage at the earliest 2 h and 4 h time points. PB and MMH samples were not exhibiting genotoxic activity after exposure to PM₁ samples.

4. DISCUSSION

The present study showed major differences in the time course of toxicological responses induced by particles from old and modern technology biomass combustion appliances. In general, the observed toxicological responses were categorised into three groups; LWB and SST emissions formed their own poor combustion quality group, and PB and MMH a good combustion quality group, whereas CMH was more or less in between these two groups. The most remarkable finding of this study was that the observed toxicological responses by different combustion qualities differed clearly between
the measured time-points. Therefore, we have divided the discussion into early- (2 – 4 h), middle- (8 – 12 h) and late-stage (24 – 32 h) responses.

4.1. Early-stage responses

In the early time points, no clear signs of inflammation were detected. In addition, genotoxic activity remained low during these 2 and 4 h exposure times, which indicates that it requires longer exposure times for the generation of DNA damage in murine macrophages. Even though some of these measured responses reached statistical significance, it seems that the early time points were not the most feasible to reveal differences between the samples in the inflammatory and genotoxic responses. However, early time points may still reveal important information of emissions potency to induce acute responses on macrophages. Thus, in contrast to our previous animal experiments (Hanno et al. 2013; 2007, Uski et al. 2012) at the early time point measurements, inflammation and genotoxicity are no good indicators of toxicity in this cell culture setup. This finding with macrophages differs from the observed increase of DNA damage in human lung epithelial cells (A549) after 4 hours exposure to wood combustion particles (Karlsson et al. 2006). Instead, the cells exhibited a clear response in some measured cytotoxicity parameters at the earliest 2-hour time point. In PI-exclusion assay, the MMH sample produced clearly its highest response, whereas the other samples produced only low responses with minor differences between the samples. Interestingly, the cells’ response to the MMH sample decreased during later time points. This rapid response indicates that the MMH sample contains some compounds, which may harm the cellular membrane of the macrophages immediately, but the induced damage is still repairable. In addition to these tests, also CMA was disturbed by the modern technology emission samples. This response remained stable towards the later time point measurements. In contrast, old technology emission samples increased CMA in the mouse macrophages at the earliest time points. This might be due to intact cell membrane based on PI-analysis that may have protected their mitochondria during the first 4 hours after the exposure.
Therefore, it is highly possible that these measured responses are related to the rapid increase of oxidative stress, which is also one of the earliest activities in the complicated chain of responses (Li et al 2002). In Moller et al. 2014, these chains have been discussed in three potential scenarios, where PM induces inflammation via oxidative stress, inflammation induces oxidative stress or these are parallel phenomena in the chain of effects that PM causes. It is known that oxidative stress increases before inflammatory responses in the first stage of the oxidative stress response (Li et al. 2002). In that model, the oxidative stress is first protective feature, which then turns into inflammation and lastly to cytotoxicity.

4.2 Responses of the middle time-points

Inflammatory responses of the mouse macrophages started to increase during the middle time points (8-12 h) (see Figures 4 and 5). Especially samples representing old technology showed increased MIP-2 concentration, whereas the response to the other samples stayed near the control level. A similar effect was seen with TNFα levels, although these responses remained overall low at these time points. The increase of MIP-2 concentrations after 12 hours exposure might indicate the potency of organic compounds in inducing proinflammatory effects in macrophages. This increase in inflammatory activity after exposure to combustion emission samples has been seen in several studies (e.g. Kasurinen et al. 2016; Jalava et al. 2012; Kochbach Bølling et al. 2012). Low inflammatory potential of wood particles has been also reported by Karlsson et al. (2006). They found that the inflammatory potential of wood smoke particles in human macrophages after 18 hours exposure were considerably lower than for those collected from street. It seems that particles from poor combustion sources with high organic content activate different pathways of toxicity in macrophages than particles from efficient combustion sources. This may be linked to the different solubility of particles from distinct combustion qualities, but also to their chemical composition as a producer of reactive oxygen species (ROS) as discussed earlier. LWB sample started to induce DNA damage in
macrophages at 8 and 12 hours middle time points, whereas genotoxic activities from the other samples were not discovered. This indicates that DNA damage by PM$_1$ samples from very poor combustion starts earlier, which is probably, associated with the organic composition. This is supported by the finding that old technology appliances samples had the highest total PAH concentrations as well as organic and elemental carbon levels. However, SST sample, which had even higher PAH content than LWB, did not induce similar increase in genotoxic response. This could be due to different reasons. The organic carbon composition of the samples may differ beyond the analysed PAH compounds. The samples may also contain more reactive oxy- and nitro-PAHs, which were unfortunately not analysed in this study. Alternatively, the bioavailability of PAH compounds may be different in the visibly sooty SST sample, compared to more tar-like LWB sample as a result of different combustion processes. The PAH content of the wood smoke particles was not associated with an increases in cytokine concentrations after 12 hours exposure nor cytotoxicity in a co-culture of A549 pneumocytes and THP-1 monocytes (Kochbach Bølling et al. 2012). Instead, high PAH-concentration has been earlier associated with increased genotoxicity in macrophages (Abbas et al. 2013; Tapanainen et al. 2012). It has also been observed that inflammation enhances the PAH initiated genotoxic effects in mouse lung (Arlt et al. 2015). However, it has been suggested that other organic compounds than PAHs, such as quinone-like compounds, are involved in the induced biological effects (Kochbach Bølling et al. 2012).

Toxic effects of PAH compounds are typically based on metabolic activation by intracellular cytochrome P450 system. However, the activity of this system varies a lot in different cell types, structures and tissues (Schirmer et al., 1998). In RAW264.7 macrophages, some P450 isoforms are present (Nakamura et al., 1998), which indicates that these cells have the ability to metabolise PAH compounds. The metabolic activation of PAH compounds might be one reason explaining the variation in the observed toxic responses that can lead to the changes seen later in mitochondrial
activity as well as cell membrane integrity. However, the PM$_1$ samples of the different combustion sources consist always of a mixture of several PAH compounds, which have both synergistic and antagonistic effects (Leme et al., 2011). Thus, the overall effects of PAHs are very difficult to estimate. In addition, it has been observed that water-soluble and heavy PAH components of urban air PM$_{2.5}$ are contributing to antiapoptotic effects in human bronchial epithelial cells via aryl hydrocarbon receptor (Ferecatu et al. 2010). Reduction of apoptosis was observed prior to secretion of proinflammatory cytokines. This response may also explain the lowered number of macrophages in SubG$_1$ phase at the middle time-points of the present study. Increased apoptotic response at the late phase time-points may be linked to the higher dose used in this study for revealing cytotoxic responses. In contrast to increased inflammatory and genotoxic activities, no clear differences between the samples were detected in the cytotoxic analyses at the middle time points. However, the MTT-test revealed a rapid decrease of metabolic activity for the old technology derived samples that displays changes in the cells metabolism. Even though there were ongoing changes in the cell viability, the middle time points turned out to be the least interesting when trying to separate the toxicological responses of the different wood combustion samples.

4.3. Late phase responses

In this study, the late time points covered 24 and 32 h points for measurements. The 24 hours time point was of special interest, since it is commonly used in cell experiments focusing on dose dependent approaches. Moreover, in some cases, there has been a discrepancy between the results of cell and animal studies, which can partly be explained by different feedback mechanisms. In animal studies, more than one measuring time has been used for the sample collection at the most feasible expression time. Instead, cell experiments that haves used only one time-point may indicate non-optimal measuring time of some of the responses.
Similar to the middle time-points, samples from old technology combustion devices had the highest inflammatory activity also at these late phase time points. Even though MIP-2 concentration to LWB sample was already decreasing from its highest concentration, SST had its highest concentration at 24 hours time point. Overall, the order between the induced responses was almost the same in the late phase time points as in the middle time points of measurement. In general, TNFα concentration was slightly increasing at the late phase time points, but all the levels remained low and there was no significant difference between the induced responses, which shows that TNFα is not a good marker for studying inflammatory responses of combustion derived PM$_1$ responses with macrophage cell line. Similarly, TNFα concentrations were at their highest level 12 hours after the exposure, but decreased at later 40 and 60 hours time points with wood smoke particle treated co-cultures of A549 and THP-1 cells (Kochbach et al. 2008a). Thus, the detected decrease of cytokine concentrations at the 24 h time point requires consideration, when the toxicity of the PM$_1$ emission samples is estimated. This change in cytokine concentrations can be a result of several mechanisms that have been reported previously. One evident explanation is the decreased amount of viable cells to produce cytokines that limits cytokine production at the late phase of measurements. Another possible explanation is the binding of cytokines to carbonaceous compounds (Kocbach et al. 2008b) or the walls of the cell culture plate. However, this phenomenon is not the explanation in cell cultures, where FBS or other protein-rich supplements are used. Moreover, it is possible that cytokines and other proteins are degraded during the time of exposure. It has been observed that cytokines are degraded rather rapidly at room temperature (Zhou et al. 2010), which means also in the conditions in the incubator. At the late 24 and 32 h time points, genotoxic responses increased clearly for the old technology derived samples, but for the other samples the level of induced DNA damage remained low. Moreover, the resolution of the genotoxic response between the samples was clearly the highest at the 24 hours time point. Thus, it seems that the organic compounds induce this increase in genotoxic activity, and this effect is further strengthened by the decreased cell membrane integrity that was
observed in PI analysis. One explanation is that PAHs can be metabolised to produce electrophilic reactive products that are able to damage DNA by forming DNA adducts. In this mixture of chemicals, genotoxic activity is usually induced both by direct-acting compounds and by compounds causing genotoxicity via metabolic activation. Furthermore, the particles themselves may mediate reactive oxygen and nitrogen (NOS) species generation through interactions with target cells, and particle-elicited inflammation in cells (Schins and Knaapen, 2007; Knaapen et al., 2004). Altogether, there are various ways through which combustion particles cause genotoxicity in macrophages directly or indirectly, one of them being oxidative DNA damage (Danielsen et al., 2009; Karlsson et al., 2006; Leonard et al., 2000).

In contrast to the old technology samples, the effect on the cell membrane integrity was minimal with the rest of the samples at the late phase time points for measurements. In the MTT-test, the rapid decrease in the metabolic activity continued with the samples representing old technology. At the late phase time points, the order of the responses to the studied PM$_1$ samples was inversed, when compared to those at the early time-points. Now the old technology samples caused clearly the largest decrease in the mitochondrial function and thus in the CMA. Overall, the response by the other samples remained even during all the measured time points, but at 24 hours the resolution of the MTT-test was the greatest. Towards the 32 hours time point, the responses started to fluctuate and the blank sample provoked higher than 100% responses. Thus, at this time point the comparison between samples is not necessarily valid anymore.

5. Conclusions

The different time-points of measurement have a strong effect on how the toxicological responses are indicated in the cell exposure studies. Thus, future studies would benefit from the use of optimised time-points for different measured parameters. It would also increase the reliability of the
interpretation of the results, if more time points were included. If the time-points of activation of
different responses are not known, the far-reaching conclusions of the toxicity of samples may not be
justified. The results from this study only apply for one cell type and it has to be taken into account
that different cell types and cell lines have their own time courses, varying with the different type of
combustion samples. The commonly used 24-hour time-point seems to be suitable for comparisons
in most of the analysis, but at least with inflammatory parameters, also earlier measurement times
should be included.

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FIGURE LEGENDS:

Fig. 1. Cellular metabolic activity (measured as percent of control) of mouse RAW264.7 macrophages in MTT-test after 2h, 4h, 8h, 12h, 24h and 32h exposure to dose 150 µg/ml of emission particles from old and new technology wood combustion appliances. Asterisks (*) indicate significant responses compared to control cells (Dunnett’s test, p<0.05). Letters indicate a statistically larger response (ANOVA and Tukey’s test, p < .05) to other samples; (a) pellet boiler (PB), (b) modern masonry heater (MMH), (c) conventional masonry heater (CMH), (d) log wood boiler (LWB), (e) sauna stove (SST).

Fig. 2. Cell death (percent of 10,000 cells) of mouse RAW264.7 macrophages in PI-exclusion test after 2 h, 4 h, 8 h, 12 h, 24 h and 32 h exposure to dose 150 µg/ml of emission particles from old and new technology wood combustion appliances. Asterisks (*) indicate significant responses compared to control cells (Dunnett’s test, p<0.05). Letters indicate a statistically larger response (ANOVA and Tukey’s test, p < .05) to other samples; (a) pellet boiler (PB), (b) modern masonry heater (MMH), (c) conventional masonry heater (CMH), (d) log wood boiler (LWB), (e) sauna stove (SST).
Fig. 3. The percentages of mouse RAW264.7 macrophages (total cell counts 10,000) in SubG₁ phase of the cell cycle after 2 h, 4 h, 8 h, 12 h, 24 h and 32 h exposure to dose 150 µg/ml of emission particles from old and new technology wood combustion appliances. Asterisks (*) indicate significant responses compared to control cells (Dunnett’s test, p<0.05). Letters indicate a statistically larger response (ANOVA and Tukey’s test, p < .05) to other samples; (a) pellet boiler (PB), (b) modern masonry heater (MMH), (c) conventional masonry heater (CMH), (d) log wood boiler (LWB), (e) sauna stove (SST).

Fig. 4. The production of tumor necrosis factor α induced by the particles (dose 150 µg/ml) emitted from old and new technology wood combustion appliances in mouse RAW264.7 macrophages after 2 h, 4 h, 8 h, 12 h, 24 h and 32 h exposure. Asterisks (*) indicate significant responses compared to control cells (Student’s t- test, *p<0.05, **p<0.01, ***p<0.001). Letters indicate a statistically larger response (ANOVA and Tukey’s test, p < .05) to other samples; (a) pellet boiler (PB), (b) modern masonry heater (MMH), (c) conventional masonry heater (CMH), (d) log wood boiler (LWB), (e) sauna stove (SST).

Fig. 5. The production of macrophage inflammatory protein 2 induced by the particles (dose 150 µg/ml) emitted from old and new technology wood combustion appliances in mouse RAW264.7 macrophages after 2 h, 4 h, 8 h, 12 h, 24 h and 32 h exposure. Asterisks (*) indicate significant responses compared to control cells (Student’s t- test, *p<0.05, **p<0.01, ***p<0.001). Letters indicate a statistically larger response (ANOVA and Tukey’s test, p < .05) to other samples; (a) pellet boiler (PB), (b) modern masonry heater (MMH), (c) conventional masonry heater (CMH), (d) log wood boiler (LWB), (e) sauna stove (SST).
**Fig.6** DNA damage induced by the wood combustion particles in mouse RAW264.7 macrophages (dose 150 µg/ml) after 2 h, 4 h, 8 h, 12 h, 24 h and 32 h exposure. Asterisks (*) indicate significant responses compared to control cells (Dunnett’s test, p<0.05). Letters indicate a statistically larger response (ANOVA and Tukey’s test, p < .05) to other samples; (a) pellet boiler (PB), (b) modern masonry heater (MMH), (c) conventional masonry heater (CMH), (d) log wood boiler (LWB), (e) sauna stove (SST).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Table 1. Chemical constituents (µg/mg) and PAH content (ng/mg) in the PM1 samples.

<table>
<thead>
<tr>
<th></th>
<th>Pellet boiler</th>
<th>Modern masonry heater</th>
<th>Conventional masonry heater</th>
<th>Logwood boiler</th>
<th>Sauna stove</th>
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Genotoxic PAHs: 3.01  704  13710  14271  42910
Total PAHs: 5.97  1464  17625  34707  83480

bdl = below detection limit.
Genotoxic PAH compounds according to WHO 1998.
Highlights

- Time dependent toxicological responses were measured from mouse macrophages
- Different combustion qualities induced different time-courses of responses
- More than one time-point for measurement should be considered in cell studies