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## Oxidative potential and in vitro toxicity of particles generated by pyrotechnic smokes in human small airway epithelial cells

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### ABSTRACT

Pyrotechnic smokes are widely used in civilian and military applications. The major issue arise from the release of particles after smoke combustion but the health risks related to their exposure are poorly documented whereas toxicity of airborne particles on the respiratory target are very well known. Therefore, this study aimed to explore the in vitro toxicity of the particle fraction of different pyrotechnic smokes.

Particles from a red signalling smoke (RSS), an hexachloroethane-based obscuring smoke (HC-OS) and an anti-intrusion smoke (AIS) were collected from the cloud. RSS particles displayed the highest organic fraction (quinones and polycyclic aromatic hydrocarbons) of the three samples characterized. AIS particles contained K and cholesterol derivatives. HC-OS particles were mainly metallic with very high concentrations of Al, Fe and Ca. Intrinsic oxidative potential of smoke particles was measured with two assays. Depletions of DTT by RSS particles was greater than depletion obtained with AIS and HC-OS particles but depletion of acid ascorbic (AA) was only observed with HC-OS particles. *In vitro* toxicity was assessed by exposing human small airway epithelial cells (SAEC) to various concentrations of particles. After 24 h of exposure, cell viability was not affected but significant modifications of mRNA expression of antioxidant (*SOD-1* and *-2*, *catalase*, *HO-1*, *NQO-1*) and inflammatory markers (*IL-6*, *IL-8*, *TNF-α*) were observed and were dependent on smoke type. Particles rich in metal, such as HC-OS, induced a greatest depletion of AA and a greatest inflammatory response, whereas particles rich in organic compounds, such as RSS, induced a greatest DTT depletion and a greatest antioxidant response.

In conclusion, the three smoke particles have an intrinsic oxidative potential and triggered a cell adaptive response. Our study improved the knowledge of particle toxicity of pyrotechnic smokes and scientific approach developed here could be used to study other type of particles.

### 1. Introduction

Pyrotechnic smokes have been systematically used since World War I and still remain effective on the modern battlefield. They are used for military exercises and operations to reduce the enemy visibility or obscure objects, or for signalling and marking positions. Law enforcement officers also use them for security and protection. In addition, civil population during festive, social, cultural, political or sports events can

use pyrotechnic smokes.

Many types of pyrotechnic smokes with various initial compositions exist but few data about the toxicity of the emitted products after pyrotechnic reactions are available. At the end of the 1990s, the first reports of smokes toxicity were published by the US National Research Council ([National Research Council \(US\) Subcommittee on Military Smokes and Obscurants, 1997; 1999a; 1999b](#)) to establish exposure guideline levels for both military in training and public residing or

**Abbreviations:** RSS, red signalling smoke; HC-OS, hexachloroethane-based obscuring smoke; AIS, anti-intrusion smoke.

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working near military-training facilities. These reports showed that smokes from different pyrotechnic smokes (hexachloroethane, red phosphorus, white phosphorus, metallic and colored smokes) caused various pulmonary damages (irritation, respiratory distress, dyspnoea, coughing, oedema, inflammation) after acute exposure and can lead to death if exposure occurs in confined spaces. However, it was highlighted a lack of data and thus, the necessity to develop new knowledge to better understand the potential toxicity of pyrotechnic smokes. Most studies on the toxicity of pyrotechnic smokes were carried out on animal models. However, for ethical reasons considering animal welfare, their use is questionable (3Rs principle: Replacement, Reduction, and Refinement; (Tannenbaum and Bennett, 2015)). Thus, the evaluation of the health impact of pyrotechnic smokes is a challenge requiring new experimental approaches close to human physiological conditions, as the use of primary cells cultivated at the air-liquid interface (ALI) (Hawley et al., 2014; Juarez Facio et al., 2022b; Lan et al., 2021). In order to understand smokes toxicity on the respiratory airways, very few in vitro studies have been carried out, by exposing pulmonary cell lines to total aerosol after smoke combustion (Hemmilä et al., 2007a; Hemmilä et al., 2007b; Hemmilä et al., 2010; van Hulst et al., 2017). Depending on the smoke's type, a moderate to strong reduction in cell viability was observed. In addition, strong genotoxicity was shown with orange smoke compared to red, yellow or purple ones.

Combustion of pyrotechnic smokes generate large amounts of particles giving to the smokes their characteristics, i.e. colored for a signalling smoke (Hemmilä et al., 2010), or with excellent hiding capacity dependent of the particle size of the aerosol for obscuring smokes (Hemmilä et al., 2007a; Koch, 2008). To our knowledge, no study was carried out to explore smoke particle toxicity whereas relationship between airborne particles exposure and health, related to cardio-pulmonary diseases, has been well-documented (Chiu and Yang, 2013; Habre et al., 2014; Madrigano et al., 2013; Nachman and Parker, 2012; Smith et al., 2020). Experimental studies have shown that smaller particles induce stronger effects than larger particles, due to their larger surface area to mass ratio (Oberdörster et al., 2005), but there is a need to consider their chemical compositions.

One of the most studied toxic mechanisms of particles is related to their ability to generate an oxidative stress by production of reactive oxygen species (ROS), when the cell's antioxidant defences are saturated (Janssen et al., 2014). High levels of ROS can then trigger various redox-sensitive signalling cascades associated with airway inflammation (Betts et al., 2013). Compounds adsorbed on particle surface, such as organic compounds or metals, are responsible for oxidative stress (Chen et al., 2021; Williams et al., 2017). ROS could be formed upon direct contact with lung lining fluid and epithelial cells (Øvrevik et al., 2015) but could also come from in vivo biotransformation of particle components.

The development of predictive tools of toxicity reflecting the oxidative potential (OP) of particles to characterize air pollution exposure have been arising (Künzli et al., 2006). The OP of particles is defined by their ability to produce ROS and/or oxidize target substrate. Different acellular methods are used but the dithiothreitol (DTT) and the antioxidant (AO) depletion assays appeared to be the most relevant assays and have demonstrated interesting complementary results (Ayres et al., 2008; Bates et al., 2019; Crobeddu et al., 2017). Thus, the tests developed for airborne particle toxicity would be very suitable for studying the toxicity of particles from pyrotechnic smokes.

This study was carried out in order to investigate the particle toxicity produced by three different types of smoke by combining acellular and cellular in vitro tests, as well as a physicochemical characterization. For this purpose, two military smokes were used, a red signalling smoke (RSS), an obscuring hexachloroethane-based smoke (HC-OS) and an anti-intrusion smoke (AIS) for civil uses. Firstly, smoke particles were collected directly from the cloud, and were characterized physico-chemically. In order to apply the 3Rs ethical principle, particle toxicity was assessed using both primary small airway epithelial cells

(SAEC) cultivated at Air-Liquid Interface (ALI) and acellular assays in order to measure the intrinsic OP of particles. The biological activity was evaluated by cell viability, antioxidant and pro-inflammatory responses 24 h post exposure.

## 2. Materials and methods

### 2.1. Chemicals and materials

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich, St Louis, MO, USA.

### 2.2. Smoke description and PM sampling

Three smoke formulations were used: a Red Signaling Smoke (RSS) used in military field and composed of potassium chlorate, lactose and red organic dye; an Hexachloroethane based Obscuring Smoke (HC-OS) used in military field and composed of hexachloroethane, aluminum oxide, iron oxide and calcium carbonate; an Anti-Intrusion Smoke (AIS) used in by civil population and composed of Potassium chlorate, lactose and organic acids.

Particles collecting from pyrotechnic smoke assays were performed in DGA Land Systems Technical Center. Particles from smokes were collected with cascade impactors placed at 1.5 m high (Staplex – Model Sierra 235) connected to a high volume pump (80 m<sup>3</sup>/h) close to the smoke source (1–2 m) and during all the smoke emitting phase (10–20 min). Cascade impactors were equipped with five impaction stages (collecting respectively particles of 7.2 – 3.0 – 1.5 – 0.95 – 0.49 μm) completed by a quartz back-up filter allowing impaction of particles < 0.49 μm. The impactor's sampling was placed to dry under a laminar flow bench for 24 h before particle collection by scrapping, stage by stage. Then, particles were weighed and collected in sterile vials and stored at 4 °C. Particles were pooled and suspended in sterile ultra-pure water for toxicological assays (1 mg/mL). Particle suspensions were placed on sterilized vials and sonicated by the Cup-Horn method (Betts et al., 2013) in order to avoid biological and chemical contaminations. They were stored at – 20 °C until use.

### 2.3. Physico-chemical characterization

Simultaneously to particle sampling by impaction, particle concentration (particles/cm<sup>3</sup>) and size distribution of aerosols were determined in real time conditions with two analysers: an optical particle sizer (OPS 3330, TSI Instrument) for aerosols between 0.3 μm and 10 μm (divided into 16 size classes) and a scanning mobility particle sizer (SMPS 3910, TSI instrument) for aerosols between 10 nm and 400 nm (divided into 13 size classes). The particles collected on the impaction stages and the back-up filter were characterized by Elementary analysis (C, H, N, S) (GC-TCD Flash 2000, Thermo instruments), ionic chromatography (ICS 5000, Thermo instruments) for anion species (Cl<sup>-</sup> and NO<sub>3</sub><sup>-</sup>) and cation species (K<sup>+</sup> and NH<sub>4</sub><sup>+</sup>), gas chromatography coupled to mass spectrometry (GC CP 3800/MS 1200MS, Varian) for organic compounds including polycyclic aromatic hydrocarbons (PAH) derivatives. Inductively coupled plasma equipped with an optical emission spectroscopic detector (ICP-OES 6300 DUO, Thermo Instruments) was used to analyse total and water soluble metals (Al, As, B, Ba, Ca Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sb, Sn, Sr, Ti, V and Zn). Soxhlet extraction with dichloromethane was performed to extract the organic components. Soluble ions and metals were obtained by lixiviation of particles in ultrapure water during 20 min in an ultrasonic bath. A microwave digestion (Xpress, CEM) was performed with nitric acid to obtain the total metal fraction.

### 2.4. Oxidative potential of smoke particles

The two assays were optimized and validated with diesel exhaust

particles (DEP) collected from a Fiat motor (2004) and TiO<sub>2</sub> nanoparticles (anatase 99%, Acros Organics, Belgique), used respectively as positive and negative control (Fig. A.1).

#### 2.4.1. Antioxidant depletion assay (AO) in a synthetic respiratory tract lining fluid (RTLFL)

Antioxidant depletion assay was based on the analysis of a synthetic respiratory tract lining fluid (RTLFL) (Ayres et al., 2008). RTLFL described by Zielinski et al. (1999) is composed by ascorbic acid (AA, 200 μM) and glutathione (GSH, 200 μM), concentrations usually present in RTLFL from normal and non-smoking subject. Antioxidant solutions were prepared in 0.9% NaCl at pH 7.4 to reflect normal human airway secretions. Particles were incubated for 4 h at 37 °C in RTLFL. Concentrations used for these assays, 70–280 μg/mL, corresponding to concentrations used for exposure, 12.5 μg/cm<sup>2</sup> to 50 μg/cm<sup>2</sup>. After incubation, samples were centrifuged for 5 min at 12,000g at 4 °C to remove particles. The supernatants were then filtered using Nylon 0.45 μM syringe filters (Cloupe, France). The final concentrations of AA, GSH and GSSG were quantified by an Agilent High Pressure Liquid Chromatography (HPLC) system 1260 Infinity II equipped with DAD detector (Agilent Technologies, Santa Clara, CA, USA). The chromatography was equipped with a Poroshell 120 EC-C18 (3 × 150 mm, 2.7 μm) with guard EC-C18 (3 mm) maintained at 25 °C. The injection volume was 1 μL. Potassium hydrogen phosphate (10 mM, pH 3) was used as mobile phase (100%), with 0.6 mL min<sup>-1</sup> as flow rate. The UV data were collected at 220 nm. The method was validated in term of specificity, response function, precision and linearity according to the ICH Q2(R1) guidelines. The LOD and the LOQ were respectively equal to 0.12 μM and 0.4 μM for AA, 0.3 μM and 0.9 μM for GSH and 0.24 μM and 0.8 μM for GSSG. The linearity was assessed by least squares regression and yielded good determination coefficients (R<sup>2</sup> = 0.9999, y = 7255.7x - 4.7647 for AA, R<sup>2</sup> = 0.9998, y = 2935.7x - 0.2493 for GSH and R<sup>2</sup> = 0.9996, y = 8884.9x - 0.3844 for GSSG). The intra-day precision showed relative standard deviation (RSD %) lesser than 2%. The inter-day precision showed RSD% better than 3%. Percentage of depletion was calculated as described by.

$OP^{AA}$  or  $OP^{GSH} = 100 - (AA \text{ or } GSH \text{ concentration in the sample} / AA \text{ or } GSH \text{ concentration in blank}) \times 100$ . GSSG production was calculated as:  $OP^{GSSG} = 100 + (GSSG \text{ concentration in the sample} / GSSG \text{ concentration in blank}) \times 100$ .

#### 2.4.2. Dithiothreitol assay (DTT assay)

This assay was commonly used to assess the progressive oxidation of DTT by particles (Cho et al., 2005). This method measures the rate of loss of DTT upon incubation with particles. Under the assay conditions, the reaction is proportional to the concentration of the redox active species of the particles. Particles (70–280 μg/mL) were incubated with 200 μM DTT in 0.10 M phosphate buffer (0.08 M Na<sub>2</sub>HPO<sub>4</sub> and 0.02 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, Chelex-treated to eliminate certain effects of metals) in 96-well plate for 1 h at 37 °C. Then, plates were centrifuged for 15 min at 2100g and 4 °C. An equal volume of 2.5 mM of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and supernatant was mixed and absorbance was measured at 412 nm (Safas Xenius, Monaco). Percentage of depletion was calculated as described by Crobeddu et al., 2017:  $OP^{DTT} = 100 - (DTT \text{ concentration in the sample} / DTT \text{ concentration in the blank}) \times 100$ .

#### 2.5. SAEC culture and particles treatment

Human small airway epithelial cells (SAEC, Lonza, Basel, Switzerland), were firstly grown for 7 days in a T-75 cm<sup>2</sup> flask with expansion medium supplemented with 0,1% (v/v) penicillin/streptomycin (10 Units/mL; GIBCO, USA), and maintained at 37 °C in 95% humidified air with 5% CO<sub>2</sub>. Due to the unavailability of Small Airway Growth Medium™ and S-ALI™ Differentiation Media (Lonza) after 2018, PneumaCult-Ex medium and PneumaCult™-ALI Differentiation medium (STEMCELL Technologies, Vancouver, Canada) were used for

SAEC expansion phase and differentiation phase, respectively, for all the experiments. The medium was replaced every two days. Between 80% and 90% confluency, SAEC were trypsinized and seeded at 110,000 cells/insert on transwells polyester permeable membrane cell culture inserts (12 mm diameter, pore size 0.4 μm, Costar, Corning®) pre-coated with a 30 μg/mL collagen solution (Rat Tail Collagen Type 1, Corning®) in PBS for 1 h at 37 °C. At confluence, cells were placed under air-liquid interface (ALI) condition by removing the apical medium and replacing the basal medium by PneumaCult-ALI medium. After 7 days of culture at ALI conditions, the exposures were performed by directly depositing particle suspensions of RSS, HC-OS and AIS (12.5; 25 and 50 μg/cm<sup>2</sup>) on apical side (200 μL) during 24 h at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. As a reference control, cells were exposed to 200 μL PBS under the same conditions.

#### 2.6. Cell viability assay

Cell viability was evaluated using the MTT assay measuring the mitochondrial dehydrogenase activity in viable cells. After exposure, basal medium was replaced by MTT reagent (5 mg/mL) diluted (1:10) in fresh PneumaCult-ALI medium. After 3 h of incubation at 37 °C, basal medium was discarded and formazan crystals were dissolved with a solution of SDS 10%/HCl 0.1 N overnight. MTT reduction was quantified by measuring the light absorbance at 570 nm (SAFAS Xenius, Safas, Monaco) using six replicates. Each test was repeated independently at least three times. Results are expressed as % viability = (Absorbance in the sample / Absorbance in the control PBS) × 100.

#### 2.7. mRNA expression measurements by quantitative real time RT-PCR

After 24 h of exposure, cells were rinsed with PBS and total RNA were extracted with TRI-REAGENT® (Sigma-Aldrich, France) according to the manufacturer's recommendations. After DNase treatment (Invitrogen, Life Technologies), RNA were quantified by Nanodrop 2000 (Thermo Scientific) and integrity was analyzed with a Bioanalyseur 2100 (Agilent Technologies, Santa Clara, USA). RNA Integrity Numbers of the samples were > 9. Reverse transcription (RT) of 1 μg of total RNA was performed using oligo (dT) primers with AffinityScript QPCR cDNA Synthesis Kit™ (Agilent Technologies). Quantitative PCR was performed with Brilliant III Ultra-fast SYBR Green QPCR Master Mix (Agilent Technologies) following manufacturer's recommendations using a thermocycler (Stratagene Mx3005P Agilent Technologies). cDNA was amplified using specific primers (Table A.1) and melting curve analysis was performed to verify the amplification of a single product following these conditions: 3 min at 95 °C; 40 cycles of 10 s at 95 °C, 20 s at 60 °C; 1 min at 95 °C; 30 s at 55 °C and 30 s at 95 °C.

Standard curves were generated for each qPCR to calculate the efficiency of the amplification and only qPCR with an efficiency of 1.8–2.1 were used for analysis. The cycle threshold (Ct) values were determined and normalized by the ddCt method with GAPDH as housekeeping gene (Livak and Schmittgen, 2001). Results are expressed by mean of 2<sup>-(ddCt)</sup> ± SD of at least four independent experiments.

#### 2.8. Quantification of cytokines released by SAEC cells

The basal cell culture media were collected directly after 24 h of exposure, centrifuged for 5 min at 13,000g and frozen at -80 °C until use. Levels of IL-8, IL-6 and TNF-α released into the culture medium were measured using commercially enzyme-linked immunosorbent assay (ELISA) kits (Cohesion Biosciences, London, UK) according to the manufacturer's instructions. The optical density of samples was measured at 450 nm with a microplate reader (SAFAS Xenius, Safas, Monaco). Cytokines concentrations were subsequently calculated from standard curves and expressed in pg/mL (mean ± SD of three independent experiments).

## 2.9. Statistical analysis

Results were obtained from at least three independent experiments. Statistical analyses were performed by a One-way Analysis Of Variance (ANOVA). Concerning data from cytotoxicity, gene expression and cytokine secretion, the differences between treatment and control groups (PBS group) were analyzed by a non-parametric Mann-Whitney test and data of oxidative potential of particles was analyzed with Dunnett's test using GraphPadPrism v9 software. Values of  $p \leq 0.05$  are considered to be statistically significant.

## 3. Results

### 3.1. Physico-chemical characterization of RSS, HC-OS and AIS particles

#### 3.1.1. Particle size analysis

Table 1 presents: 1) the average of the total amount of particles (particles/cm<sup>3</sup>) emitted during the peak of emission 2) the part of the main size of particles detected for each type of smoke.

An example of particle number profile for HC-OS is given in Fig. A.2. If we compare the number of particles detected by both OPS and SMPS, it appears that most of the particles are below 400 nm. AIS is the smoke grenade that generates the largest amount of particles/cm<sup>3</sup>. The data of the particle counters are consistent with the samplings performed on the impactor's stages: most of the particles are collected on the last stages of the impactors (0.95 = -0.49 μm) and on the back-up filters.

#### 3.1.2. Elemental analysis and metals

The elemental analysis showed a high concentration of carbon (70.1% and 68.3%), hydrogen (5.5% and 9.4%) and nitrogen (8% and 0.3%) respectively for RSS and AIS particles whereas sulphur was not detectable (< 0.5%). Particles from HC-OS are very poor in carbon (2.3%) compared to RSS and AIS particles, they contain 4.9% of hydrogen and there is no detectable concentration of nitrogen and sulphur.

The metal analysis (Table 2) indicated that high levels of potassium were observed for all smoke particles. HC-OS particles were highly concentrated in metals since we measured very high levels of Al, Fe and Ca as well as moderate levels of Zn, Sn, Mg, S, Na and Cu. RSS particles contained very high levels of Sb as well as Ba and S. AIS particles were mainly composed of Al, Na, S and Sb.

Concerning water-soluble metals in particle suspension (Table A.2), we noticed the presence of the same major metals than in the total fraction but not always in the same proportion. K and Na were the main water soluble metals (1–10 μg/mL) detected in RSS and AIS particle suspension in addition with Sb in RSS particles suspension and B in AIS particles suspension. Traces of Ca, Al, Mg, S, Ba, Fe, Pb, and Zn were also measured in RSS and AIS particle suspensions. For HC-OS particles, very high levels of Al (saturated), Ca, Fe, K and Sn (>10 μg/mL), Mg, Zn, Na, S (1–10 μg/mL) were measured and numerous other metals in the concentration range 0.1–1 μg/mL (B, Ba, Cr, Cu, Mn, P, Pb, Ti) were

**Table 1**

Real time number of particles emitted in the cloud.

particles / cm <sup>3</sup>		RSS	AIS	HC-OS
10–400 nm	total	250,000–860,000	450,000–1 200,000	430,000
(SMPS data)	for all size sizes			
	main sizes	65,000–270,000 (154 & 274 nm)	131,000–269,000 (154 nm)	130,000 (205 nm)
0,3–10 μm	total	38,000	62,000	45,000
(OPS data)	for all size sizes			
	main sizes	5 000–12 000 (0.5 & 1 μm)	20,000 (0.3 μm)	11,500 (0.3 μm)

**Table 2**

Total metals concentrations in RSS, AIS and HC-OS particles collected on impactor.

Total metals concentrations (mg/g)	RSS	AIS	HC-OS
> 1	K (7.6), Sb (3.4)	K (3.0)	Al (105.9), Fe (12.1), Ca (9.6), K (1.8)
0.1–1	S (0.5), Ba (0.2)	Al (0.6), Na (0.2), S (0.2)	Zn (0.94), Sn (0.9), Mg (0.8), S (0.8)
0.01–0.1	Al (0.07), Zn (0.05), Na (0.03), Cr (0.02), P (0.02), Cu (0.01), Pb (0.01), Sn (0.01)	Sb (0.08), Cu (0.08), Sn (0.03), Fe (0.02), P (0.02), Pb (0.01), Zn (0.01)	Na (0.1), Cu (0.1), Cr (0.09), Pb (0.09), Sb (0.04), Mn (0.03), P (0.03), Ba (0.02), Ni (0.02), Ti (0.02)

detected.

#### 3.1.3. Ions analysis

The ionic chromatography analysis exhibited that RSS and AIS particles were highly concentrated in nitrates (NO<sub>3</sub>: RSS, 19.5 mg/g; AIS, 5.5 mg/g). Potassium was abundant in the three samples (K<sup>+</sup>: RSS, 11.4 mg/g; AIS, 3.4 mg/g; HC-OS, 1.6 mg/g) as well as chlorides, which are extremely high in HC-OS sample (Cl<sup>-</sup>: RSS, 8.1 mg/g; AIS, 4.5 mg/g; HC-OS, 215.8 mg/g).

#### 3.1.4. Organic components detection

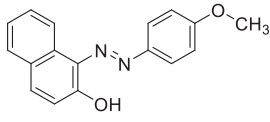
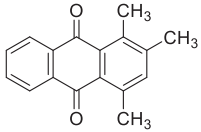
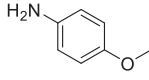
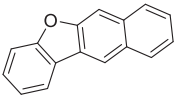
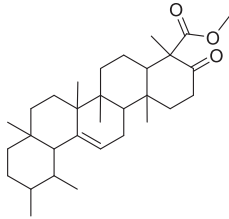
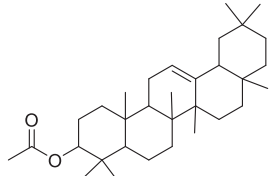
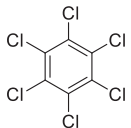
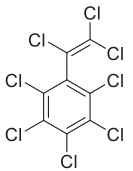
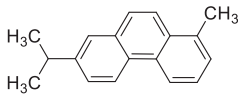
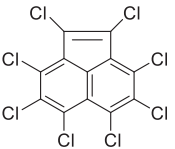
The organic compounds detected in the smoke particles were quite different from one pyrotechnic smoke to another. Numerous organic molecules were detected in RSS and AIS. HC-OS particles that were poor in carbon contain fewer compounds, the main one being hexachlorobenzene. Several chlorinated aromatic or polyaromatic compounds (i.e. octachlorostyrene, octachloronaphthalene), methylated derivatives of PAH, alkanes and phthalates were also detected in HC-OS particles (Table 3). RSS particles contained a large set of aromatic molecules containing nitrogen (aromatic amines and nitriles), which is consistent with its elementary analysis (N, 8%) and also oxygenated aromatic molecules. These molecules are for example oxygenated or nitrogenous PAHs such as quinolines, naphthalene-naphthalenyloxy, naphthalenol, methyl-naphthalenol, amino-methyl-naphthalenol, methyl dibenzofurane, benzo(b) naphtho(2,3-d) furan and/or isomers, hydroxycarbazole, benz(cd) indol-2(1H) one and/or isomers, dimethyl phenantrolinone, benz(a) or (c) acridine and isomers, benz(a) or (c) acridinone, methyl benz(a) fluorenone and/or isomers. RSS contains also diazenes, alkanes and quinones such as trimethyl-anthraquinone (Table 3). Compounds identified in AIS are mainly polycyclic compounds such as cholesterol derivatives (mass: 218, 250, 273). Examples of molecular structures detected in the particles are presented in Table 3.

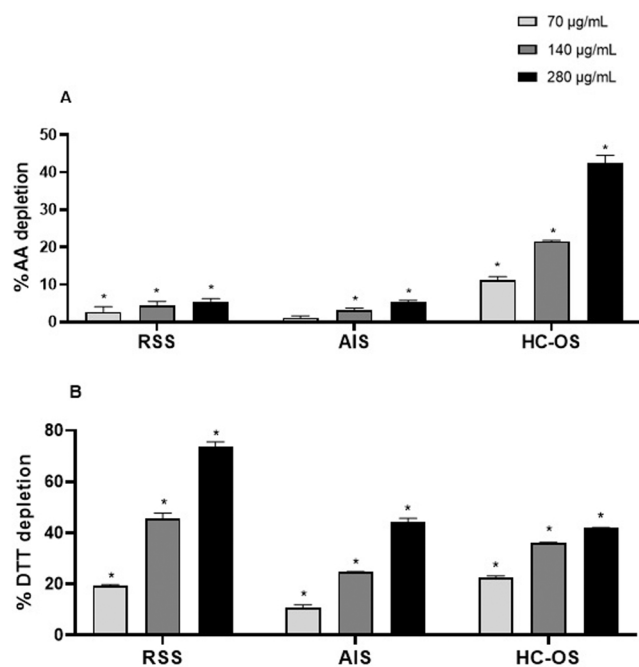
In smoke particles suspension, no solubilization of organic compounds in any smoke was observed suggesting that organic molecules remained adsorbed on the particulate fraction.

## 3.2. Oxidative potential

Antioxidant depletion assay (AO) was realized in a synthetic respiratory tract lining fluid (RTLFL). As shown in Fig. 1A, among all smoke particles, HC-OS particles were the most reactive sample by providing 11%, 22% and 42% of ascorbic acid (AA) depletion at 70, 140 and 280 μg/mL (or 12.5, 25 and 50 μg/cm<sup>2</sup>) respectively, depleting in a dose-dependent manner compared to the control. Smoke particles from RSS and AIS induced a low AA depletion, 3% (70 μg/mL) to 6% (280 μg/mL) and 3% (140 μg/mL) to 5% (280 μg/mL), respectively. Smokes particles did not cause any depletion of GSH and any production of GSSG

**Table 3**  
Type of organic compounds detected in RSS, AIS and HC-OS particles collected on impactor.

RSS	 1-[(E)-2-(4-methoxyphenyl) diazenyl]-2-naphthol	 1,2,4-trimethylanthracene-9,10-dione	 4-anisidine	 Benzo [b] naphto [2,3-d] furan
AIS	 Methyl 3-oxours-12-en-24-oate		 (3α)-Olean-12-en-3-yl acetate	
HC-OS	 Hexachlorobenzene	 Octachlorostyrene	 Phenanthrene, 1-methyl-7-(1-methyl ethyl)	 Acenaphthylene, octachloro ethyl



**Fig. 1.** Percentage of AA depletion relatively to free-particles control after 4 h of incubation in RTLF with RSS, AIS and HC-OS particles (A). Percentage of DTT depletion relatively to free-particles control after 1 h of incubation with RSS, AIS and HC-OS particles (B). Data are expressed as mean value  $\pm$  SD of at least three independent experiments carried out in triplicates. \* $p \leq 0.05$  (Dunnett's test).

(Fig. A.3).

Dithiothreitol assay (DTT) was dose dependently depleted by all smoke particles (Fig. 1B) but RSS seemed to be the most reactive sample. At 280  $\mu\text{g/mL}$ , RSS particles showed a maximum depletion of 74% whereas AIS and HC-OS particles exhibited a lower but similar maximum of  $\text{OP}^{\text{DTT}}$  around 44%. At lower doses, we noticed that depletion of HC-OS particles (22%, 70  $\mu\text{g/mL}$ ; 36%, 140  $\mu\text{g/mL}$ ) was

significantly higher than depletion from AIS particles (11%, 70  $\mu\text{g/mL}$ ; 25%, 140  $\mu\text{g/mL}$ ).

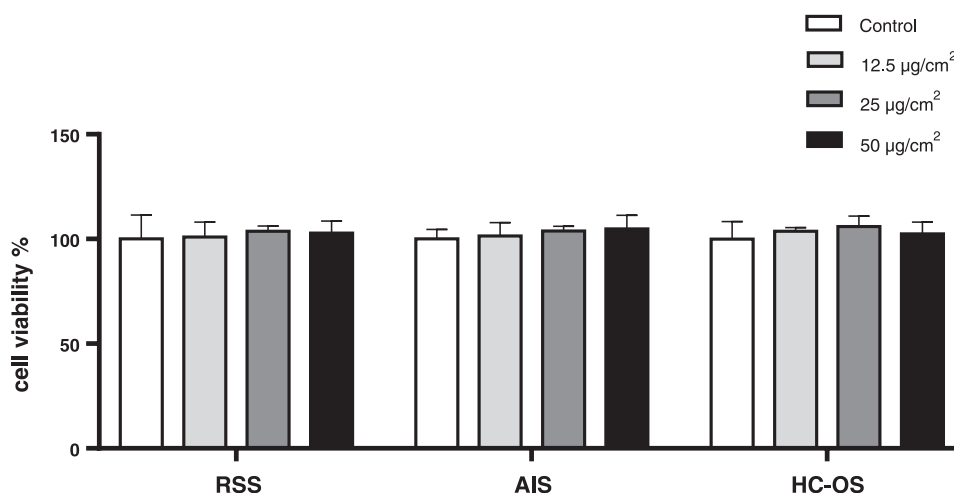
### 3.3. Cell viability

Small airway epithelial cells (SAEC) were exposed for 24 h to suspended particles from RSS, AIS and HC-OS at 12.5, 25 and 50  $\mu\text{g/cm}^2$ . The results of cell viability assessed on SAEC cells using the MTT assay are shown in Fig. 2. None of the three types of smoke particles induced any decrease in cell viability whatever the concentration tested.

Since particles had an intrinsic oxidative potential and despite the absence of a cytotoxic response after exposure, we chose to further characterize the cellular response by analyzing both antioxidant and inflammatory gene expression.

### 3.4. Antioxidant response

Fig. 3 presents the modulation of the mRNA expressions of superoxide dismutases-1 (*SOD-1*) and -2 (*SOD-2*), *catalase*, *NADPH quinone oxidoreductase-1* (*NQO-1*) and *heme oxygenase-1* (*HO-1*) after 24 h of exposure of SAEC to RSS (Fig. 3A), AIS (Fig. 3B) and HC-OS particle suspensions (Fig. 3C). For all concentrations, particles from RSS induced a significant increase of *SOD-2*, *HO-1* and *NQO-1* mRNA levels. At 50  $\mu\text{g/cm}^2$ , expressions of *HO-1* and *NQO-1* were strongly increased by 14.4-fold and 10.0-fold respectively while *SOD-2* expression was moderately increased by 3.0-fold. Expressions of *SOD-1* and *catalase* were not altered compared to the control. An exposure to AIS particles generated a significant increase of *HO-1* and *NQO-1* expressions at all tested concentrations (1.5-fold to 3.3-fold, *HO-1*; 2.5-fold to 7.4-fold, *NQO-1*) compared to PBS control. At the highest dose of exposure, a statistically significant but moderate increase expression of *SOD-1* (1.3-fold) and *SOD-2* (2.1-fold) expressions was observed while *catalase* expression was slightly reduced (0.64-fold). HC-OS particles exposure induced overexpression of *NQO-1* at 25  $\mu\text{g/cm}^2$  (2.3-fold) and 50  $\mu\text{g/cm}^2$  (3.1-fold). For all concentrations, we noticed an increase of *SOD-2* expression (2.5-fold to 3.4-fold) and a reduction of *catalase* expression (0.66–0.54-fold<sup>2</sup>). No effects on *SOD-1* and *HO-1* mRNA levels were observed.



**Fig. 2.** Cell viability results from the MTT assay. SAEC were exposed during 24 h to RSS, AIS and HC-OS particles to a range of concentrations (12.5; 25; 50  $\mu\text{g}/\text{cm}^2$ ). The results are presented as percentage of cell viability compared to control (cells cultured with PBS). Data were expressed as mean value  $\pm$  SD of at least three independent experiments carried out in replicates.

### 3.5. Inflammatory response

The pro-inflammatory response was characterized by measuring mRNA expression changes (Fig. 4A) and secretion (Fig. 4B) of IL-6, IL-8 and TNF- $\alpha$  cytokines 24 h after exposure to smoke particles from RSS, AIS and HC-OS.

Concerning RSS and whatever the concentrations, IL-8 mRNA level was highly induced by 7.0-fold. IL-8 cytokine secretion was significantly increased only at the highest concentration reaching a maximum level of 11,000 pg/mL. IL-6 mRNA level was significantly reduced by 0.6-fold at 25  $\mu\text{g}/\text{cm}^2$  and 50  $\mu\text{g}/\text{cm}^2$ . But, the cytokine secretion did not decrease significantly. We noticed a non-significant increase of TNF- $\alpha$  expression. In addition, we did not detect TNF- $\alpha$  secretion after any smoke particles exposure. AIS particles generated a significant increase of IL-8 expression (5.2-fold) at 50  $\mu\text{g}/\text{cm}^2$  but a non-significant effect on the cytokine secretion compared with control. About IL-6 mRNA and cytokine secretion, no modification was observed. AIS particles caused a slight significant rise of TNF- $\alpha$  expression by 2-fold at all concentrations tested. Concerning HC-OS particles, they produced a strong inflammatory response in SAEC. Indeed, after 24 h of exposure, expressions of all cytokines were significantly induced by all concentrations, especially IL-8 (13.5 fold, 12.5  $\mu\text{g}/\text{cm}^2$ ; 9.4 fold, 25  $\mu\text{g}/\text{cm}^2$ ; 13.5 fold, 50  $\mu\text{g}/\text{cm}^2$ ). The secretion of this cytokine was also significantly increased by 3-fold. A moderate but significant increase of IL-6 gene expression was noticed by 2.0-fold but the cytokine secretion was only observed at the highest dose tested (2.0-fold) compared to control. Expression of TNF- $\alpha$  is significantly increased by 3.4–4.0-fold but the increase in cytokine secretion could not be confirmed.

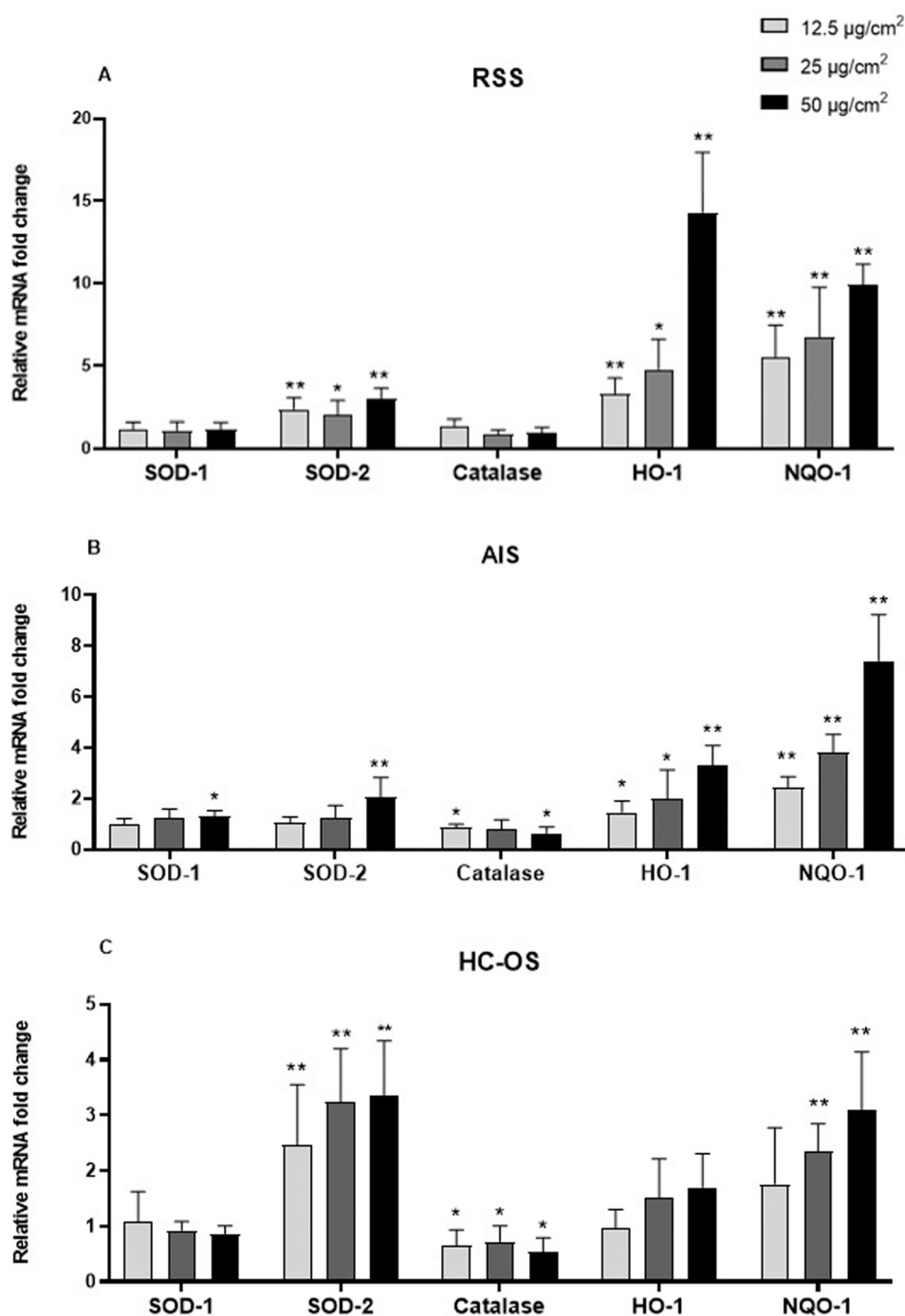
## 4. Discussion

While the evidence for the health adverse effects of atmospheric particles has been growing, there is very few data on the toxicity of particles generated by pyrotechnic smokes. Yet exposure to such smoke particles can occur in military, professional, festive, sporting and civilian fields. In order to fulfill this lack of knowledge, we intended to gain information on the physicochemical characterization, intrinsic oxidative properties (OP) and biological responses of human small airway epithelial cells (SAEC) after exposure to particles from two military smokes (RSS and HC-OS) and one used by civilians (AIS).

The physicochemical characterization shown that, for the three types of smokes, the most majority of the formed particles had a diameter lower than 400 nm leading to the capacity to penetrate deeply into the airways after inhalation and to reach small airway region (Geiser and

Kreyling, 2010). This small diameter justified the relevant choice of SAEC model to study toxicity of the smoke particles. Other studies on particles from colored signaling smokes (red, orange, violet and yellow) exhibited larger sizes than us, smaller than 2.1  $\mu\text{m}$  (vanHulst et al., 2017) or 1  $\mu\text{m}$  (Hemmilä et al., 2007b). To our knowledge, except analysis of particle size, chemical compositions of smoke particles have never been studied. The most noticeable findings are that each smoke exhibits a specific chemical signature. In RSS particles, we noticed high level of Sb, which could be related to the grenade propellant composition (Dalby et al., 2010) that may differ between smokes. RSS and AIS particles presented metal levels in accordance with other studies on combustion particles (Danielsen et al., 2011; Deng, Zhang et al., 2013; Visentin et al., 2016) whereas metals levels of HC-OS were exceptionally high (Al, Fe, Zn and Cu). Furthermore, RSS and AIS particles appeared to be more organic than HC-OS particles. Indeed, RSS particles contain a large set of aromatic molecules containing nitrogen and oxygenated aromatic molecules related to initial chemical compositions and especially to the red dye of the smoke, such as diazenes. AIS particles contained mainly polycyclic compounds like cholesterol derivatives whereas HC-OS particles contained low amounts of chlorinated molecules.

Oxidative stress triggering pro-inflammatory response is considered as the main mechanism of particle toxicity and oxidative potential (OP) of the smoke particles could be a relevant indicator to characterize this effect. The OP has the advantage to integrate the physicochemical properties of particles such as size, chemical composition and/or surface reactivity. Results showed that ascorbic acid (AA) was only depleted by HC-OS particles compared to RSS and AIS particles and no type of smoke particles affected glutathione depletion. Our results of OP<sup>AA</sup> were consistent to other studies showing that metals drove the AA response (Bates et al., 2019; Visentin et al., 2016; Yu et al., 2018) since HC-OS particles exhibited the highest OP<sup>AA</sup> value. Among metals composition of HC-OS particles, Al (Godri et al., 2011), total Fe (Godri et al., 2010; Janssen et al., 2014) and soluble Fe (Koehler et al., 2014) as well as total Zn (Godri et al., 2011) have been reported to impact OP<sup>AA</sup>. OP<sup>DTT</sup> results demonstrated that DTT was higher depleted by RSS particles compared to HC-OS and AIS particles. About the DTT assay, numerous studies reported high sensitivity to metals, especially Cu, Mn, Co, Vi, Ni and Pb (Koehler et al., 2014) but also sensitivity to quinones (Calas et al., 2018; Cho et al., 2005; Fang et al., 2016; Verma et al., 2015). However, usually typical ambient PM<sub>2.5</sub> samples are richer in metals than in organic compounds, so it suggests that metals dominate the DTT response (Conte et al., 2017; Fang et al., 2016; Janssen et al., 2014). The singular response of RSS particles is probably related to the numerous



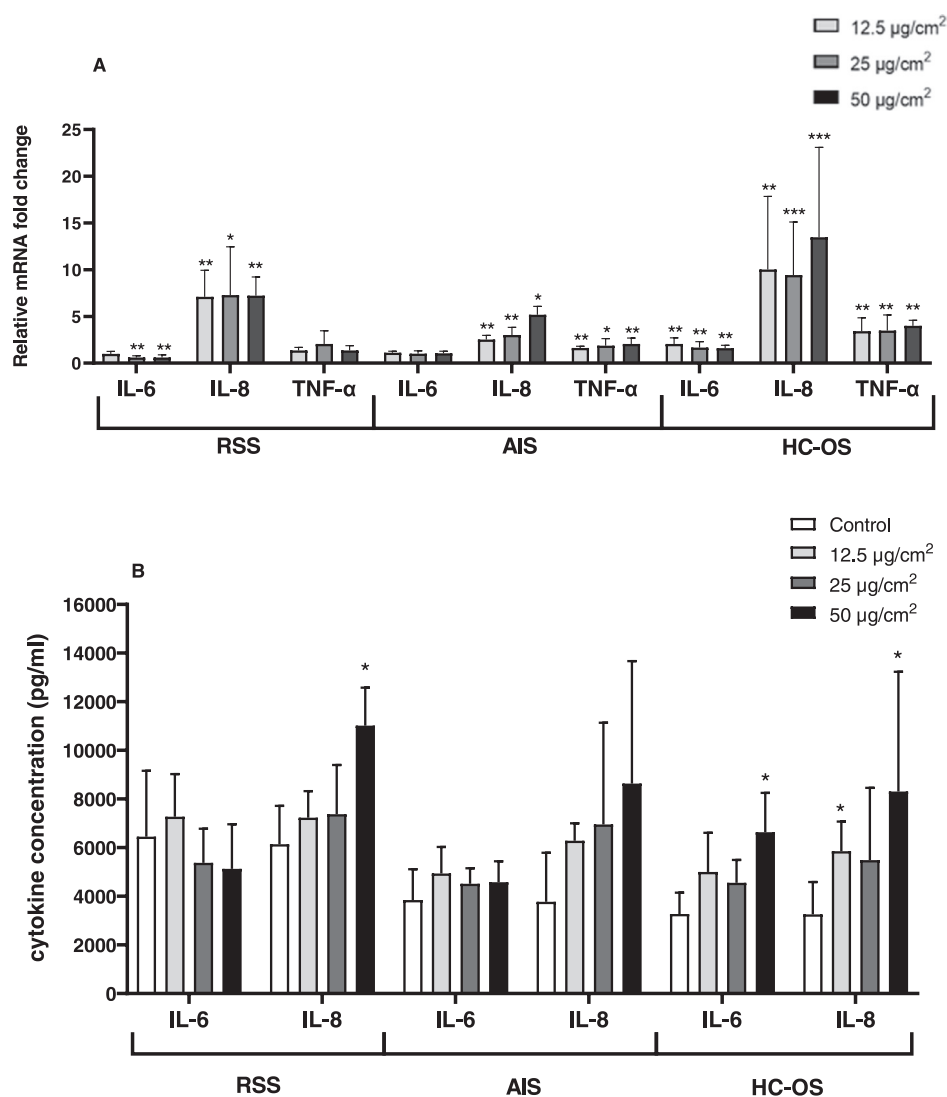
**Fig. 3.** Antioxidant mRNA expression after 24 h of exposure to RSS (A), AIS (B) and HC-OS (C) particles (12.5, 25, 50 µg/cm<sup>2</sup>). SOD-1 and -2, superoxide dismutases-1 and -2; NQO-1, NADPH quinone oxydoreductase-1; HO-1, heme oxygenase-1. Data were expressed as mean value ± SD of at least three independent experiments carried out in replicates. \* $p \leq 0.05$ , \*\* $p \leq 0.01$  (Mann-Whitney test) indicate significant differences to the control (PBS).

organic compounds and metals included in the particle composition. Indeed, some studies reported correlations between total PAH or some specific molecules such as anthracene, pyrene, benzo(a)anthracene, chrysene and indenopyrene with OP<sup>DTT</sup> (Calas et al., 2018; Crobeddu et al., 2017; Gao et al., 2020). According to literature and our results, it seems difficult to identify the specific components that drive the DDT assay. Thus, the difference in sensitivity of each OP to certain types of chemical components provide complementary information on the link between OP and particle composition. Our results point out the need to use several assays to assess the OP of particles, which is in accordance with several studies on OP (Conte et al., 2017; Fang et al., 2016; Janssen

et al., 2014).

In this study, for the first time the biological effects of smoke particles were evaluated on a human pulmonary model cultivated at ALI. The exposure of SAEC for 24 h to RSS, AIS and HC-OS particles did not influence cell viability. Several Finnish studies (Hemmilä et al., 2007a, 2007b, 2010) were carried out by exposing bronchial cells (BEAS-2B) for 5 min with a time recovery of 48 h to total aerosol of different types of colored smokes (red, orange, blue, green, violet, purple and yellow) and screening smokes (red phosphorus), an hexachloroethane (HC) based smoke used as a reference. These studies revealed that red signalling and HC smokes decreased cell viability to 16% and 37% respectively as





**Fig. 4.** mRNA expressions (A) and cytokines secretion (B) of cytokines in PBS control and after 24 h of exposure to RSS, AIS and HC-OS particles (12.5, 25, 50 µg/cm<sup>2</sup>). Data were expressed as mean value ± SD of at least three independent experiments carried out in replicates. \**p* ≤ 0.05, \*\**p* ≤ 0.01, \*\*\**p* ≤ 0.001 (Mann-Whitney test) indicate significant differences to the control (PBS).

compared with unexposed cells. Furthermore, two types of red phosphorus were studied, one did not affect cell viability and the second one induced a decrease to 65% of viability. An another in vitro study evaluated the cytotoxic effects of three red smokes from different manufacturer on alveolar cells (A549 cells) cultivated at ALI (van Hulst et al., 2017), only one generated a cytotoxic effect (>10%). Because we worked only on the particulate fraction of smokes, it was difficult to compare our results with these previous studies, which performed exposure to total aerosols, including gas phase. However, numerous studies on particles toxicity reported various cytotoxicity responses. Regarding SAEC model, an exposure of 1 h (Svensson et al., 2016) or 24 h to metallic nanoparticle (Wu et al., 2020) or 24 h to surgical smoke (Sisler et al., 2018) reduced cell viability in SAEC while 48 h of exposure to diesel exhaust particles (DEP) did not induce any cytotoxicity (Zhang et al., 2019). Time of exposure and physicochemical characteristics of particles could explain the different biological responses. In addition, concentrations of particles used have to be considered. In works cited above, concentrations used are lowest than ours. However, concentrations of PM<sub>2.5</sub> used in the work of Lan et al., 2021 on SAEC cells are closed to those tested in our study and results on viability are very

similar. Exposure of 24 h could be considered as a long time of exposure but it could represent a residual particles deposition in the lower respiratory tract (Geiser and Kreyling, 2010; Lan et al., 2021). Our results showed that smoke particles tested exhibit an intrinsic oxidative potential at the lowest concentration tested without inducing any cytotoxicity after 24 h of exposure. However, it seems important to explore key events such as antioxidant and inflammation responses because these mechanisms could be the earliest steps in the hierarchical biological effect (Xiao et al., 2003) after exposure to smokes particles.

Our results highlighted an induction of an antioxidant response by over expression of *HO-1* mRNA after exposure to RSS and AIS particles as well as over expression of *NQO-1* expression after exposures to the all types of smoke particles. These results are consistent with other studies that showed over expression of *HO-1* and *NQO-1* in pulmonary cells exposed to DEP, PM<sub>2.5</sub> or ultrafine particles (Crobbeddu et al., 2017; Deng, Rui et al., 2013; Frias et al., 2020; Juarez-Facio et al., 2022b). Organic components are able to cross the membrane barrier and activate Nrf2 pathway that regulates expression of *HO-1* and *NQO-1* (Hawley et al., 2014). The antioxidant response generated by RSS and AIS particles may be explained by their rich composition in PAH. Indeed, the

phenanthrene, naphthalene and quinones, that also composed RSS particles have been reported to induce expression of *Nrf2* (Chowdhury et al., 2018; Klein et al., 2017) and subsequently *HO-1* expression (Li et al., 2004). Compelling evidence showed that PM<sub>2.5</sub> or DEP compromised antioxidant protective capacity by reducing expression of *SOD-1* and *SOD-2* (Cui et al., 2020; Seriani et al., 2016; Wang et al., 2019). These data are not in line with our findings since *SOD-2* expression is over expressed by all smoke particles. However, it is well known that antioxidant response is time-dependent (Deng, Zhang et al., 2013; Hawley et al., 2014), therefore studying the expression of genes in a time-dependent manner seems necessary. In addition, enzymatic activities of these antioxidant proteins might be realized to confirm efficient antioxidant response. Nevertheless, RSS particles seem to induce a stronger antioxidant response than AIS and HC-OS particles. That suggests a potential link with its high capacity to deplete DTT. Indeed, significant correlations between the DTT assay and genes expression of antioxidants (*HO-1* and *SOD-2*) were reported (Crobbeddu et al., 2017; Karavalakis et al., 2017; Li et al., 2003). Overexpression of these antioxidant genes could be interpreted as an adaptive response to oxidative stress induced by smokes particles (Ballweg et al., 2014; Cantin, 2010; Marchini et al., 2013).

Smoke particles exposure has been shown to activate an antioxidant response in SAEC, which could be a consequence of ROS production. This key event could then trigger inflammation by increasing the synthesis of pro-inflammatory cytokines. *IL-8* expression was significantly induced by all smoke particles type whereas *IL-6* expression was only induced by particles from HC-OS. Several studies using different cell models have demonstrated that different types of particles induced various cytokines expression and secretion, which is in line with our findings. Increased mRNA levels of *IL-6*, *IL-8* or *TNF- $\alpha$*  is observed after DEP, PM<sub>2.5</sub> or ultrafine particles exposures (Bengalli et al., 2019; Juarez-Facio et al., 2022b; Luo et al., 2021; Totlandsdal et al., 2010; Vattanasit et al., 2014). Expression of *IL-6* and *IL-8* could also be down regulated as it was previously reported with ultrafine particles exposure of BEAS-2B cells (Juarez-Facio et al., 2022a). Comparing our results with in vitro studies using SAEC grown at ALLI, the occurrence of PM<sub>2.5</sub> surrogates to over-secrete *IL-6* but not *IL-8* was recently reported (Lan et al., 2021). Moreover, it was shown that several metallic nanoparticles were able to induce different cytokines secretion (Svensson et al., 2016) and that printer-emitted-particles were able to produce *IL-6* and *IL-8* (Sisler et al., 2018). Our results showed moderate secretion of cytokines that seems to be in accordance with the activation of antioxidant responses suggesting an adaptive cell response (Cao et al., 2015; Fattman et al., 2003; Marchini et al., 2013). Overall, HC-OS particles appeared to induce a stronger inflammatory response and they were the only particles able to oxidize AA. The high content of metals in HC-OS particles probably drive the inflammatory response. Indeed, Fe, Al and Mn levels have shown to be crucial for the pro-inflammatory effects on the respiratory system (Shao et al., 2018) as well as Cu, Ni and Zn, which are able to induce cytokines release in BEAS-2B cells (Van Den Heuvel et al., 2016). Our results indicated a link between OP and the inflammatory response. However, various studies reported that in vitro inflammatory reactions is not associated with any OP assay (Gerlofs-Nijland et al., 2013; He et al., 2018; Øvrevik, 2019). To what extent particle composition is responsible for OP and biological responses as well as the link between OP and biological markers needs to be further characterized.

## 5. Conclusion

In conclusion, to our knowledge this is the first study characterizing the physico-chemical properties of smoke particles, assessing their oxidative potential and studying the biological effects on a small airway cell model grown at ALLI. This study highlighted the importance to consider differences in chemical composition of smoke particles, when evaluating OP and in vitro biological effects. The combination of two

assays for OP assessment could provide complementary information on particle composition and relative contribution of chemical components on oxidative properties of smoke particles. Our results showed that particles triggered an adaptive antioxidant response leading to a moderate pro-inflammatory response. Additionally, we observed that OP<sup>DTT</sup> and OP<sup>AA</sup> could be good indicators for in vitro antioxidant and inflammatory responses, respectively. Finally, our study pointed out the need to further investigations on smoke particle toxicity in order to establish exposure guidelines in the scope of professional recurrent exposures. Scientific approach developed here could be used to study particles from other pyrotechnic activities such as domestic and commercial fireworks or gun fires at shooting ranges.

## CRediT authorship contribution statement

**Violaine Martin de Lagarde:** Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Tiphaine Rogez-Florent:** Data curation, Formal analysis, Methodology, Resources, Software, Supervision, Validation, Writing – review & editing. **Fabrice Cazier:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – review & editing. **Dorothee Dewaele:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – review & editing. **Francine Cazier-Dennin:** Conceptualization, Methodology, Writing – review & editing. **Alexane Ollivier:** Investigation, Methodology. **Marion Janona:** Investigation, Methodology. **Sophie Achard:** Conceptualization, Writing – review & editing. **Véronique André:** Conceptualization, Writing – review & editing. **Christelle Monteil:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing. **Cécile Corbière:** Conceptualization, Data curation, Formal analysis, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2022.113637](https://doi.org/10.1016/j.ecoenv.2022.113637).

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