TIME DEPENDENCE OF TOXICOLOGICAL RESPONSES *IN VITRO* INDUCED BY PARTICLES EMITTED FROM SMALL-SCALE WOOD COMBUSTION

Pro gradu -research
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ABSTRACT:

Small-scale wood combustion has significant health effects both in individual and population level. Increase in $PM_{2.5}$ (particles, that have diameter $\leq$ 2.5 µm) concentration in air increases the risk to die prematurely. Mortality rate rises especially due to respiratory- and cardiovascular diseases. Adverse health effects are stronger with group at risk including children, elderly people and those suffering cardio respiratory diseases.

Frequency of wood combustion makes it a global issue. Increase in the producing of renewable energy will raise wood combustion also in the developed Western countries. Different combustion appliances, techniques and fuels have effect on the chemical and physical features of the forming particles and thus the health effects. Combustion is more efficient with new technology appliances and the burning of the fuel is more complete than in old technology appliances, which produce more for example polyaromatic hydrocarbons which have been connected with inflammation and cytotoxicity in immunological cells. Macrophages are part of body's immune defense and they are usually the first defense cells in the lungs to face the alien particles. Macrophages are able to phagosytate for example particles from small-scale wood combustion. This enables the use of them in toxicological research.

In the present study, particles emitted from three different combustion appliances were used to assess their ability to induce cytotoxicity in mouse macrophage cell line RAW264.7. Used appliances were sauna stove, pellet boiler and modern masonry heater. Toxicological properties of formed particles were studied by measuring cell viability with MTT-test, cell number with counts and the relative amount of apoptotic and necrotic cells and cell cycle progress with flow cytometry. Study was done by using six different time points; 2h, 4h, 8h, 12h, 24h and 32h.

This study shows that particles from different combustion appliances have health related toxicological properties that clearly differ from each other. Sauna stove, that represents the old technology, caused strong toxic reaction in cells in later time points, while modern masonry heater caused fast toxic reaction in early time points. Pellet boiler did not cause any major responses in cells. Difference was visible in the entire tests used in this study.

The present results suggest that adverse health effects of the small particles are not unambiguous, because the chemical and physical features of the particles vary a lot. It is noteworthy that wood combustion is not properly regulated, which increases the variation of exposure. Further study on this matter is needed.

ITÄ-SUOMEN YLIOPISTO, Luonnontieteiden ja metsätieteiden tiedekunta
Ympäristötiede
Karppela Maria-Pia: Puun pienpoltossa syntyvien hiukkaspäästöjen toksikologisten soluvasteiden aikariippuvuus
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TIIVISTELMÄ:

Puun pienpoltolla on merkittäviä terveyshaittoja sekä yksilö- että väestötasolla. $PM_{2.5}$ (hiukkaset, jotka ovat halkaisijaltaan $\leq$ 2.5 µm) kokoisten hiukkasten pitoisuuden lisääntyminen ilmassa lisää riskiä kuolla ennenaikaisesti. Erityisesti kuolleisuus erilaisiin hengitys- ja sydänsairauksiin lisääntyy. Terveysvaikutukset ovat voimakkaampia riskiryhmiin kuuluvilla, kuten lapsilla, vanhuksilla ja hengityselin- ja sydänsairauksista kärsivillä.

Puunpolton yleisyyden takia kyseessä on globaali ongelma. Uusiutuvan energian tuoton kasvattaminen tulee lisäämään puunpolttoa myös kehittyneissä maissa. Erilaisilla polttolaitteilla, tekniikoilla ja polttoaineen valinnalla voidaan kuitenkin vaikuttaa syntyvien hiukkasten kemiallisiin ja fysikaalisiin ominaisuuksiin ja sitä kautta haitallisuuteen. Uuden polttotekniikan avulla palaminen on tehokkaampaa ja polttoaine palaa perusteellisemmin kuin vanhemman tekniikan laitteissa, joissa syntyykin enemmän esimerkiksi polyaromaattisia hiilivetyjä, jotka on liitetty erilaisiin haittavaikutuksiin.

Makrofagit ovat osa kehon immuunipuolustusta ja ne ovat keuhkoissa ensimmäisiä puolustussoluja jotka kohtaavat ulkopuolelta tulevat altisteet. Makrofagit pystyvät fagosytoimalla eli solusyömällä ottamaan sisäänsä esimerkiksi pienhiukkasia. Tämä mahdollistaa niiden käytön toksikologisessa tutkimuksessa.

Tässä tutkimuksessa käytettiin kolmen erilaisen polttolaitteen tuottamien pienhiukkasten haitallisuuden tutkimiseen hiiren makrofagisolulinjaa RAW264.7. Käytettävät polttolaitteet olivat saunan kiuas, pellettipoltin sekä moderni takka. Syntyneiden hiukkasten terveysvaikutuksiin liittyviä toksikologisia vasteita tutkittiin mittaamalla solujen elävyyttä MTT-testillä, solujen määrää solulaskuilla ja apoptoottisten ja nekroottisten solujen osuutta sekä solusyklien etenemistä virtaussytometrillä. Tutkimus tehtiin aikavastekokeena käyttäen kuutta aikapistettä; 2h, 4h, 8h, 12h, 24h ja 32h.

Tutkimuksessa selvisi, että erilaisten polttotekniikoiden synnyttämillä hiukkasilla on selkeästi toisistaan poikkeavia toksikologisia vaikutuksia. Vanhan polttotekniikan laitetta edustavan saunan kiukaan hiukkaspäästöt aiheuttivat voimakkaan toksisen reaktion soluissa viimeisissä aikapisteissä kun taas modernin takan päästöt tuottivat akuutin toksisen vasteen. Pellettipolttimen hiukkaspäästöt eivät tuottaneet merkittäviä vasteita soluissa. Erot ovat nähtävissä kaikissa käytetyissä testeissä.

Pienhiukkasten aiheuttamat terveyshaitat eivät ole yksiselitteisiä, sillä hiukkasten kemiallinen ja fysikaalinen koostumus vaihtelee suuresti. Puunpolttoa ei ole säädelty laissa kunnolla, mikä lisää altistumisen kirjoa. Lisää kokeellista tutkimusta puun pienpolton terveysvaikutuksista tarvitaan.

ABBREVIATIONS AND DENIFITIONS

A-MuLV	Adelsons murine leukemia virus
ANOVA	one directional analysis of variance
ATP	adenosine triphosphate
CDK	cyclin-dependant kinase
DGI	Dekati® Gravimetric Impactor
DNA	deoxyribonucleic acid
FSC	forward scatter
G_1	Resting phase of the cell cycle
IAP	inhibitors of apoptosis
LPS	lipopolysaccaharide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NO	nitric oxide
PAH	polyaromatic hydrocarbon
PBS	phospate buffered saline
PI	propidium iodide
PRD	porous tube diluter
RAW264.7	mouse monocyte macrophage cell line
RNA	ribonucleic acid
PM	particulate matter
PM1	particles that have diameter ≤ 1 µm
PM2.5	particles that have diameter ≤ 2.5 µm
PM10	particles that have diameter ≤ 10 µm
PM100	particles that have diameter ≤ 100 µm
S-G_2/M	cell cycle phases from DNA synthesis to mitosis
SSC	side scatter
sub G_1	apoptotic cell fragments

TABLE OF CONTENTS

PREFACE

I was on my second study year when I started the research on my Master of Science Thesis. Now –when graduating and finishing this paper- I'm on my fourth study year. I am graduating relatively fast and this Thesis has been with me almost my whole student time. All my studying has been somehow related to this Thesis, because when you start the research, you'll be thinking it even in the Christmas holidays –like I did in 2012. Even my friends and family have been exposed to this ever going studying about small-scale wood combustion and $PM_{2.5}$ particles. Thank you for patience and support on this matter. And thank you for your sabotage in everything else. Without you or Huone (same thing) I wouldn't be here.

I want to thank my supervisor Maija-Riitta Hirvonen who led me through this writing process. Somehow all the text I wrote became science. My other supervisor Maija Tapanainen was irreplaceable when I was in laboratory doing the test for this Thesis.

For my friends and family

7.6.2013 in Kuopio

Maria-Pia Karppela

INTRODUCTION

Wood combustion is a health risk that hasn't been studied enough. Epidemiological data has shown that particles from wood combustion are associated with adverse health effects and they have a huge effect on life quality and economics. Different respiratory and cardiovascular diseases shorten the life expectancy and cause extra cost on health care system (Amann et al. CAFE D, IIASA, 2005). Toxicological findings, so far, support these epidemiological associations. However, further toxicological studies are needed to identify the causative constituents in PM behind the reported health impacts.

It has been recently shown that used combustion technique has a significant impact on the chemical and physical properties of the formed particles. Moreover, the used fuel and user practices affect the characteristics of PM emissions. Globally, wood combustion in open fires is common, whereas in the developed countries different furnaces and fireplaces are used for wood combustion. The amount of wood combustion is also rising because of the need to increase the amount of renewable energy. Consumers in Finland use both old and new technology combustion appliances. These different techniques may produce very different health effects which is the result of very different combustion particles.

Several murine or human cell types, including key target cells of the respiratory tract (e.g macrophages, epithelial cells) can be used for toxicological assessment of PM induced activation of cellular mechanisms behind the observed harmful effects. In lungs, macrophages are usually the first ones to face the material that has been inhaled. These cells can intake alien material by phagocytating it. These features make lung macrophages a good test object to study the effects of wood combustion particles.

Many *in vitro* –inhalation toxicology studies are based on the 24 hour exposure. Cell cycle of mouse macrophage cells is normally approximately 24 hours, thus this is reasonable. However, there is need for identification of time dependence of the induced responses, since using only one time point may hide the progression of different toxicological mechanisms. Thus, there is need for studying also earlier time points to identify the development of impairments and get more information about the activated pathways.

This pro gradu was designed to study time dependency of activated cellular processes in a mouse macrophage cell line which may lead to different damages in cells. Analysis of the amount of apoptotic and necrotic cells, cell cycle progression, viability and the number of cells were used in assessment harmfulness of the particles emitted from different combustion techniques.

1.0 REVIEW OF THE LITERATURE

1.1 INTRODUCTION

The amount of small scale wood combustion is increasing in Europe due to European Union's energy policy aiming to increase the use of renewable energy up to 20% from the total energy consumption by 2020 (Directive 2009/28 of the European Commission). The use of renewable energy is seen as an efficient way to decrease carbon dioxide emissions and subsequently to slow down the climate change. This will increase biomass combustion in residential small-scale systems, which are responsible for a substantial burden of the total $PM_{2.5}$ (particulate matter $D_p < 2.5$ µm) emissions. However, from the public health point of view, these decisions have raised concern that increased biomass combustion may substantially increase the health risks, since biomass is known to be a major source of gaseous and particulate air pollutants in the atmosphere. It is also noteworthy, that small scale wood combustion is the only significant particulate emission source that is not properly regulated (Jalava 2008).

Health related toxic effects of small scale wood combustion emissions vary a lot depending on combustion appliances, used technology and fuels. For example the amount of oxygen in combustion process has effect on particle size and formation clearly (Bølling et al. 2009). Thus, these properties should be taken into account in all the regions where wood and other biomass is burned (Naeher et al. 2007). Different kind of combustion produces particles with different physicochemical and toxicological properties. In wood combustion, carbon dioxide and carbon monoxide are the most common gases (Naeher et al. 2007). Instead, organic and elemental carbon, sulphuric acid and fly ash are the most common components in the emission particles (Lighty et al. 2000).

Epidemiological research shows that when $PM_{2.5}$ concentration grows to 10µg/m^3, mortality rate increases 4 to 14 % (Dockery et. al. 1993, Pope et. al. 2002, McDonnell et. al. 2000). Health effects induced by small particles are mainly based on inflammatory responses, cytotoxicity, oxidative stress and genotoxicity (Bølling et al. 2009). Regardless of the evidence showing the association between particulate exposure and adverse health effects, the causative constituents in PM emissions are not known. In addition, toxicological properties of emissions from different heating appliances, and fuels are poorly understood. Thus, there is need for identification of the key components and properties of emissions that cause the

observed toxicological responses responsible for the associated health effects. (Bølling et al. 2009)

1.2 SMALL-SCALE WOOD COMBUSTION

The most important sources of biomass combustion derived particles are long-range transported emissions from wild fires as well as domestic cooking and heating. Open fires in houses can produce huge amount of particles in different sizes. Fine particles originate mostly from primary combustion (e.g. small scale wood combustion), but these particles change in atmosphere through coagulation with other particles and condensation with water on their surface. Wood combustion produces mostly $PM_{2.5}$ sized particles, so the research on this particle size and its health effects is reasonable (Jalava 2008). Energy production uses increasingly amounts of wood-based biomass, which leads subsequently to growth of $PM_{2.5}$ emissions. Biomass combustion is also increasing because of global efforts to decrease carbon dioxide emissions. Thus, the emissions cause problems also in society level (Bølling et al. 2009).

In Finland, wood combustion is traditionally more common in rural than in urban areas. However, recently wood combustion is becoming more prevalent also in urban areas, which has raised health concerns. In Finland, annually about 8,4 million cubic meters of wood is burned to produce energy both in industry and in households and about 6,7 million cubic meters of that is used for residential heating (METLA 2011). Currently wood combustion covers over 20 % of energy consumption in Finland and over 40 % of that is used for residential heating (METLA 2011). Finland's environmental center (SYKE) estimates that about 30 % of $PM_{2.5}$ emissions are from stray heating and small scale combustion. Although modern technology appliances have become more common, 25 % of stationary combustion emissions originate from residential wood burning in Finland based on primary $PM_{2.5}$ (Karvosenoja et al. 2008).

1.2.2. Combustion appliances

Wood combustion appliances do not have any efficiency standards or regulations in Finland (Jalava 2008). Thus, devices and user practices vary a lot. Wood combustion is going to be challenging in the future, and the goal to achieve clean combustion technologies requires development of new technologies and fuels.

Although used combustion appliances vary a lot, there are some major types that describe the general situation. Roughly speaking, appliances can be classified to old and modern technology devices. Typical old technology device in this study was sauna stove, while pellet boiler and modern masonry heater represented modern technology. The main differences between these appliances are the amount of oxygen, burning temperature and fuel input (Pettersson et al. 2011). Sauna stove's burning temperature vary a lot more than for example that of pellet boiler and modern masonry heater. Irregular fuel input and air flow cause this. Modern appliances have more leveled combustion conditions because these factors are stabilized. However, fuel input is irregular when it's manmade.

Grate and burner affect temperature and atmosphere in combustion appliances. In addition, wood fuel can be a mixture of different origins. These factors effect on combustion conditions. Smouldering combustion increases significantly the fine particle emissions and amount of organic compounds in the particles (Tissari et al. 2008, Hytönen et al. 2009). Low oxygen levels are also part of poor combustion.

The amount of air necessary to maintain combustion is theoretically stoichiometric ratio. In this ideal situation all the fuel reacts with air and combustion is complete, which is never the situation with wood combustion. The amount of excess air is dependent on the burner design. Also the amount of air needed for dilution of flue gas is dependent on specific drafting device (Utiskul et al. 2012). The amount of air and oxygen is dependent on used technology. The old combustion appliances and new technology can have a huge difference with air amount, which affects particulate formation and their chemical and physical features.

1.2.4. Physicochemical properties of emission particles

Particle properties depend strongly on combustion appliance and conditions. Such properties are particle size, morphology, number and mass concentration as well as chemical composition. Particle size and mass range are dependent on the combustions type: slow combustion produces different range than fast combustion. Ignition phase produces larger particles in slow combustion (particle size 50-500 nm) while fast combustion produces smaller particles (particle size 20-200 nm). In flaming phase particles are mostly size PM 10-100 in both slow and fast combustion. In smouldering phase particle size is almost the same as in flaming phase, but the number of the particles is smaller. The greatest number of particles is forming in flaming phase. Slow combustion produces more particles in ignition

but less in smouldering (Wardoyo et al. 2005). This size and mass range is from vegetation combustion, but it can vary a lot depending on fuel and appliance.

In good combustion conditions, the fine particles consist mostly of alkali metal compounds. For example pellet boiler represents these combustion conditions. Fine particles include also organic and elemental carbon. Fine particles from modern masonry heater contain small amounts of carbons. Smouldering combustion e.g. from sauna stove, the main compounds in fine particles are organic and elemental carbon (Tissari et al. 2009). Thus, amount of carbon can be used as an indicator of poor combustion.

Fine particles can include different ions including K^+, SO_4^{2-}, Cl^- and Na^+. These ions end up in fine particles from ash, where they vaporize in hot temperatures (TEKES Project 40296/07 report).

Water-solubility of particles depends on combustion appliance. Burning in good combustion conditions produce more water-soluble material than in poor combustion circumstances. These water-soluble particles are mostly ash-forming ions. The non-water-soluble particles from poor combustion conditions are mostly organic and elemental carbon (Lamberg et al. 2011).

Good combustion conditions produce more inorganic ions than organic. However, the amount of ions is relatively small in good combustion conditions compared to those in poor combustion. Typical inorganic ions are potassium, sulphate, sodium, chloride and nitrate. The organic ions are equally common both in good and poor combustion. Relatively poor combustion particles include more organic ions. Study from Lamberg et al. 2011 showed that secondary formation has a role in organic ion formation. Organic ions are formed in particles when the combustion emissions are mixed with the ambient air.

1.2.5. Toxicological properties of particles

Toxicological properties of inhalable combustion particles and their dose have significant impact on their ability to cause adverse health effects (Happo et al. 2010, Jalava et al. 2007, Uski et al. 2012). It has been shown recently that the particles derived from poor wood combustion are more potential to trigger a cascade in cells leading to programmed cell death (apoptosis) than the particles derived from good combustion process (Tapanainen et al. 2011).

Health related toxicological properties of combustion emissions can be assessed as activation of cellular pathways leading to cytotoxicity, inflammation, oxidative stress and genotoxicity.

Cytotoxicity and inflammation

According to Holgate et al. 2003, cytotoxicity is related to chronic respiratory diseases and to development of cardiovascular diseases. Same study shows that inflammation caused by epithelial damage is connected with asthma. Thus, cytotoxicity and inflammation cause remarkable portion of health effects caused by small scale wood combustion particles.

The particulate matter sized 2.5-10 µm has been identified to be potent cytotoxicity and inflammation inducer. The fine particulate matter has been suggested in epidemiological studies to be more harmful to human health than coarse particles (Jalava 2008). Exposure to wood combustion particles triggers production of inflammatory mediators. This effect is also dose-dependent, but high doses can cover the inflammatory reaction due to increasing cell death (Tapanainen et al. 2011). Although wood combustion particles cause inflammatory reactions the, amounts of inflammatory mediators have been moderate comparing to amounts in cells that were exposed to particles derived from urban air or traffic (Jalava et al. 2007; 2010, Kocbach et al. 2008).

While inflammation and cytotoxicity are often simultaneous, some compounds can increase another and decrease other. Compounds that are from incomplete combustion (such as PAH compounds) caused negative correlation with inflammatory mediators but not with cytotoxicity, according to Jalava (2008). These inflammatory and cytotoxic mechanisms are the main mechanisms with health effects that are caused by particulate matter in air (Jalava 2008).

Recent study has shown that wood combustion particles have moderate inflammatory potential, but cytotoxic and genotoxic properties are more important (Kocbach et al. 2008).

Oxidative stress

Oxidative stress is connected with local and systemic inflammation, cytotoxicity and genotoxicity (Jalava 2008). Adverse health effects are thought to be mediated by these variable mechanisms.

Oxidative stress is one of the mechanisms effecting e.g. cancer origin. Air pollution has been shown to have a connection with oxidative DNA-damage and lung cancer (Møller et al.2008). There is evidence that high PAH-concentrations cause also oxidative DNA damage (Singh et al. 2007). Oxidative stress is a common denominator for many effects that particulate matter is causing (Jalava 2008). Wood combustion particles have been shown to increase lipid peroxidation, to generate free radicals and to cause oxidative DNA damage (Leonard et al., 2000, Karlsson et al., 2006 and Danielsen et al., 2009).

Genotoxicity

PAHs (polyaromatic hydrocarbons) are organic compounds, which concentrations increases during smoldering combustion. The genotoxicity of the particles also increases when PAH concentration grow (Sevastyanova et al. 2007). Increased PAH-compound concentration in particulate samples have increased also the amount of apoptotic cells (Tapanainen et al. 2011). Combustion appliances and combustion conditions have significant effect on forming particles and thus physicochemical characteristics of the emission particles as well as their health related toxicological properties. Incomplete combustion produces particles with more genotoxic potential than particles from complete combustion (Leonard et al., 2000, Karlsson et al., 2006 and Danielsen et al., 2009).

1.3 CYTOTOXICITY

Different exposure factors induce different responses in host defense cells. Wood combustion emissions include particles with various different physicochemical properties. Therefore the detected effects on host defense cells (macrophage cells in this study) can be various depending on combustion technology, appliance, fuel quality and exposure time. According to current view, the most important toxicological mechanisms activated by PM are related to inflammation, cell death (apoptosis/necrosis), genotoxicity and oxidative stress. Inflammatory and cytotoxic activities of emitted particles are anticipated to be linked with the non-carcinogenic respiratory and cardiovascular effects, whereas genotoxic activity has been linked with the lung cancer risk among human subjects. (Jalava 2008)

The present review of literature is focused on mechanisms of cytotoxicity.

1.3.1 Cytotoxicity as an immunotoxic mechanism

Cytotoxic compound is a compound that is toxic to cells and can cause damage or death. Immunotoxicity is the discipline of toxicology which studies the interactions of xenobiotics (drugs, chemicals, or environmental agents) with the immune system resulting in adverse effects. (Luxenburg 1984 *International Seminar on the Immunological System as a Target for Toxic Damage / Comission of the European Community)*. There is wide variety of immunotoxic effects because of immune systems physiological processes and pathological conditions. The scope of immunotoxicology includes immunosupression, immunostimulation, hypersensitivity and autoimmunity. Each area requires different modalities of clinical and nonclinical evaluation to identify the corresponding immunotoxic effects involved, to understand the underlying mechanisms and to predict the risk of possible adverse effects.

1.3.1.1. Immune system

Immune system consists of biological structures and processes which primary goal is to protect host organism from diseases and damages. This goal is done by detection of self (i.e. all tissues organs and cells of the body) from nonself (i.e. variety of opportunistic pathogens (bacteria, viruses) and transformed cells or tissues such as tumors. In order to defend the host, the immune system utilizes either independently or simultaneously a wide variety of mechanisms which are either nonspecific lacking immunological memory (innate immunity) or specific (adaptive immunity). The immune system is composed of different organs and tissues that can act autonomously or in concert with peripheral blood, multiple lymphocyte effector cells (whose function can be modulated by hormones, growth factors and messengers e.g cytokines) and redundant effect or mechanisms.

Immune system includes numerous lymphoid organs and different cellular populations with a variety of functions. It can be divided in three main parts. In the primary immune system includes central (generative) organs i.e. thymus and bone marrow, in which lymphocytes mature. The secondary immune system is formed from peripheral organs i.e. spleen and lymph nodes where naive lymphocytes are activated by antigens. The tertiary immune system includes 1) skin-associated lymphoid tissue, 2) mucosal associated lymphoid tissue 3) gut associated lymphoid tissue 4) bronchial associated lymphoid tissue and 5) the cells lining the genitourinary tract.

Immune system contains several kinds of cells that are in charge of immune defense. These immunocompetent cells include lymphocytes (T lymphocytes, B lymphocytes, Natural Killer cells) which are cellular vectors of adaptive immunity. Secondly, phagocytes (monocytes/macrophages, neutrophils) which destroy internalized pathogens. Polynuclear leukocytes including a) eosinophils which engulf and destroy large particles and participate in allergic and inflammatory responses and b) basophils/Mast cells which are source of vasoactive amines and other inflammatory mediators and participates in allergic and inflammatory responses. Moreover, among the immunocompetent cells are dendrite cells which are professional antigen presenting cells. (Ross et al. 2011)

One of the ways is to stop pathogens and other potentially harmful organisms and components to enter the system. Different surfaces e.g. skin and mucous membrane in respiratory system are forming these barriers.

These barriers can also contain cellular and chemical barriers instead of only physical block. Phagocytes are body's innate way to dispose harmful objects. These cells patrol all around body, but they can specialize to work in certain organ. Lung macrophages are lung specific and are the first ones to face the external threats that are coming in with the air we breathe.

Two important types of immune systems defense cells are granulocytes and mononuclear leukocytes (Ross et al. 2011). These both main types are produced in bone marrow from the same original form. Both leucocytes and granulocytes have several original forms that develop in to mature cells. This development happens usually outside the bone marrow, typically in different lymphatic tissue, e.g. thymus. Monocytes and lymphocytes have same origin form, from which both granulocytes and monocytes are formed. Monocytes next form is monoblast and then promonocyte, which develops in to ready monocytes (Ross et al. 2011). Monocytes work as macrophages and dendrite cells in body. This happens through inflammation signals, which make the monocytes to move to the site on infection. Monocytes can differentiate into macrophages to prevent harmful compounds from entering system (Swirski et al. 2009).

These immune system basic principles are very similar with mammals. For example human and mouse have 99 % same genome (Rosenthal & Brown 2007). This enables the parallel use of mouse and human cells in *in vitro*-experiments. Moreover, for risk assessment *in vitro* responses can be compared to those induced in a whole animal *in vivo*. Thus, this important

tool for risk assessment - although extrapolation from mice vs. human and *in vitro* vs. *in vivo* - is always needed while estimating exposure reactions induced in human being.

1.3.1.2. Macrophages and phagosytosis

Macrophages are target cells in PM induced immunotoxicity. They are within tissues where they can phagosytate cellular debris and pathogens either as stationary or as mobile cells. They also stimulate lymphocytes and other immune cells to respond to the pathogen.

Monocytes are major type of blood white cells. Promonocytes and monoblasts travel in blood and lymphatic system and then move to tissues, where they mature in to tissue macrophages. Macrophages are white blood cells within tissues. Typical tissues are lungs, liver, spleen and central neural system. Macrophages are named after the tissue they are staying: e.g.. macrophages in lungs are alveolar macrophages, in liver they are Kupffer cells and osteoclasts in skeletal system. (Ross et al. 2011)

Macrophages are found both in organs and blood circulation. In lungs macrophages face first arriving molecules and organisms. They have lysosomes in their cytoplasm and also structures, that make them able to move. The structure of these cells can vary in consequence of external stimulus, but nuclear is bean shaped and structure is granulated in macrophages (Abbas et. al. 2007). Macrophages participate to body's immune defense by phagosyteting (engulf and then digest) and destroying alien material e.g. cellular debris and pathogens either as stationary or as mobile cells. Lung macrophages move in the alveoli surface where they can get in touch with air that is breathed in and collect a great number of particles phagosyteting them. (Fels et. al. 1986). They are often the first cells that make contact with antigens. Subsequently they present this antigen material to lymphocytes, after they have processed it (Ross et al. 2011).

Phagocytosis is systems way to get rid of harmful objects. In phagocytosis alien object clipped on phagosyteting cells surface receptors. This adhesion stimulates signal routes in cell, which leads to intake of the alien object. Cell starts to reach over the object with actine fibres. This phagosome stenosis is moved inside the cell and actine fibres are removed from around of the vesicle. Phagosome turns into phagolysosome when lysosomic enzymes are inserted in to the vesicle. These enzymes break down to the content of the phagolysosome. (Abbas et. al. 2007)

1.3.1.3. Cell death

Cell death is part of cells normal life cycle. It can be programmed (apoptotic) or caused by external accident (necrotic). Different reasons of cell death lead to different kind of deaths. Programmed cell death can happen just for single cell (e.g.. in the end of its life cycle) but external irradiation usually causes damage in wider area. Normal cell can adapt to changes or then it can go through injury. Some cell changes are reversible, but after certain point cell go through either necrosis or apoptosis. (Kumar et. al. 2010)

Difference between apoptosis and necrosis is that apoptosis is an active process and it needs energy, while necrosis does not. Necrosis is usually happening because cells can't produce enough energy (ATP), which leads to cell death. (Eroschenko et al. 2008)

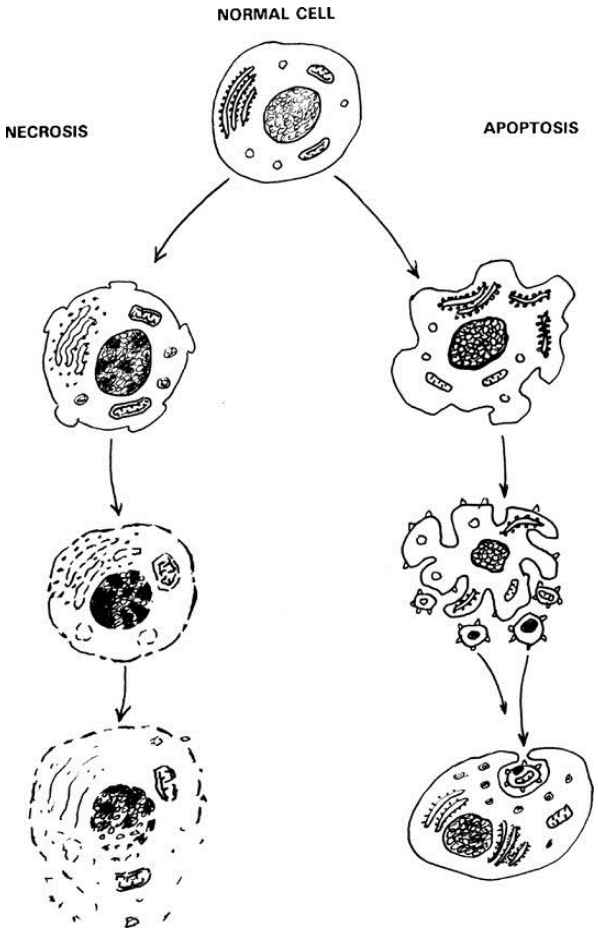

Fig. 1. Schematic representation of the different stages of necrosis (left) and apoptosis (right). In apoptosis nuclear the degrading of the cell is controlled but in necrosis the cell degrades uncontrollable which can lead to inflammation. (van der Meer et al. 2010)

Macrophages can go through apoptosis or necrosis, just like other cells in body. Caspase complex and complement complex are part of apoptosis and cells can regulate them to certain level. Macrophages have also caspase-independent cell death. Lysosomes take part in phagosytosis by fusing with phagosome, but lysosomes have only limited lifespan. Therefore they need to be replaced continuously. This function is called autophagy. (Eroschenko et al. 2008) Macrophages caspase-independent cell death is controlled by autophagy. This reaction was visible in cells that were exposed to lipopolysaccaride (LPS) and pan-caspase inhibitor (Xu et. al. 2006).

Apoptosis

Apoptosis is normal in every individual and it is also essential in normal fetal development. Apoptosis controls cell number in adults. Apoptosis is also cell reaction to something harmful, that doesn't kill the cell directly, but causes damage in the whole system. Apoptosis is induced either by some external signal (membrane receptors bind signal molecule) or it may also arise from intracellular signals (for example DNA damage). Apoptosis is also inhibited by inhibitors of apoptosis (IAP). These inducing signals and inhibiting signals form together caspase cascade. These are set of enzymes that are found in every cell in inactive form. These enzymes active the next enzyme in the series, so the avalanches of short protein sequences are causing thorough effect in the cell. (Eroschenko et al. 2008)

Apoptosis is usually typical to cell type (e.g. epithelial cells have limited lifetime, so apoptosis is part of their life cycle) or cells destroy themselves if they behave uncharacteristic way (e.g. T lymphocytes that react to normal body components are triggered to self-destruct). Apoptosis is typical in cells that are infected by viruses or in cells that have genetic mutation. (Eroschenko et al. 2008)

When apoptosis starts in the cell, its nuclear chromatin forms one or more dark-staining masses. Different size DNA fragments are formed and cell loses its DNA. At the same time cell loses cell-cell contacts because of shrinking and eosinophilia increases. When apoptosis comes along, the nuclear material breaks into fragments. Nuclear membrane goes through dissolution in this stage. These cell fragments form apoptotic bodies that are phagosytated by macrophages. (Eroschenko et al. 2008)

Necrosis

Necrosis differs from apoptosis in many ways. Necrosis is not controlled process and cell organs don't fragment and orientate organized. Individual cells don't usually go through necrosis. If whole tissue or area is somehow injured, it can lead to necrosis in this location. Necrosis is always pathological process. (Proskuryakov et al. 2003) Apoptosis is usually beneficial to body or organ but necrosis is harmful or even fatal.

In necrosis, cell swell and plasma membrane gets disrupted. Also endoplasmic reticulum and mitochondria swells. Cells can't keep up their normal metabolism and thus necrotic cells are incapable to maintain e.g. membrane integrity. Cells own lysosomes digest the cell, but leukocytes can also participate. Necrotic cells leak in the surrounding tissue, because of their inactive membrane. This leads to inflammation in tissue. (Kumar et. al. 2010)

Apoptotic fragments are phagosytated usually very soon after apoptosis but in necrosis phagocytes are prevented from locating the dead cells and phagocytating the remains. This can lead to large areas of dead tissue and cause severe damage in the organ (Kasper et al. 2001).

Inflammation is involved in necrotic conditions and thus associated with increased production of inflammatory mediators such as cytokines. Also nitric oxide (NO) and different reactive oxygen species are detected in intense necrotic cell death (Raffray et al. 1997). Nitric oxide has also been shown to kill cells by necrosis and not by apoptosis (Mitrovic et al. 1995).

1.3.2. Cell cycle progression

Cell cycle can be separated into two different stages; mitotic phase (M phase) and interphase, which is a non-dividing phase. Interphase takes most part of the cell cycles time. Interphase can be separated in three phages; G_1 phase, synthetic phase (S phase) and G_2 phase. G_1 phase takes longest time in cell cycle. It is the first gap phase and cell differentiates and goes through its typical functions during this phase. After G_1 phase cell goes through S phase. In S phase nuclear DNA is replicated before mitosis. G_2 phase takes place after S phase and before mitosis. It is relatively short gap comparing to first gap and during it cell prepares to mitotic division. (Cooper et al. 2000) Some cells can also leave the cell cycle after M phase and enter to G_0 phase. There cells are terminally differentiated and can return to cycle after suitable stimulation. Each phase takes few hours to complete, but G_1 phase can take several days or

even weeks, depending on cell type. G_0 phase can last even a lifetime of organism (Telser et al. 2007).

Mitosis can be separated into four stages: prophase, metaphase, anaphase and telophase. In prophase, chromosomes became visible within nucleus. Chromosomes have already duplicated in S phage. In prophase also two pair of centrioles wanders to opposite side of the cell and interpolar microtubules are formed between them. Metaphase is next phase in mitosis and in this phase chromosomes arrange in the metaphase plate, which means that chromosomes are all aligned in the cell equator. Chromosomes also get attached to mitotic spindle at kinetochore site. (Cooper et al. 2000) In next phase, anaphase, centromere splits and centrioles are pulled apart. This leads to two identical groups of chromosome clusters on either side of the cell. In the final phase, telophase, chromosomes began to uncoil to their interphase stage. Cytoplasm divides (cytokinesis) and cell membrane forms two daughter cells. (Telser et al. 2007)

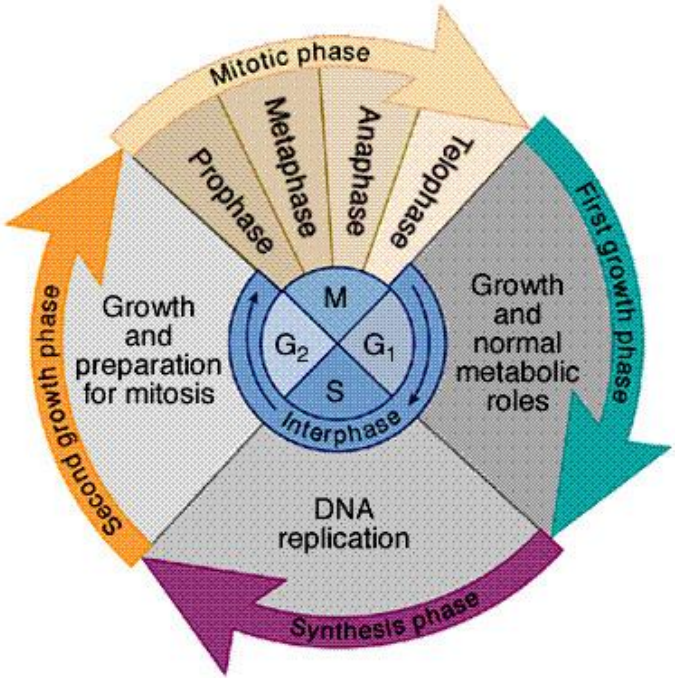

Fig. 2. Cell cycle phases and basic processes during cell duplication and growth (St. Rosemary Educational institute 2013)

Cell has several checkpoints to ensure that only viable cells will duplicate and continue life. Either this mechanism stops the dividing until the damages has been repaired, or targets cells to apoptosis. Three basic check points that are usually classified are in G_1 phase, intra-S-phase

check point and G_2 check point. G_2 point is for checking DNA replication. If DNA replication is not complete, cycle will be arrested in this point (Koshland 1989). There are also other check points, such as spindle check point. This cell cycles check points is metaphase checkpoint. Mitosis doesn't progress until all the chromatides are aligned in the cell equator (Telser et al. 2007).

Cell cycle check points work because of cyklins and cyclin-dependant kinases (CDKs). Cyklins are proteins, which are produced only in certain state of cell cycle. Appearance of these proteins in different stages of cell cycle activates CDKs because cyklins bind themselves with CDKs. Progressing of the cell cycle depends on each stage, because cyklins start always the next phage, and they are not produced, cycle will not continue. Some environmental factors (like grow factors) can also effect on cell cycle progress. (Solunetti 2006)

Amount of DNA varies in the stages of cell cycle. Amount is single in the G_0-G_1 stage but it doubles in the S (synthesis) –stage. Amount stays doubled in G_2-stage and in M (mitosis) – stage it turns into single again when chromatins are divided with daughter cells. (Vermes et. al. 2000)

Many factors effect on cell's life and division, inner factors and outside factors. Cell protects itself and whole organism by controlling the health of dividing cells. This mechanism can be used to estimate damages that some outside factors cause to cells. Severity of exposing factor compares on the damage it causes; cell arrest, apoptosis or necrosis.

1.4. FLOW CYTOMETRY

1.4.1. Basic principles of flow cytometry

Flow cytometry is currently widely used in research aiming to discover size, shape and density of cells as well as to study DNA, RNA and protein content in cells. Moreover, cell structures including membrane receptors and structure, internal and external receptors can be studied and cells state and features such as apoptosis, necrosis, inner pH and calcium flux. (Alfonso & Al-Rubeai 2011)

Flow cytometry is a method that can be used to discover both biological and physical properties of cells. For the analysis, cells are suspended in fluid and the fluid is passed

through the laser beam with high-velocity. Particles go through laser as a single file, which makes it possible to observe individual cells and structures (Alfonso & Al-Rubeai 2011). Sample fluid is absorbed to capillary from test tube and into very narrow tube system. Sheath fluid is flowing through this tube system more quickly than the sample. This makes it possible for particles to go through laser as a single file. When particle meets laser, it absorbs the light from the source and either emitters or fluorescent it to its medium. This light is gathered by optical mirrors, which direct it to detector, which then modifies light into electrical signals whose amount is the same as the particles in detected sample fluid. It is possible to detect several parameters in one sample run, if suitable detectors are installed around the fluid stream. (Shapiro 2001)

Individual cell stream is created by right size flow chamber, which is selected due to cell size. Single cells are observed in interrogation zone, where lenses focus light bundle to cell. Basic flow cytometry is based on diffraction of this light, but there are many other applied methods available. Those include e.g. possibility to use different wave lengths of light to observe signal of the cell or to use biologically active colors, which can show the state of cells activity and damages (Mandy et. al. 1995). The most common excitation source in flow cytometry is argon laser, which uses 488 nm wave length and it is usable for most fluorescent compounds (Shapiro 2003).

It is also possible to use biologically active colors, which can show the state of cells activity and damages (Mandy et. al. 1995). Particles under research are also possible to mark with fluorescent compound. This allows examining particles metabolic products, amount of DNA and surface proteins by using fluorescent light (Shapiro 2003).

Two parameters are important while observing changes in cells. Forward scatter (FSC) – detector collects light which is parallel to optical axis. This lights intensity tells the size of the particle. Side scatter (SSC) –detector collects light which is in 90 º angle to optical axis. This lights intensity tells about the shape and structure of the particles. (Shapiro 2001)

1.4.2. Flow cytometry application in this study

In the present study, flow cytometry was used to identify particulate exposure induced effects on cells in different phages of cell cycle. For this it is possible to identify $G_{0/1}$ phase, S phase and G_2/M phase cells from each other. Moreover, it is possible to evaluate whether cells have

been arrested to some cell cycle check point by analyzing the relative amounts of cells on each stage (Darzynkiewicz et. al. 2001). Apoptotic and necrotic cells can be detected by using propidium iodide (PI), which binds only on damaged cell's DNA and RNA due to the damages in cell membrane.

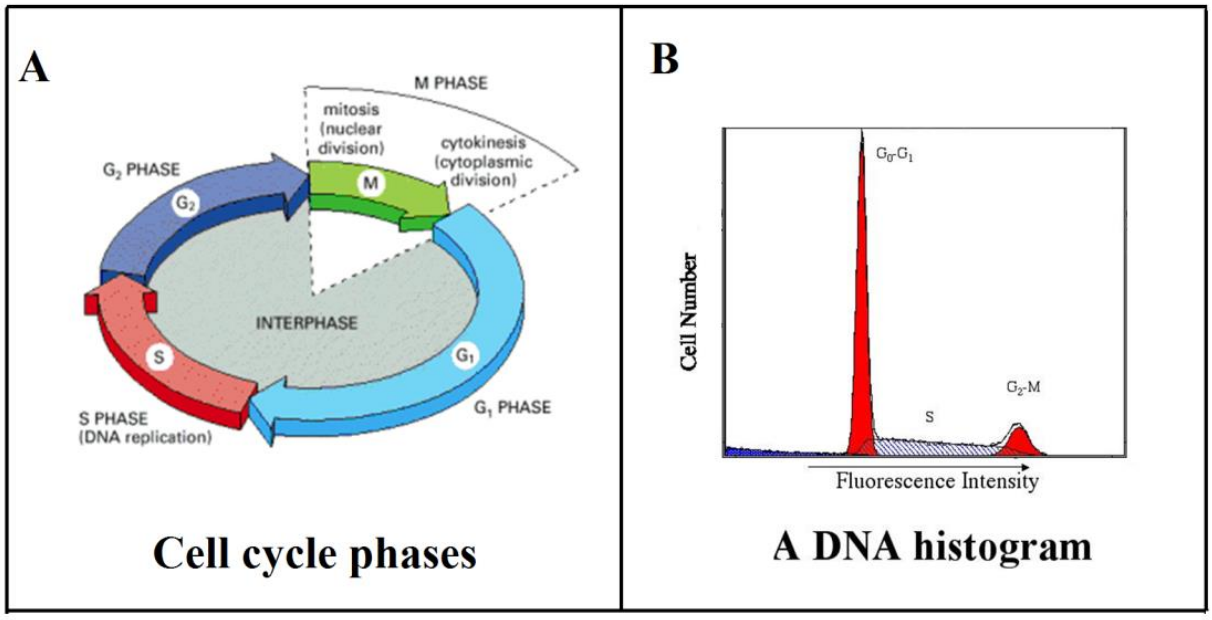

Fig. 3. Cell cycle phase relation with DNA histogram. Different cell cycle phases can be seen in histogram and cell amounts in these phases can be measured. (Tabll & Ismail 2011)

1.4.2.1. Analysis of cell damages

Different states of cell damages can be investigated by analyzing changes in permeability of cell membrane using PI, a fluorescent compound which binds with double-stranded DNA and RNA. Shortly, cell membrane begins to leak in the necrosis and in the final stages of apoptosis which makes possible for PI to attach with DNA. Instead, in apoptotic and living cells PI cannot pass the membrane. When PI is attached with DNA it fluorescents red light in 608 nm wave length. By collecting this information about fluorescent particles, it is possible to measure the amount of necrotic and late apoptotic cells. (Belloc et. al. 1994)

Very simple way to present the amount of these signals is to show on x-axis the intensity of the fluorescence and on the y-axis the amount of particles. Then it is possible to define certain

groups inside this population and to announce the amount as percents of the whole population. (Shapiro 2003)

1.4.2.2. Cell cycle analysis

Cell cycle analysis with flow cytometry is based on different amounts of DNA in cells in different stages of the cell cycle. Cells are fixed with ethanol and formaldehyde, which makes their cell membrane more permeable. This treatment also prevents cells from disintegrating and it stops harmful microbial action in samples (Shapiro 2003). Using the same PI dying method as for analysis of cell damage, it is possible to separate G_0/G_1 –stages and S-G_2/M –stages with flow cytometry. Amount of DNA varies in these stages and it is also possible to show eg. apoptotic cells, whose fluorescence is lower.

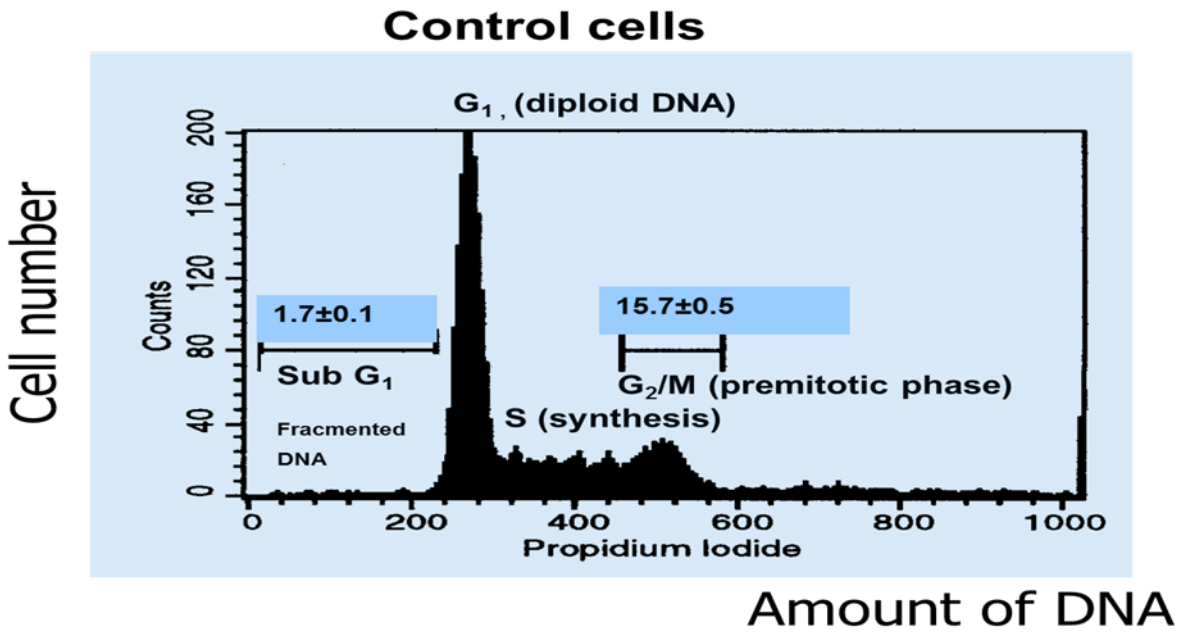

Fig. 4. Normal cell cycle of unexposed immunological control cells.

Figure 4 shows normal cell cycle of unexposed immunological control cells used in the preset study. X-axis represents the amount of DNA in cells, which means that on the right side of the histogram are the cells that contain the highest amount of DNA. Y-axis indicates the number of cells. Higher peak means greater number of cells in that stage of cell cycle. In the normal circumstances these control cells proliferate every 22 hours. In this example picture control sample contains less than 2 % of apoptotic cells and less than 16 % of cells that are in the G_2/M –phase.

Sub-G_1 –stage reflects the amount of apoptotic DNA fragments and vacuoles. In histogram this shows wider than G_0/G_1 –point area (Nicoletti et. al. 1991). Apoptotic cells can be indentified because of this fragmented DNA.

G_1-stage shows in picture as G_1 peak. Amount of DNA is normal diploid after mitosis, which shows as a peak. Amount of DNA does not vary a lot in this stage. Percentual amount of cells is great in this stage.

In **S-G_2/M –stage** the amount of cellular DNA has doubled during S-phase. This leads cells to premitotic G_2-phase and finally mitosis.

2.0 AIMS OF THE STUDY

The present study aimed at comparing of toxicological responses of particles emitted from a sauna stove, a modern masonry heater and a pellet boiler in the mouse macrophages (RAW264.7). The studied effects were focused on responses in cell cycle and apoptosis activated by particles with aerodynamic diameter less than 1µm (PM_1) since the most of the particles emitted from residential wood combustion appear in that size range (Tissari et al., 2008). Cells were exposed at different time points (2h, 4h, 8h, 12h, 24h and 32h) to reveal different reactions and damages that exposures cause.

3.0 MATERIALS AND METHODS

3.1 WOOD COMBUSTION APPLIANCES USED IN THIS STUDY

3.1.1. Sauna stove

Sauna stove represented old technology combustion in this study. The used fuel was 2 batches of birch from which the 1st batch was 5 x 0.31 kg and the 2nd batch was 6 x 0.53 kg. Sample was collected from diluted flue gas for 20-35 minutes from the ignition batch and the 2nd batch (Tissari et. al. 2007).

3.1.2. Modern masonry heater

In this study, modern masonry heater represented new technology combustion. The fuel was also birch batches in this appliance. The combustion was four-phased: 1st batch was 10 x 0.4 kg, and other batches were 4 x 1 kg. Samples were collected from diluted flue gas during 3rd and 4th batches and collection time was 50 minutes (Tissari et. al. 2007).

3.1.3. Pellet boiler

The fuel for the pellet boiler was commercial wood pellets. This boiler worked with top-feed fuel input. The combustion was continuous with this appliance and the sample collection time was 2-3 hours (Lamberg et. al. 2011).

3.2 SAMPLE COLLECTION AND PREPARATION

All the samples used in this study for toxicological analyses were provided by Fine Particulate Laboratory at UEF.

The samples were collected by using particulate sampling system for toxicological and chemical analysis as previously described by Lamberg et al. 2011. Briefly, PM_1 was collected from diluted flue gas of each heating appliance on polytetrafluoroethylene (PTFE) filters (Millipore Corp., Billerica, MA, USA) with Dekati® Gravimetric Impactor (DGI, Dekati Ltd, Tampere, Finland). Blank control substrates were collected from all the sampling campaigns and treated equally with other substrates. The DGI system consists of a heated cyclone (cut diameter: 10 μm), a porous tube diluter (PRD), the gravimetric impactor and a pump. Mass flow controllers were used to control the flow rates of the pre-cleaned particle free dilution air and the diluted flue gas. The temperature of the diluted flue gas was measured with Pt100 temperature sensors. The DGI itself consisted of four impaction stages with cut-off diameters of 2.5, 1, 0.5 and 0.2 μm as well as a backup filter (< 0.2μm).

Sample preparation was made with validated procedures (Jalava et al. 2005). Sample particles were extracted from filters with methanol by cutting filters in to pieces and by placing them to test tubes with methanol. Tubes were put in to ultrasonic water bath for 30 minutes. PM_1 samples were pooled in a flask. After this the methanol was evaporated in a rotary evaporator (Heidolph laboratory 4000). Vacuum (Vacuubrand) was used in the same time with chiller

(Lauda WK500). Particulate mass was cooled with nitrogen (99,5%) and then samples were stored to -20 ºC.

Before the cell exposure, particulate sample tubes were sonicated for 30 min in a water bath sonicator (FinnSonic m03) to suspend the size-segregated particulate samples into sterile water (Sigma W1503, St. Louis, MO, USA) at a concentration of 5 mg/ml. The blank sample tubes were treated similarly.

3.3. TOXICOLOGICAL ANALYSIS

3.3.1. Cell line

Cell line used in this experiment was RAW264.7 macrophages, which are originally from American cell bank (ATCC, American Type Culture Collection). Cells are mouse macrophage cells that are transformed into cancer cells with Adelsons leukemia virus (A-MuLV). This virus is murineleukemia retrovirus type.

RAW264.7 cells were cultured in RPMI 1640 (Roswell Park Memorial Institute) medium in which were added 10 % of deactivated FBS (Fetal Bovine Serum), 2 mM of L-glutamin and 100 U/ml penicillin-streptomycin (all made by Gibco, UK). Cells were cultured in +37 ºC and in 5 % carbon dioxide concentration (Binder CB 210, Binder, Germany). Cells were sustained twice a week in 75 cm^2 cell culture bottles (Nalge Nunc International, USA): on Mondays as 1:30 and on Fridays as 1:20. Growth medium was replaced on Wednesday (15 ml/bottle). The measurement of amount of viable and death cells in the cell cultures were performed by using Bürkers chamber and Trypan Blue –colour (Sigma-Aldrich Corp., USA). Briefly, 30 µl sample of cell suspension was taken from the control well and mixed with 30 µl of filtered Trypan Blue –colour. This suspension was added on Bürkers champer under the glass plate. Cells were calculated with 20x objective (Olympus IMT-2, Olympus Optical Co., Japan). Viable and death cells, which were dyed blue, were counted from three to four A-square of Bürkers chamber. Cell viability was controlled by counting the percentage of viable cells in the cell culture, before preparing cells for exposure research. Cell counts were taken also from control sample after exposure in each time point.

3.4. EXPOSURE

Exposure was performed on 6-well plates (Costar, Corning, USA) at a concentration of 500 000 cells/ml. Cells were plated 24 hours before exposure and growth medium was replaced (2 ml/well, RPMI medium) one hour before exposure.

3.4.1. Doses and exposure time

The RAW 264.7 macrophages were separately exposed to particle samples in a time dependent manner for 2h, 4h, 8h, 12h, 24h and 32 hrs. The particulate dose used in these experiments was 150 µg/ml, which was selected according to previous studies (Jalava et. al. 2005). Three independent experiments were made in duplicate. To rule out possible contamination of particulate samples and methodological artifacts, each plate had also a blank sample in volume corresponding to the dose of 150 µg/ml. These blank sample cells were exposed to sterile water (W1504, Water for Embryo Transfer). For positive control sample, the cells were exposed to lipoplysaccharide (LPS) from *E.coli* (50 µg/ml). The LPS is gram-negative bacteria originated cell wall component, which leads to defense reactions such as production of inflammatory mediators in mammalian cells.

After the exposure, the cells were scraped and resuspended into cell culture medium. Viability of the cells was measured with the MTT-test from cell suspension (2×100 µl) of each well. Cell count sample (30 µl) was taken from the control well. The remaining cell suspension was centrifuged (6082 x g, 5min, +4 ºC, Biofuge Fresco, Heraeus, Germany) and the supernatants were stored at -80°C for cytokine analysis (Jalava et al., 2005). The cells were washed, suspended into 1 ml of Phosphate Buffered Saline (PBS) and fixed in 70% (v/v) ethanol for subsequent propidium iodide staining (Penttinen et al., 2005).

3.5. ANALYSIS OF CELL VIABILITY (MTT test)

Cell viability was tested by spectrophotometric method called MTT test. In this test, the cells that are functional can transform MTT to colored formazan in their mitochondria (Mosmann 1983). Cell suspension (100 µl) was added as duplicate to 96- well plate right after exposure time was up. Thereafter 25 µl of MTT-solution (50 mg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma-Aldrich Corp., USA; 10 ml of PBS, Gibco, UK) was added to the plate. The 6-well plate was covered with parafilm and the samples were incubated for 2 hours at + 37 ºC (Binder, Germany). This incubation enabled MTT solution to

penetrate into cells. After the incubation, 100 µl of SDS-solution (20g natriumdodecylsulphate, 50 % dimethylformamide, Sigma-Aldrich Corp., USA) was added to the sample wells as well as to the two blank wells, and incubation was continued overnight at + 37 ºC. Absorbances were measured next day at wavelength of 570 nm with a multilabel plate reader (Victor$^{3}_{TM}$, PerkinElmer Corp., Waltham, MA, USA) and the results were analyzed using WorkOut 2.0 software (Dazdaq Ltd., Brighton, UK).Viability of cells was indicated as percentage from control sample. Sample particles cause changes in spectrophotometric results because of their absorbance ability. This variation between growth medium and medium with sample particles was measured with same multilabel plate reader. The responses induced by the studied particulates alone, were reduced from those activated by activated in the cells.

3.6. FLOW CYTOMETRIC ANALYSIS

Flow cytometry CyAn ADP (Dako, USA) was used to examine necrosis, apoptosis and cell cycle stages of the exposed cells. This device uses 488 nm argon laser as an excitation source. Before the analysis of the samples, devices optical sensitivity and functions were confirmed with the control particles (SpectrAlign^{TM} Particles,Dako, USA,3.0 µm, 1 x 10^{7} particles/ml).

Flowing speed was controlled during the sample flow and this was kept as stable as possible (200 cells/s). Between the different samples, the device was washed with cleaning solution (Dako, USA) and Milli Q –water.Results of flow cytometry were analyzed with Summit v. 4.2. (Dako, USA)

3.6.1.1 Cytotoxicity (PI exclusion test)

Necrosis and late onset of the apoptosis was measured in fresh, stained cells. Propidium iodide (PI, Sigma-Aldrich Corp., USA) was used to detect necrotic and those apoptotic cells, whose cell membrane was damaged. These cells were regarded PI-positive. Cell suspension from the exposure was moved to the flow cytometry tube (500 µl), sample was centrifuged (370 x g, 5 min, Megafuge) and supernatant was decanted. Cells were washed once with 1 ml of PBS and centrifugation was repeated. Supernatant was decanted and cell pellet was resuspended to 500 µl of PBS. Thereafter, 50 µl of PI (10 µl/ml) was added to the tubes and the samples were incubated for 15 min in the dark at room temperature. PI concentration in the samples was 1 µg/ml. Right after the incubation, the samples were analyzed with flow

cytometry counting 10 000 cells/sample. In the flow cytometry analysis only the whole cells were counted, cell fractions were eliminated from results.

Section of death cells was measured from the histogram. Living cells (PI-negative cells) were excluded and the amount of PI-positive cells could be regarded as percentage from the fluorescence on x-axis and amount of cells on y-axis.

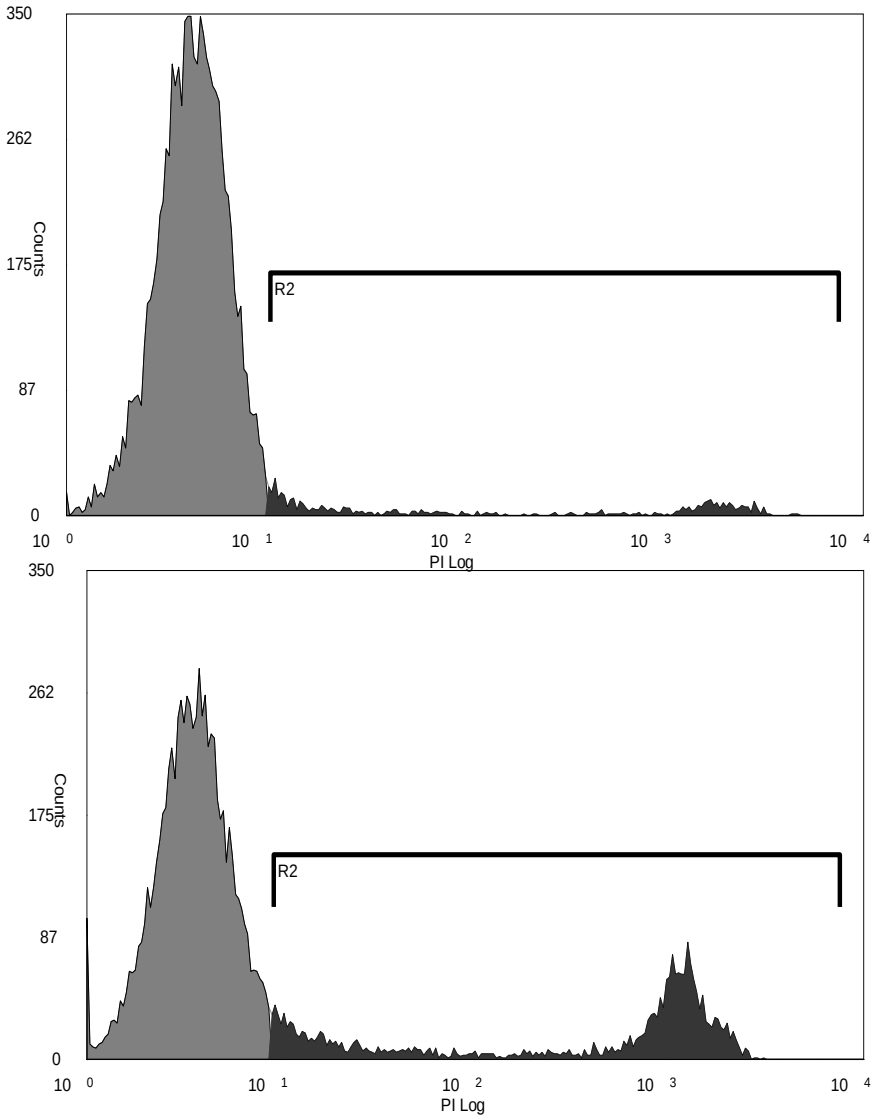

Fig. 5. Flow cytometry analyze picture from PI-exclusion test in24 hour time point. X-axis represents the strength of the fluorescence of PI and y-axis the amount of cells. Top picture shows the control test cells and the bottom picture modern masonry heaters test cells. The amount of non-viable cells has been outlined on the right side of the histogram.

3.6.2. Apoptosis and phases of the cell cycle (DNA content analysis)

Flow cytometry and PI was used to analyze cell cycle phases of the macrophages. Cells were fixed with ethanol to make the cell membrane more permeable for PI. After the exposure, cell suspension was centrifuged (6082 x g, 5 min, + 4 ºC, Biofuge Fresco, Heraeus, Germany) and the supernatant was removed (and saved for later use at -80°C). Cell pellet was resuspended into 600 µl of cold PBS. This cell suspension was pipeted drop by drop into the ice cold 70 % ethanol (Altia, Finland), which was constantly mixed (Vortex Genie 2, Scientific Industries Inc., USA). Prepared samples were stored at + 4 ºC. The samples could be analyzed next day at the earliest.

PI binds both with double stranded DNA and double stranded RNA. Therefore the samples must be treated first with RNase-enzyme. Ethanol treated cells were centrifuged (400 x g, 10 min, Megafuge) and then ethanol was removed from the tube. The cells were suspended into 1 ml of PBS and suspension was moved to flow cytometry tube. Thereafter, 15 µl of 10 mg/ml RNase (Ribonuclease A, Sigma-Aldrich Corp., USA) was added to tube so that the final concentration in the tube was 0.15 mg/ml. The samples were incubated for one hour at + 50 ºC (Digital Heatblock, WR International, USA). After the incubation, 1 ml of PBS and 16 µl of PI (1mg/ml) were added. The final PI concentration was 8µg/ml in the flow cytometry tube. Cells were incubated at + 37 ºC for two hours in the dark and analyzed right after incubation with flow cytometry counting 10 000 cells/sample.

Analysis of the cell cycle phases and the amount of apoptotic cells were made from the same histogram. This histogram showed DNA amount (strength of the fluorescence of PI) on an x-axis and cell count on a y-axis. Apoptotic cells have less DNA due to DNA fragmentation (sub G_1 –stage) and that is why they can be separated from other cells. In G_1 –stage cells have single DNA amount whereas in S-stage (synthesis) the amount is doubled. Amount of DNA stays doubled in G_2 –stage, before the cell divides (M-stage). Based on the difference in the amount of DNA, S-G_2/M –stage is possible to separate from G_1-stage. Control samples were used to define the fluorescent areas in histogram for the samples. The area of the histogram peak is percentual amount of the 10 000 cells tested.

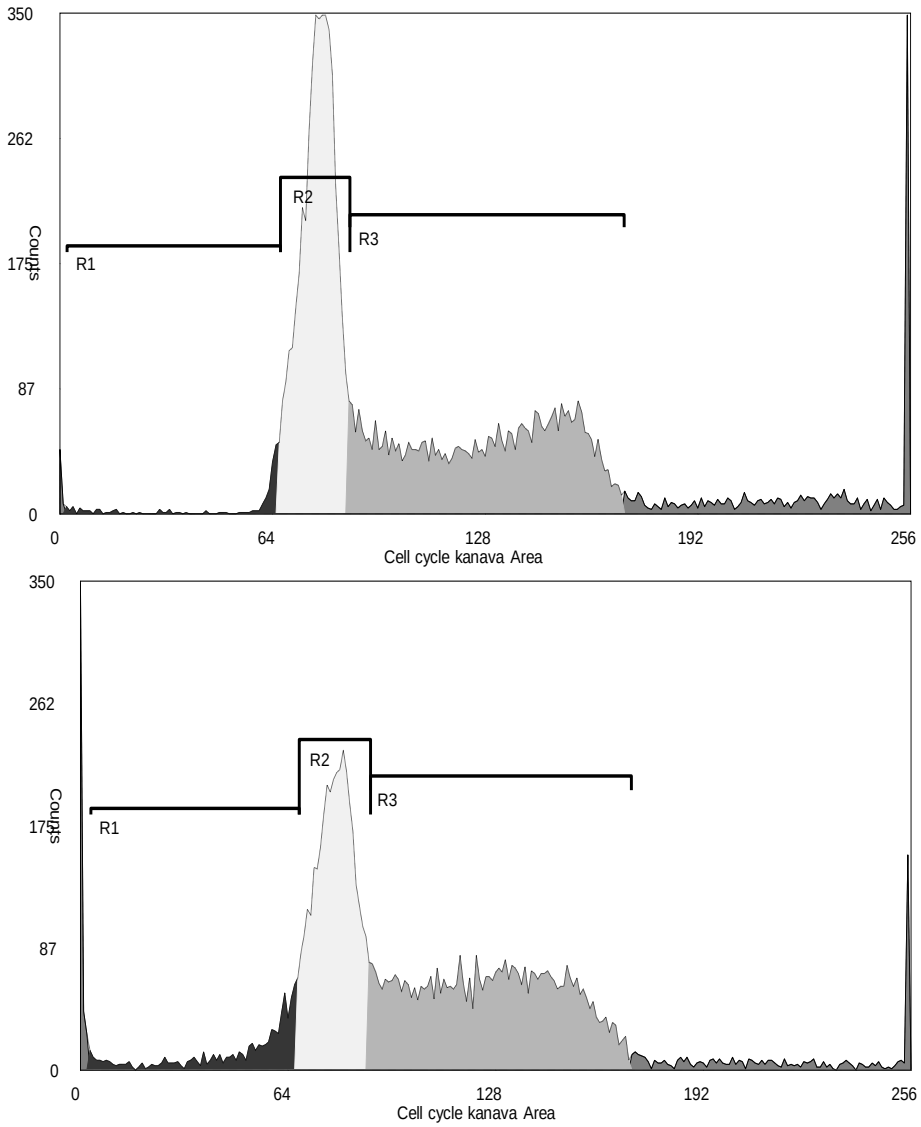

Fig. 6. Flow cytometry analysis picture of cell cycle in 24 hour time point. X-axis represents the strength of the fluorescence of PI and y-axis a cell count. Top picture shows the control sample test cells and the bottom picture the modern masonry heater. Sub G_1 phase is on the left side of the picture, G_1 phase in the middle and S-G_2/M phase on the right.

3.7. STATISTICS

The data was statistically analyzed in PASW statistics version 17.0 (SPSS Inc., Chicago, IL, USA) using analysis of variance (ANOVA). The responses of the control cells were utilized in the statistical analysis, since they did not differ from those induced by the blank substrates. Comparisons between particulate samples were made by Dunnett's post hoc test considering the first level as control (number of replicates, n=3). All the differences were considered to be statistically significant at $p<0.05$.

4.0 RESULTS

4.1. CELL COUNT ANALYSIS IN CONTROL CELLS

Amount of death cells in culture phase (average of the used bottles every week) varied during the test. The average amount of death cells was 17.3 % ±3.7 % and the mount of alive and death cells varied also between different exposure times. The total cell number was greatest at the 24 hour time point (3.28 x 10^6 cells ±1.7 x 10^6 cells) and the number of death cells was the lowest also at this time point (9.83 % ±4.9 %). Total cell number increased until 24 hour time point and decreased slightly after that until 32 hour time. Percentage of death cells decreased until 24 hour time point down to 9.83 % and increased after that being 12.96 % at 32 hour time point. (Figure 7)

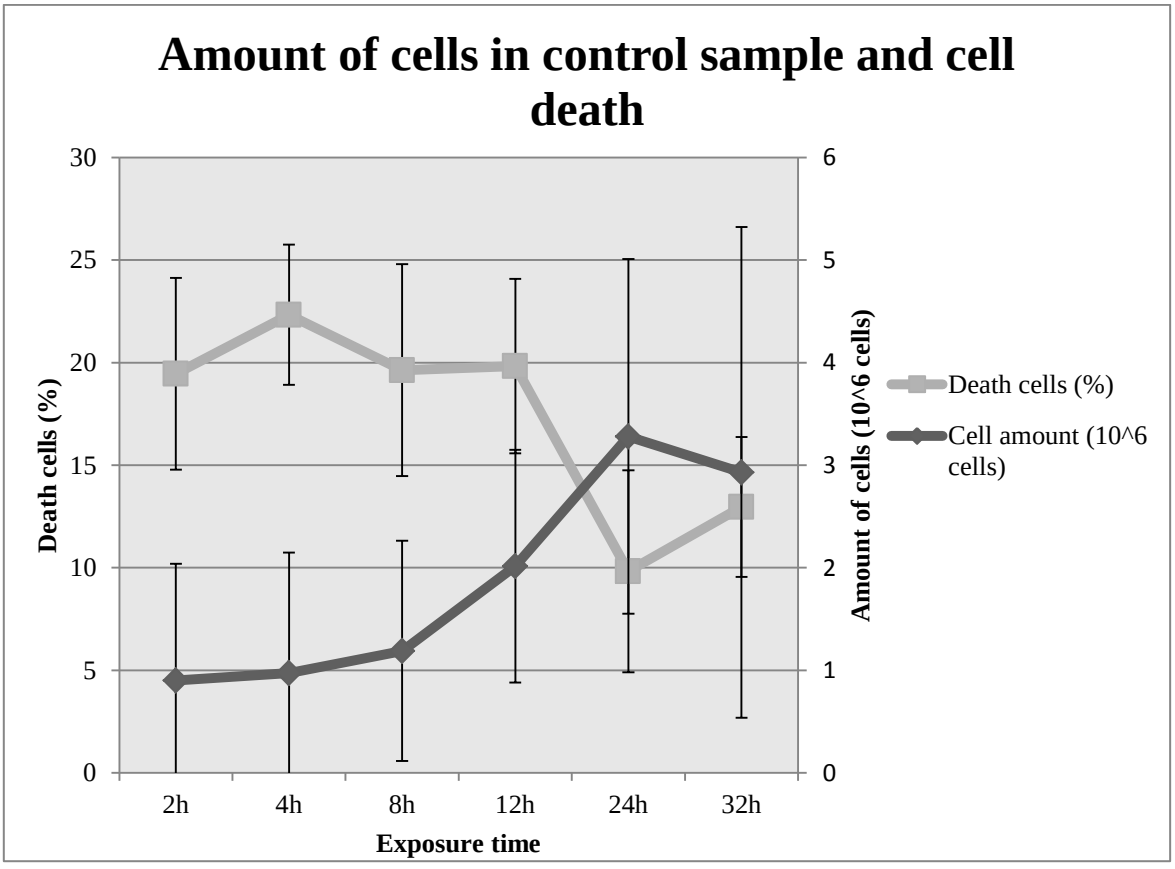

Fig. 7. Total cell number (10^6 cells) and death rate (%) of RAW264.7 cells in control samples in different time points, n=3.

4.2. CELL VIABILITY (MTT)

PM samples from pellet boiler emission increased cell viability between the time points from 2hrs to 4hrs t from 68.15 % (±2.52 %) to 98.29 % (±11.29 %). In later time points the cell viability leveled to 80 %. Cell exposure to PM samples from the modern masonry heater caused significant cytotoxicity at 2 hrs (55.05 % (±6.7 %) and 4 hrs (48.77 % (±2.57 %) time points and but after that the cell viability was at stabilized at 8, 12, 24 and 32 hour time points to same level as seen with the samples from pellet boiler emissions.. The viability of the cells after the exposure to the samples emitted from the sauna stove was at the three first time points at the control level or even slightly more. After that the cell viability decreased at 12 hour time point to 66.89 % ±23.05 % and further down to 21.68 % ±26.52 % at 24 hour time point. At 32 hour time point the cells were slightly recovered the cell viability being 67.04 % ±19.19 %.

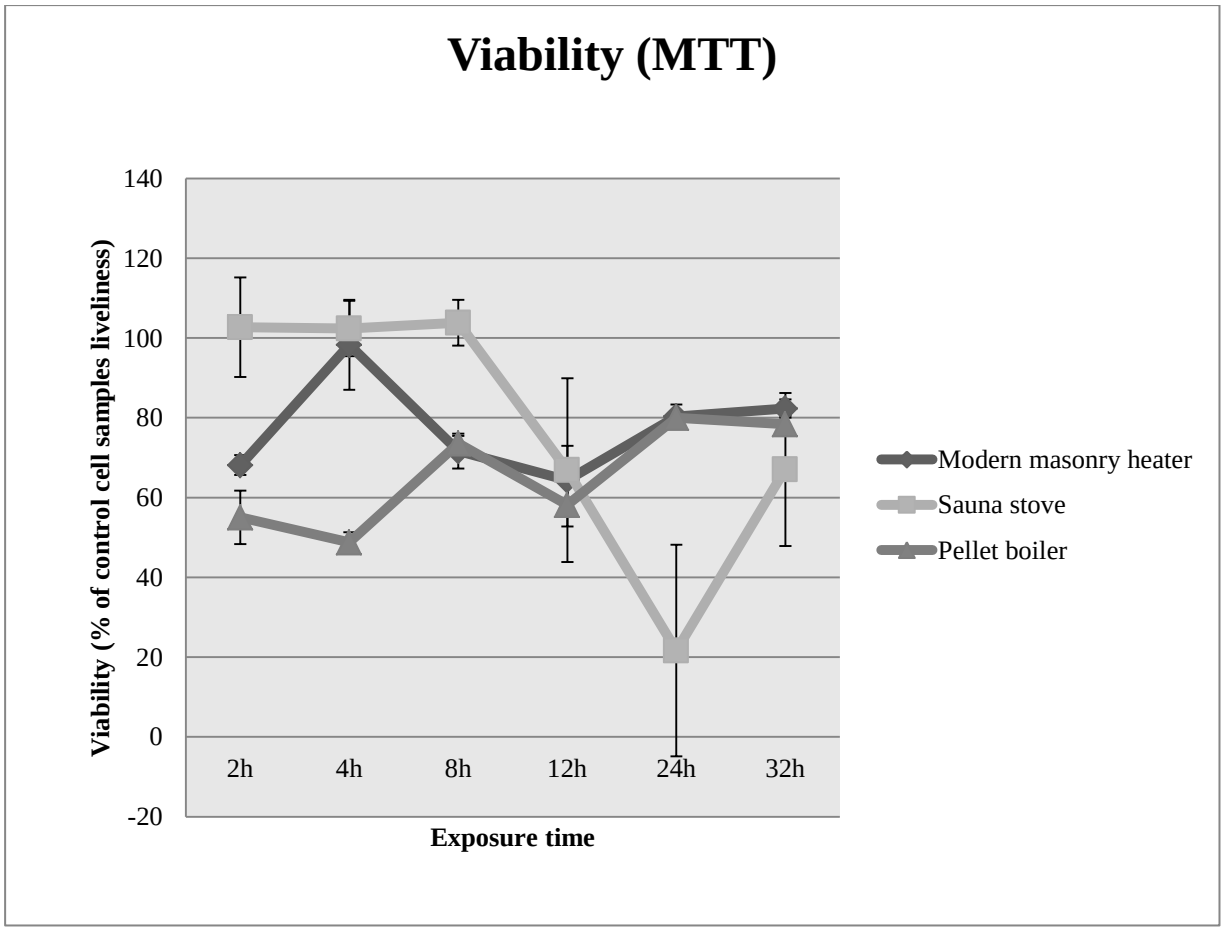

Fig. 8. Cell viability after the exposure to PM emissions from different combustion appliances (150 µg/ml), analyzed with MTT-test, n=2.

4.3. DNA CONTENT ANALYSIS

In sub G_1 phase: Percentage of cells in the sub G_1 phase after the exposure to emission PM from the modern masonry heaters was greatest at 2 hour and 4 hour time points decreasing after that until 32 hour time point. Instead, the amount of sub G_1 cells after the exposure to sauna stove PM was still significantly increased at 24 hour and 32 hour time points. Pellet boilers emission PM induced the lowest level of apoptotic cell death at all the time points. (Figure 9A)

G_1 phase: The cell amount in G_1 phase, after the exposure to emission PM from the modern masonry heaters was aligned with control samples in all the other time points but at 2 and 4 hours. G_1 phase cells after the exposure to pellet boilers PM were aligned with control sample cells in all the time points. Sauna stove PM induced G_1 cell amount was lower than in control sample and the difference grew in 24 hour and 32 hour time points. (Figure 9B)

S-G_2/M –phase: The amounts of cells in S-G_2/M –phase after the exposure to the PM from the modern masonry heater increased in every time point over the control sample until 8 hour time point. After that, at 12 hour and 24 hour time points, the amount of S-G_2/M –phase test cells decreased comparing to control cells, but at 32 hour time the amount was increased again. In case of sauna stove emissions, the S-G_2/M phase cells amount was slightly higher than in control sample in all the other time points but at 2 and 24 hours. Pellet boilers PM exposure induced effect on S-G_2/M –phase cell amount was aligned with control samples. (Figure 9C)

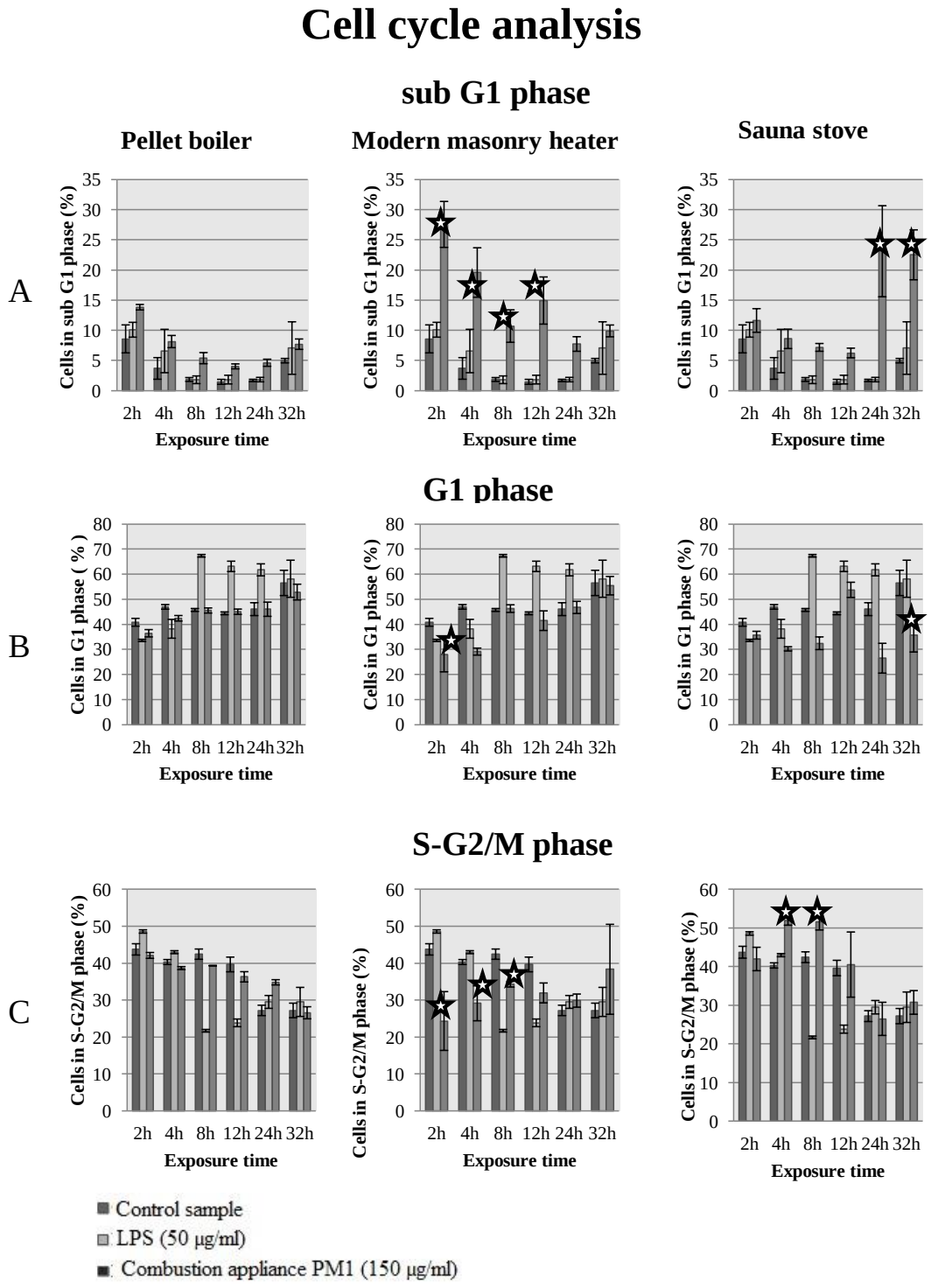

Figure 9. The percentages of mouse RAW264.7 macrophages in the Sub G_1 phase of the cell cycle (apoptosis), G_1 and S-G_2/M phase after exposure to (150 µg/ml) of emission particles from wood combustion appliances and control samples (LPS 50 µg/ml). Asterisks (*) indicate statistically significant responses compared to control cells (Dunnett's test, $p<0.05$).

4.4. PI EXCLUSION TEST

In the control samples, the amount of necrotic and late apoptotic cells varied from 4.93 % to 21.39 % in the studied time points. LPS exposure, which was used as a positive control, induced a time dependent decrease in the amount of necrotic and late apoptotic cells starting from the 2 hour time point. The level at the 2 hour time point was 23.8 % ±2.3 % and it reached s at 32 hour time point the level of 17.47 % ± 1.45 %.

Amount of necrotic and late apoptotic cells after the exposure to emissions from pellet boiler (150 µg/ml) was slightly higher than in the corresponding control samples in all the other time points but 32 hour point. At that time point the control sample contained more apoptotic and necrotic cells than pellet boiler emission exposed cells (control sample 14.21 % ±3.80 % and pellet boiler PM exposed cells 12.01 % ±1.84 %). Amount of necrotic and late apoptotic cells in the positive control (LPS, 50 µg/ml) was higher than in pellet boiler PM exposed cells at all the time points. (Figure 10A)

Exposure to the sauna stove emission samples had only slight effect on the amount of late apoptotic cells and necrotic cells in time points 2-12 hours. Instead, at 24 hour time point, the amount of the late apoptotic cells and necrotic cells increased over control cells and at the 32 hour time point the amount was significantly increased compared to corresponding control (control sample 14.21 % ±3.80 % and sauna stove test cells 50.43 % ±20.94 %). When compared to positive control (LPS) exposure, the cell response to sauna stove PM was smaller until 12 hour time point than that of LPS, but after that, the amount of late apoptotic cells and necrotic cells increased over the LPS induced response. (Figure 10B)

Modern masonry heater emission PM exposed cells had greater amount of both late apoptotic and necrotic cells than in control sample until 32 hour time point. The amount of necrotic cells decreased from time point 2 hour to time point 32 hour. In the control sample cells, the late apoptotic and necrotic cell amount was at 2 hour time point 12.21 % ±0.31 % and at 32 hour time point 14.21 % ±3.80 %, while modern masonry heaters test cells late apoptotic and necrotic cell amount was 41.46 % ±11.62 % in 2 hour time point and 11.63 % ±0.46 % in 32 hour time point. (Figure 10C)

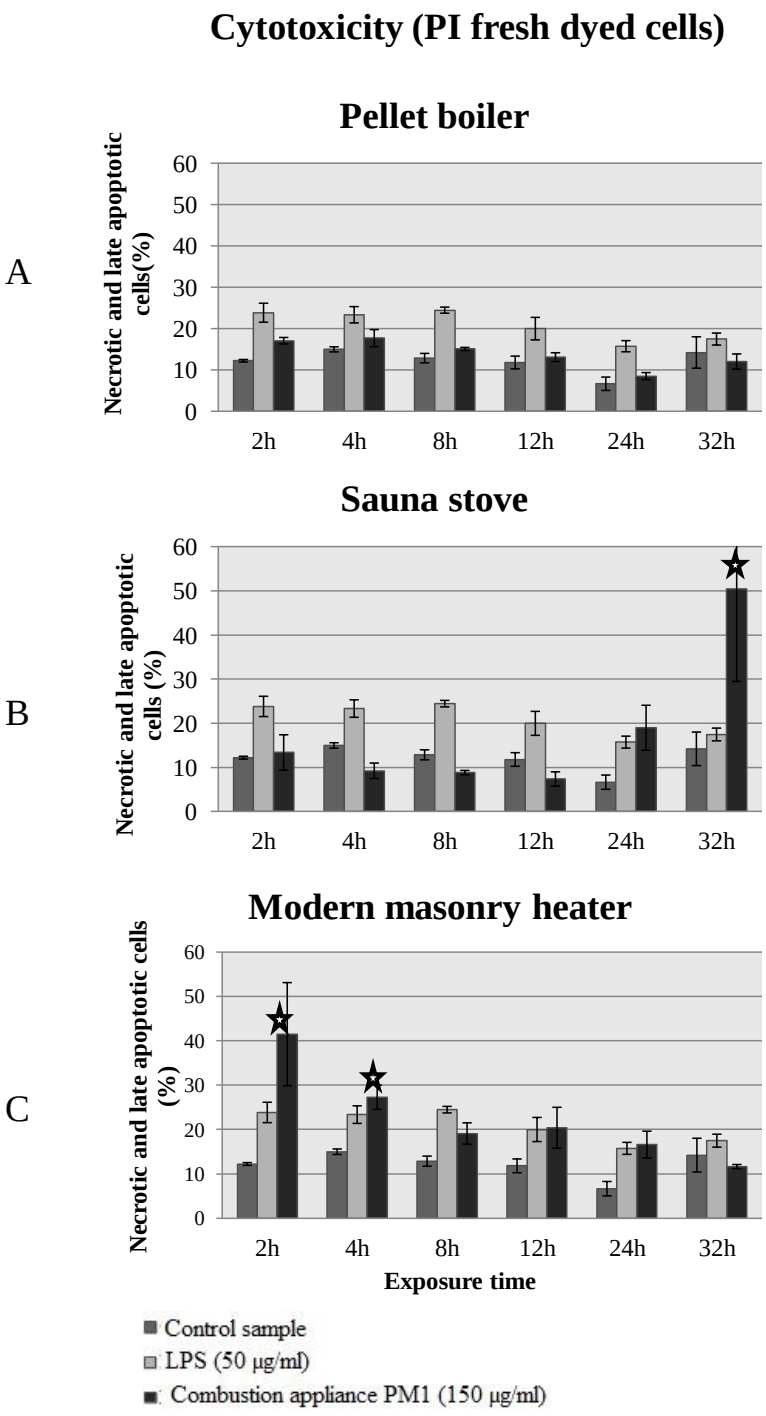

Fig 10. The percentages of mouse RAW264.7 macrophages in late apoptotic or necrotic phase after exposure to (150 µg/ml) of emission particles from wood combustion appliances and control samples (LPS 50 µg/ml). Asterisks (*) indicate statistically significant responses compared to control cells (Dunnett's test, $p<0.05$).

5.0 DISCUSSION

Cytotoxicity assessed as MTT test and cytometry test for PI fresh dyed cells showed that the emission samples from modern combustion appliances can cause fast toxic reaction in RAW264.7 macrophages whereas the samples from old technology combustion appliances activated proliferation and cause strong toxic reaction in later time points. Cell cycle analysis indicated that the cells which were exposed to PM from pellet boiler had almost the same proportions of cells in different cell cycle phases. Exposure to the emission PM from modern masonry heater caused increase in the amount of cells in sub G_1 phase in early time points and sauna stove in later time points which is well in line with PI and MTT test results.

5.1. CYTOTOXICITY (MTT)

In the present study, the PM emissions from the modern masonry heater caused a fast, toxic reaction which leads to low cell viability in the early time points. In the later time points, the cells slightly recovered but the cell viability was still significantly decreased while compared to corresponding control cells. Overall, the acute cytotoxic effect by the emissions of pellet boiler followed the response caused by the masonry heaters PM. This is well in line with previous reports showing that PM samples from the new technology appliances are less cytotoxic to macrophages (Jalava et. al 2012). Fast toxic reaction has also been seen in good combustion quality appliances (Tapanainen et. al 2011, Jalava et. al 2010). Modern combustion appliances PM can contain inorganic ash and metal more than old technology samples (Jalava et. al 2012), which could explain the acute reaction.

On the contrary, the sauna stove PM emissions caused different acute cytotoxicity response pattern in cells while compared to those induced by PM from modern masonry heater or pellet boiler. Sauna stove PM emissions increased cell dividing during first three time points. This was also indicated as higher amount of cells than in control samples in these time points. After this the amount decreased. In earlier studies, old technology appliances have shown a certain threshold level to cause a major decline in the viability (Jalava et. al 2012). This threshold emerges in later time points.

Sauna stove PM has some components including polyaromatic hydrocarbons, volatile organic carbon, carbon monoxide and fine particle mass (Tissari et. al 2007) that seem to increase cell proliferation. This may explain the great amount of sauna stove PM exposure cells in first three time points (Tapanainen et al. 2011). After these time points the amount decreases

significantly, which can be explained by the sauna stove PMs toxicological properties. Thus, it is important to use different time points in experimental set up to show this phenomenon.

5.1.2. Effects on necrosis and apoptosis

In this study, pellet boilers PM emissions were the least cytotoxic, which was shown as low necrosis and apoptosis levels. Modern masonry heaters PM caused strong cytotoxic reaction in cells after short exposure but effect decreased in later time points. Sauna stove PMs increased cytotoxic reaction up to 32 hour time point significantly in PI test.

New technology stoves, such as pellet boiler and modern masonry heater caused relatively low toxicological responses in RAW264.7 macrophages. However, acute toxicological effects from samples representing good combustion quality have been reported (Tapanainen et al. 2011, Jalava et al. 2010). This may be caused by the inorganic ash which causes cell damages. These samples have also had greater metal ratio, which can be partially responsible for this reaction (Jalava et al. 2012).

Sauna stove PM caused low cytotoxic response in cells in early time points. Flow cytometry test for PI fresh dyed cells measures necrotic and late apoptotic cells and not already dead cells and cell particles. Thus, the low cytotoxic responses from sauna stove PM may be explained by the fact that cells were dead and degraded. Low cytotoxic responses by pellet boiler PM may be due to PM cytotoxic properties, which are lesser than with sauna stove. Cytotoxic reactions by the PM from modern masonry heater within first two time points suggests that PM matter induces effect on macrophages, but effect is relatively fast but transient .

5.2. EFFECTS ON CELL CYCLE

In sub G_1 –phase: cell proportions in different cell cycle phases after the exposure to pellet boilers PM followed those of control samples. Instead, in modern masonry heaters PM emissions, the cell amount was greater in sub G_1 phase's at the first time points than in control cells. This supports the findings in the other test showing acute toxic reaction. Furthermore, the amount of cells in sub G_1 phase after the exposure to sauna stove PM increased in later time points, which is also well in line with the PI and MTT test results.

Studies have shown earlier that incomplete biomass burning causes DNA damage (Leonard et al., 2000, Karlsson et al., 2006 and Danielsen et al., 2009). This damage causes sub G_1 cell proportions to grow, which was discovered with modern masonry heater and sauna stove, only in different time points.

G_1-phase: Amounts of cells that were exposed to pellet boilers PM were similar as those in control samples. Exposed cells to modern masonry heaters emitted PM, showed smaller proportion of G1-phase cells in early time points than unexposed control cells, but reached the control level in later time points. This effect was not seen in case of sauna stoves PM samples, i.e. the cell amount in this cell cycle phase decreased in late time points. These results support earlier observations.

Proportion of cells in G_1 phases at late time points after the exposure to sauna stove particles was smaller because cell cycle was stopped to sub G_1 phase in some cells. This indicates cell and DNA damage. Particles that were formed in sauna stove contain insoluble components, which have shown to cause inflammatory and cytotoxic activities *in vitro* (Huang et. al. 2004 and Kocbach et. al. 2008).

S-G_2/M –phase: Pellet boiler emission PM did not cause changes in cell amount comparing to control sample in this cell cycle phase. Instead, exposure to the samples collected from the modern masonry heater, induced lower cell proportions in this phase with early time points. In contrary, sauna stove PM emissions caused greater S-G_2/M –phase cell proportions in the middle time points.

The greater cell proportion in middle time points after the exposure to sauna stove derived samples can be explained by the compounds that increase proliferation (Tapanainen et. al 2011). Earlier studies have also shown that masonry heaters combustion particles have higher potency to cause DNA damage than pellet boilers. This is caused by the PAH concentration differences in these particles (Karlsson et. al 2006). This can explain why modern masonry heater derived sample leads to lower proportions of dividing cells in early time points.

Cell proportions after exposure to emission particles from pellet boiler in all the cell cycle phases were in line with control samples cell proportions, which means that those particles did not cause major changes in cell cycle. Changes in cell cycle could indicate damages in DNA, thus pellet boiler emitted PM does not distract normal cell dividing. Modern masonry

heaters PM exposure induced cell proportions in G_1 phase were similar as those in control samples, but in sub G_1 cell amount was greater in first time points. Moreover, the corresponding cell amounts in S-G_2/M phase were lower than in the control sample. This indicates, that cells are not dividing in first time points, but they are apoptotic or necrotic and therefore in sub G_1 phase. Similarly exposure to sauna stove emitted PM increased the amount of cells in sub G_1 in last two time points, which indicates apoptotic and necrotic cells. Also the amount of G_1 cells was decreased in these time points, because the cells had not proceeded to G_1 phase. In S-G_2/M phase the sauna stove PM exposed cell amount were increased in 4 hour and 8 hour time, which can indicate DNA damage. Thus, the cell cannot proceed to mitosis because of the damages in DNA, which leads to accumulation of cells in this phase. These unfixable DNA damages stop cell cycle or they may induce apoptosis (Branzei & Foiani 2008).

6.0 SUMMARY AND CONCLUSIONS

The present pro gradu –research aimed to study time dependency of activated cellular processes in a mouse macrophage cell line leading to different damages in cells. These damages indicate the harmful effects of particles from different combustion appliances. Different appliances produce particles whose properties vary; size, chemical and physical features, morphology, number and mass. In this study the comparison of health related toxic effects between three different appliances (sauna stove, pellet boiler, modern masonry heater) showed, that damages in cells depend on the chosen appliance and on the exposure time.

The results show that old technology combustion produces particles, which increase first proliferation and then cause strong toxic reaction in later time points. Proliferation may indicate genotoxicity, because the cell number was greater than in control samples, and this uncontrollable dividing is typical for cells which have genotoxic features. Particles from modern masonry heater caused a fast toxic reaction in cells but the effect reduced when exposure time increased. This shows that these particles have some features that cause apoptosis and necrosis in cells. Earlier studies suggest that inorganic ash and higher metal ratio may be the cause (Tapanainen et al. 2011, Jalava et al. 2010 & 2012). Nevertheless, the emission particles from modern masonry heaters caused lower toxicological responses in cells than sauna stoves particles. Pellet boiler derived particles caused relatively low toxicological responses in cells. Combustion in this appliance was continuous and thorough, which is reflected in the toxicological effects of the produced particles.

This experiment can be seen as a successful test. Test results were in line with hypothesis and with earlier studies. Using of the different time points in this study revealed the becoming damages and their mechanisms in cells. Exposure in real life can be anything from short term to lifetime, thus the knowledge of the toxicological effects in very different exposure times is necessary. Moreover, in real life the exposure to emission particles do not directly occur from the combustion appliance. In this test the particles were processed for longer storage and to enable *in vitro* –testing. Direct exposure would give more realistic results and then the effects of processing and preserving of the particles could be ruled out. Also for proper risk assessment both toxicological *in vivo* –testing and epidemiological data needs to be connected with in vitro data to create a whole picture of the effects of small scale combustion, the chemical and physical properties of the particles needs to be further studied. The identifying of different chemical compounds in these particles would create a preferable picture of the

reasons responsible for toxicological effects. Also the combustion technology and the various use of it need to be studied more. Nevertheless, in this pro gradu –research scale even the toxicological effects on cellular level were a challenging totality.

Small-scale wood combustion is important source of energy but it may have significant impact on human health not only in the developing countries but also in the developed countries. Inadequate legislation and knowledge of the health effects of the combustion particles may lead to situation were public health suffers and the mortality rate and harm for individuals are rising. If wood combustion is going to be a way to produce energy more sustainable, further studies of health effects are frantically needed.

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