Nose-only inhalation exposures to alumina nanoparticles / hydrogen chloride gas mixtures induce strong pulmonary pro-inflammatory response

Alexandra Bourgois

Institut de recherche biomédicale des armées

Dominique Saurat

Institut de recherche biomédicale des armées

Alexandre Boyard

Institut de recherche biomédicale des armées

Nathalie Guitard

Institut de recherche biomédicale des armées

Suzanne De Araujo

Institut de recherche biomédicale des armées

Sylvie Renault

Institut de recherche biomédicale des armées

Francisca Fargeau

Institut de recherche biomédicale des armées

Christine Frédéric

Institut de recherche biomédicale des armées

Emmanuel Peyret

Institut National de l'Environnement et des RISques

Emmanuel Flahaut

Université Fédérale Toulouse Midi-Pyrénées

Aurélien Servonnet

Institut de recherche biomédicale des armées

Anne-Laure Favier

Institut de recherche biomédicale des armées

Ghislaine Lacroix

Institut National de l'Environnement industriel et des RISques

Sabine François

Institut de recherche biomédicale des armées

Samir Dekali (✉ samir.dekali@gmail.com)

Institut de recherche biomédicale des armées <https://orcid.org/0000-0002-9662-0284>

Research

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Abstract

Background: Combustion processes, especially in aerospace and defense fields, can lead to complex aerosols emission containing gases and nanoparticles (NPs). Alumina (Al_2O_3) NPs and hydrogen chloride gas (HCl_g) are for instance present in high concentrations after solid composite propellants use. Exposure to these pollutants mixtures by inhalation is thus possible but literature data towards their pulmonary toxicity are missing. To specify hazards resulting from these combustion aerosols, an inhalation study was implemented.

Male Wistar rats were exposed by nose-only to Al_2O_3 NPs (13 nm) and/or HCl_g aerosols for 4h (unique exposures ; UE) or 4h a day for 4 days (iterative exposures ; IE). Bronchoalveolar lavage fluids (BALF) content and lungs histopathology were analyzed 24h after exposures.

Results: Iterative co-exposures (IE) increased total proteins and lactate dehydrogenases (LDH) concentrations in BALF indicating alveolar-capillary barrier permeabilization and cytolysis. Early pulmonary inflammation was induced after IE to Al_2O_3 NPs $\pm$ HCl_g resulting in polymorphonuclear neutrophils (PMN) and pro-inflammatory cytokines increases (TNF- α , IL-1 β , GRO/KC) in BALF. Moreover, after exposure to Al_2O_3 NPs $\pm$ HCl_g aerosols, both exposure scenarios induced early pulmonary histopathological lesions, among which vascular congestions, bronchial pre-exfoliations, vascular and interalveolar septum edemas. Lung oxidative damages ([8-hydroxy-2'-deoxyguanosine](#) ; 8-OHdG) were observed *in situ* following UE in each experimental condition, suggesting early oxidative stress induction by aerosols inhalation. However, no 8-isoprostane concentration increase was simultaneously found in animals BALF.

Conclusions: Biological effects of the studied aerosols are dependent on both aerosol content and exposure scenario. Results showed an important early pro-inflammatory effect of Al_2O_3 NPs/ HCl_g mixtures on rats lungs following iterative inhalations (IE). Taken together these data raise concerns towards potential long term pulmonary toxicity of combustion mixtures aerosols, and highlight the importance for workers to wear individual protections.

Background

Combustion reactions from different sources can produce high volumes of complex aerosols containing various components as gases and particles in the environment [1, 2]. Solid composite propellants use in aerospace and defense fields lead to emission of alumina (Al_2O_3) particles and hydrogen chloride gas (HCl_g) [3, 4]. Moreover, these pollutants can also be emitted respectively by industrial activities such as aluminum manufacturing [5], and wastes incineration [6] increasing the risk of workers exposure.

Alumina (Al_2O_3) is the oxidized form of aluminum and exists under several crystalline phases (γ , δ , θ and α) [7]. Among them, a majority of γ then δ polymorphs were found following solid composite propellants use [4, 8]. Alumina is mainly used for aluminum production and enters in the composition of many

everyday products (paper, plastics, ceramics, flame retardants and coatings) due to its hardness. Otherwise, alumina can also be found in foods and cosmetics [9]. To determine health hazard related to alumina particles exposure, only few studies were conducted *in vivo* by inhalation. 28 days nose-only inhalation of Al₂O₃ NPs (11.94 nm, 0.2 to 5 mg/m³, 5 days a week) showed pulmonary pro-inflammatory properties of Al₂O₃ NPs on rats. This was characterized by total cells, polymorphonuclear neutrophils (PMN), lymphocytes, lactate dehydrogenase (LDH), TNF-α and IL-6 increases in bronchoalveolar lavage fluids (BALF). Alveolar macrophages accumulation was also observed in lungs after 28 days exposure [10]. In mice, pulmonary pro-inflammatory effects were also recorded after 7 days exposure to 40 nm Al₂O₃ NPs (0.4 mg/m³) by inhalation. Increased concentrations of IL-6 and IL-33 were measured in BALF associated with emphysema and airway remodeling appearance. However, in this study, BALF PMN concentration was not increased following Al₂O₃ NPs inhalation [11]. Al₂O₃ NPs intratracheal (IT) instillation on rats (6.3 nm, 0.5 to 300 cm²/mL) also induced acute pulmonary inflammation resulting in PMN concentrations increases in BALF [12]. Moreover, mice IT instillation exposures to Al₂O₃ particles (4.37 μm, 40 mg) increased macrophages, PMN and fibronectin concentrations in BALF until 12 months after exposure suggesting pulmonary inflammation persistence over time [13]. Another study, carried out by nasal instillation on rats highlighted dose dependant inflammation and alveolar-capillary barrier permeabilization after Al₂O₃ particles (size unknown, 1 to 40 mg/kg) exposure [14]. Besides pro-inflammatory properties, several *in vitro* Al₂O₃ particles toxicity studies results suggest oxidative stress induction and genotoxic potential (DNA single and double-strand breaks induction) of these particles [15, 16]. These effects were also found *in vivo* after oral, intravenous or peritoneal injection exposures to Al₂O₃ particles [16, 17, 18, 19].

Hydrogen chloride gas is a known irritant and caustic agent able to cause severe ocular and cutaneous lesions [20]. Once in water, HCl_g dissolves to form hydrochloric acid solution (HCl). HCl_g and HCl exposure risk is non negligible as these compounds are widely used in industries and laboratories and as they can be found in some household products [21]. After HCl_g inhalation by rats and mice, eyes, mucous membranes and skin irritations were observed associated with respiratory system lesions (emphysema, atelectasis and edema) [22]. Respiratory system necrosis as well as PMN increases were also observed on rats after 30 min inhalation (1284 and 1293 ppm of HCl_g) [23]. Nevertheless, HCl_g long term inhalation (10 ppm ; 6h a day 5 days a week) performed during rats entire life did not induce neoplastic or preneoplastic lesions [24]. Similar respiratory symptoms were found in Guinea pigs and rabbits associated with severe and persistent inflammatory reactions [25]. Mitogenic effect of HCl (40 mM) was also showed on rabbit esophageal mucosa after 1h incubation [26]. Moreover, respiratory rate increase and oxygen partial pressure in blood decrease were observed after HCl_g head-only inhalation in baboons (500, 5 000 and 10 000 ppm ; 15 min). However, no alteration of pulmonary function was reported 3 months post-exposure [27]. Pro-inflammatory effect of HCl was also demonstrated *in vitro* on esophageal mucosa and mice macrophages (cell line RAW 264.7), resulting in substance P, IL-8, IL-6, IL-1β, platelet-activating factor (PAF) and nitric acid levels increases [28, 29, 30, 31].

Taken together, these toxicity data regarding Al_2O_3 particles and HCl_g raise questions towards their potential combined toxicity due to possible workers exposure. Mixtures effects study is an actual major concern in toxicology. Only few scientific data are available in literature, especially for mixtures of compounds with different physical forms (solid and gas for example). However, population is daily exposed to multiple pollutants simultaneously and a recent study highlighted mixtures effects (synergy, addition, antagonism or inhibition) unpredictability [32]. Therefore, it is currently necessary to study mixtures effects in a case-by-case basis. Biological effects and resulting hazard of complex combustion aerosols exposure are poorly described in literature. To date only one study was published concerning combined effect of Al_2O_3 particles and HCl_g . This study was carried out *in vivo* on rats and mice by whole body exposure [33]. However, during exposures, HCl_g was always associated with hydrogen fluoride gas (HF_g) and the only studied endpoint was mortality. Conclusions did not highlight any additive or synergistic effect of Al_2O_3 particles addition to the gases mixture on animals mortality.

As the main route of exposure to these mixtures is inhalation, this study objective was to assess pulmonary toxicity after inhalation of Al_2O_3 NPs / HCl_g mixture *in vivo*. Wistar rats were exposed by nose-only inhalation to aerosols containing Al_2O_3 NPs (size and crystalline phase: 13 nm ; γ/δ) and/or HCl_g . Blood, BALF and lungs were collected 24h after exposures. Cells counts, pro-inflammatory cytokines, lactate dehydrogenases, total proteins and 8-isoprostane concentrations were measured in bronchoalveolar lavage fluids (BALF). Lungs histopathological lesions were analyzed and presence of oxidative nucleic acids damages (8-OHdG) was studied *in situ* in lung tissue.

Results

Aerosols physico-chemical characterization

Transmission Electron Microscopy (TEM) observations showed micronic spherical particles agglomerates on Al_2O_3 bulk powder (fig. 1 A). To characterize alumina crystalline structure, X-Ray diffraction (XRD) experiments were performed on bulk powder. Results confirmed majority of γ and δ polymorphs as indicated by the supplier (Fig. 1 B).

Once nebulized, Al_2O_3 NPs formed spherical agglomerates (Fig. 1 C). Presence of HCl_g did not modify agglomerates shape in aerosols (data not shown). Energy Dispersive X-ray (EDX) analysis confirmed aluminum and oxygen atoms presence without chemical impurities. The copper spike on EDX spectra correspond to TEM grids used for aerosol sampling (Fig. 1 D). Results obtained with Sioutas (SKC Inc.) cascade impactor revealed that a majority of particles agglomerates in generated aerosols had a size below 250 nm, and presence of HCl_g 5 ppm did not significantly modify particles concentrations and size distribution (Fig. 1 E).

To deepen and complete these analytical data, aerosol particles content was assessed using mass measurements (ICP-OES) after filter samplings. Mean concentrations of $22.1 \pm 4.5 \text{ mg/m}^3$ and 20.0 ± 2.9

mg/m³ were respectively measured for aerosols containing Al₂O₃ NPs and Al₂O₃ NPs associated with HCl_g 5 ppm (Table 1 A). Therefore, we confirmed that addition of HCl_g to aerosol did not significantly modify NPs concentrations. HCl_g concentrations in generated aerosols were estimated through chlorine (Cl⁻) levels measurements carried out on filters (particulate fraction) and bubbler (free fraction) samples. Results showed 10 times smaller Cl⁻ levels than target concentrations (5 ppm = 7.4 mg/ m³). Addition of Al₂O₃ NPs to the HCl_g aerosol did not significantly modify Cl⁻ levels. However, it has to be noted that, in presence of Al₂O₃ NPs, Cl⁻ in the aerosols was exclusively under particulate fraction whereas it was exclusively under free fraction when alone (Table 1 B).

Table 1 - **Generated aerosols alumina and HCl_g contents.** Table A shows alumina (Al₂O₃) mean mass concentrations in the generated aerosols. Aluminum mass was measured on three different filters (µg/filter). Based on these results, aluminum and alumina concentrations in aerosols (mg/m³) were calculated and alumina mean mass concentrations (mg/m³) were determined for each experimental aerosol.

Table B shows chlorine mean concentrations in the generated aerosols. Chlorine particulate and free fractions were respectively measured on three filters and bubbler samples (µg/filter or µg/bubbler). Corresponding chlorine mass concentrations were calculated (µg/m³) and were added to determine total chlorine levels (µg/m³). Total chlorine mean mass concentrations (µg/m³) were calculated for each experimental aerosol.

A

Aerosol	Al (µg/filter) ^a	Al concentration (mg/m ³) ^b	Al ₂ O ₃ concentration (mg/m ³) ^c	Al ₂ O ₃ concentration mean ± SD (mg/m ³)
Al ₂ O ₃ 13 nm	137	9.1	17.3	22.1 ± 4.5
	183	12.2	23.0	
	207	13.8	26.1	
Al ₂ O ₃ 13 nm + 5 ppm HCl _g	185	12.3	23.3	20.0 ± 2.9
	147	9.8	18.5	
	144	9.6	18.1	

^a After white deduction ; ^b Sampling at 1L/min for 15 min (/ 0.015) ; ^c x 1.889

B

Aerosol	Particulate fraction (filters)		Free fraction (bubblers)		Cl ⁻ total concentrations (µg/m ³) ^b	Cl ⁻ total concentrations mean ± SD (µg/m ³)
	Cl ⁻ (µg/filter)	Cl ⁻ (µg/m ³) ^a	Cl ⁻ (µg/bubbler)	Cl ⁻ (µg/m ³) ^a		
5 ppm HCl _g	-	-	32.2	2145	2145	791 ± 1178
	-	-	3.4	228	228	
	-	-	0	0	0	
Al ₂ O ₃ 13 nm + 5 ppm HCl _g	8.9	596	0	0	596	617 ± 32
	9.0	601	0	0	601	
	9.8	653	0	0	653	

^a Sampling at 1L/min for 15 min (/ 0.015) ; ^b Particulate fraction + free fraction

Lungs aluminum content increasing after iterative exposures

Pulmonary aluminum load was measured by ICP-OES after aerosols inhalation. Following unique exposures (UE) to Al₂O₃ NPs alone or associated with HCl_g 5 ppm, concentrations around 100 µg of aluminum per g of lung were measured (Fig. 2). However, these mean quantities were not significantly different from air exposed animals. Iterative exposures (IE) to Al₂O₃ NPs alone or in association with HCl_g 5 ppm induced significant aluminum quantities increases in lungs compared to air exposed rats or to those exposed to the same conditions in UE (Fig. 2). IE to Al₂O₃ NPs with HCl_g 5 ppm significantly increased pulmonary aluminum load compared to iterative exposures to each component alone (Fig. 2).

Clinical symptoms and body weight

Animals' clinical follow-up did not highlight any treatment-related clinical symptom following exposures. Significant weight losses were observed after each 4-hour exposure compared to weight before exposure (Table 2). Rats did not always recover their initial weight between two exposures, especially after IE, even in air exposed group. However, these weight decreases were always less than 10% of rats' initial weight.

Table 2 – Rats body weight monitoring during the experimental period. Rats were exposed uniquely or iteratively to air, HCl_g 5 ppm, Al₂O₃ 13 nm or to the mixture Al₂O₃ 13 nm + HCl_g 5 ppm. Rats body weight (in gram, mean ± SD) was measured before and after each aerosol exposure. Obtained results during unique exposures and during iterative exposures are respectively presented in table A and table B. Tables show initial body weight before exposures (BE, t0, t24h (B), t48h (B), t72h (B)), after exposures (AE, t4h, t28h (B), t52h (B), t76h (B)) and before sacrifice (BS, t24h (A) and t96h (B)).

Two-way ANOVA and Dunnett's post-test (n=12, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001, α risk = 0.05) were performed as statistical analysis (SA) to compare measured body weights during the study with initial body weights (t0).

A Unique exposure

		t0	t4h (AE)	t24h (BS)
Air	Mean $\pm$ SD	289.0 $\pm$ 10.7	272.9 $\pm$ 10.5	287.8 $\pm$ 10.6
	SA		****	ns
HCl _g 5 ppm	Mean $\pm$ SD	289.2 $\pm$ 8.1	272.7 $\pm$ 9.0	285.8 $\pm$ 8.5
	SA		****	*
Al ₂ O ₃ 13 nm	Mean $\pm$ SD	333 $\pm$ 7.7	319.9 $\pm$ 6.2	328.5 $\pm$ 8.2
	SA		****	****
Al ₂ O ₃ 13 nm + HCl _g 5 ppm	Mean $\pm$ SD	324.8 $\pm$ 16.7	309.6 $\pm$ 14.9	320.9 $\pm$ 17.9
	SA		****	ns

B Iterative exposure

		t0	t4h (AE)	t24h (BE)	t28h (AE)	t48h (BE)	t52h (AE)	t72h (BE)	t76h (AE)	t96h (BS)
Air	Mean $\pm$ SD	296.4 $\pm$ 9.6	280.7 $\pm$ 9.8	291.5 $\pm$ 8.7	275.3 $\pm$ 9.2	287.4 $\pm$ 7.4	272.9 $\pm$ 8.8	286.5 $\pm$ 7.4	275.4 $\pm$ 9.6	286.4 $\pm$ 7.9
	SA		****	***	****	****	****	****	****	****
HCl _g 5 ppm	Mean $\pm$ SD	303.0 $\pm$ 10.6	284.4 $\pm$ 9.9	301.6 $\pm$ 9.7	282.1 $\pm$ 8.9	299.6 $\pm$ 9.3	280.5 $\pm$ 8.2	298.6 $\pm$ 9.1	280.6 $\pm$ 8.4	300.2 $\pm$ 9.8
	SA		****	ns	****	**	****	***	****	*
Al ₂ O ₃ 13 nm	Mean $\pm$ SD	305.3 $\pm$ 17.1	292.5 $\pm$ 16.4	300.7 $\pm$ 17.8	289.9 $\pm$ 18.4	297.8 $\pm$ 20.3	286.6 $\pm$ 19.4	295.6 $\pm$ 20.5	283.5 $\pm$ 19.3	296.6 $\pm$ 21.2
	SA		****	*	****	****	****	****	****	****
Al ₂ O ₃ 13 nm + HCl _g 5 ppm	Mean $\pm$ SD	286.2 $\pm$ 9.0	270.9 $\pm$ 9.7	283.7 $\pm$ 9.5	271.0 $\pm$ 8.8	283.8 $\pm$ 9.9	271.0 $\pm$ 10.1	283.4 $\pm$ 10.6	272.4 $\pm$ 11.2	283.1 $\pm$ 12.1
	SA		****	ns	****	ns	****	*	****	*

AE: After exposure

BE: Before exposure

BS: Before sacrifice

SA: Statistical analysis

ns: not significant

Alveolar-capillary barrier and pulmonary inflammation

No change in total proteins or lactate dehydrogenases (LDH) concentrations was observed following UE compared to air exposed rats (Fig. 3 A and B). Only IE to Al₂O₃ NPs alone or associated to HCl_g 5 ppm induced significant concentrations increases of total proteins and LDH in rats bronchoalveolar lavage fluids (BALF) compared to air exposed control animals (Fig. 3 A and B). Total proteins concentrations of 0.18 ± 0.03 g/L and 0.22 ± 0.04 g/L and LDH concentrations of 99 ± 43 U/L and 112 ± 42 U/L were measured in rats BALF following respectively IE to Al₂O₃ NPs and to Al₂O₃ NPs + HCl_g 5 ppm.

Total cells number increased in rats BALF after IE to Al₂O₃ NPs. Moreover, significantly higher total cells numbers were counted following IE than UE to Al₂O₃ NPs and to Al₂O₃ NPs + HCl_g aerosols (Fig. 3 C). IE to same aerosols also induced significant polymorphonuclear neutrophils (PMN) number increases in BALF compared to air exposed control animals (Fig. 3 D). In contrast, no significant change in macrophages and lymphocytes numbers was highlighted by the carried out counts (Fig. 3 E and F).

IE to Al₂O₃ NPs or Al₂O₃ NPs + HCl_g aerosols leaded to significant TNF- α and IL-1 β concentrations increases in rats BALF (Fig. 4 A and B). TNF- α concentrations of 42.86 ± 13.07 pg/mL and 45.73 ± 11.77 pg/mL, and IL-1 β concentrations of 456.2 ± 163.2 pg/mL and 341.6 ± 58.11 pg/mL were respectively measured. Significant increased concentrations of GRO/KC and MIP-2 were also measured in rats BALF after respectively IE to Al₂O₃ NPs (Fig. 4 C) and UE to Al₂O₃ NPs (Fig. 4 D) compared to air exposed control rats .

Histopathology observations

Lung parenchyma observations revealed the presence of pathological lesions following experimental aerosols inhalation (Fig. 5). However, no fibrosis, resorption hyperplasia or pleura inflammation was reported in this study.

In control rats lungs, vascular congestions were occasionally found and considered as physiological lesions (Fig. 5 A and B). HCl_g 5 ppm exposures mainly induced vascular lesions. UE increased vascular congestions (Fig. 5 C), while IE induced vascular edema and, in some cases, velamentous epithelium was observed (Fig. 5 D). Al₂O₃ NPs inhalation mainly induced interstitial and inflammatory lesions (Fig. 5 E and F). Indeed, marked interalveolar septum edemas were found within rats pulmonary epithelium after UE and IE. Bronchial pre-exfoliation phenomena (Fig. 5 E) and vascular edemas (Fig. 5 F) were also observed. In some cases, localized inflammatory areas, composed of polymorphonuclear cells and lymphocytes, were also found in rats lungs (Fig. 5 F). Co-exposures to Al₂O₃ NPs and HCl_g 5 ppm induced

vascular and interstitial lesions at a time (Fig. 5 G and H). Bronchial pre-exfoliation phenomena (Fig. 5 G), vascular and interalveolar septum edemas (Fig. 5 G and H) were found in rats lungs after both UE and IE.

Lesions scoring (Novotec society) was performed in order to compare early pulmonary lesions induced by the different experimental aerosols and exposure scenarios (Fig. 6). Obtained results showed higher mean lesion scores in animals exposed to aerosols containing Al_2O_3 NPs (alone or associated with HCl_g). Results did not suggest major influence of exposure scenario towards histopathological lesions induction as close scores were calculated for UE and IE to the same aerosol. Moreover, adding HCl_g to Al_2O_3 NPs aerosol did not seem to worsen lesions comparing to those resulting from Al_2O_3 NPs inhalation alone.

Oxidative stress analysis

To assess oxidative stress induction following experimental aerosols exposures, 8-OHdG (8-hydroxy-2'-deoxyguanosine, oxidative nucleic acids damages marker) was labeled *in situ* in rats lung tissue using immunofluorescence. Microscopic observations revealed 8-OHdG fluorescent signal after UE (Fig. 7 A-D), whereas no specific signal was observed after IE to the different aerosols. After UE to the experimental aerosols, cytoplasmic and nuclear labeling was found both in alveolar (Fig. 7 B and D) and bronchial epithelia (Fig. 7 C). These labeling localizations referred respectively to RNA (cytoplasmic) and DNA (nuclear) oxidations.

To complete our investigations on oxidative stress induced by inhalations, 8-isoprostane (lipid peroxidation marker) concentrations were measured in rats BALF 24h after the last exposure. Results did not reveal any significant increase in 8-isoprostane concentrations following inhalations (Fig 7 E). Significant 8-isoprostane concentrations decreases were measured after IE to Al_2O_3 NPs and to Al_2O_3 NPs + HCl_g 5 ppm compared to control, with respective mean values of 26.6 ± 1.5 pg/mL and 24.4 ± 1.7 pg/mL versus 34.9 ± 4.7 pg/mL for control group (Fig. 7 E).

Discussion

Due to the lack of scientific data in literature concerning biological effects of mixtures containing NPs and other environmental pollutants, leading *in vitro* and *in vivo* studies is a major challenge to improve knowledge on toxicological impact on human health. Therefore, our research focused on exposure to mixture aerosols containing hydrogen chloride gas (HCl_g) and alumina nanoparticles (Al_2O_3 NPs). These two pollutants of interest are especially retrieved in high concentrations after solid composite propellants use in aerospace and defense fields. Inhalation is the main route of exposure to these combustion aerosols, and little is known about combined effects of HCl_g and Al_2O_3 NPs on the respiratory tract. Consequently, our objective was to assess early pulmonary effects of model aerosols mimicking for instance inhalation exposure to combustion aerosols from solid composite propellants on rats. To investigate toxicological effects, animals were nose-only exposed 4h (unique exposures ; UE) or 4h a day for 4 days (iterative exposures ; IE) to aerosols. Wistar rats were selected for this project as they are often used in inhalation studies [34] and recommended by different OECD guidelines [35, 36, 37] for pulmonary

toxicity assessment. Nose-only inhalation was used to insure better control of the inhaled dose. To assess pro-inflammatory response and oxidative stress induction, bronchoalveolar lavage fluids (BALF) content and lungs histopathology were analyzed 24h after exposures.

Specific physico-chemical characterization is a mandatory step to better understand the biological effects of mixture aerosols. Their characterization revealed the presence of spherical Al_2O_3 NPs agglomerates with sizes smaller than 250 nm. Al_2O_3 NPs concentrations in aerosols were high and comprised between 20.0 mg/m^3 and 22.1 mg/m^3 . Indeed, Paur and colleagues reported in their literature review that 5 mg/m^3 could be considered as an upper limit for workplace NPs mass concentration, according to Occupational Safety and Health Administration (OSHA) standard for respirable nuisance dust [38]. Based on this value, they estimated a mass of NPs per surface area deposition in human lungs of $5 \times 10^{-3} \text{ } \mu\text{g/cm}^2/\text{h}$, which would correspond in our exposure scenarios to theoretical particles deposition in human lungs of $0.02 \text{ } \mu\text{g/cm}^2$ after UE and of $0.08 \text{ } \mu\text{g/cm}^2$ after IE. Considering alveolar surface in rats of 4000 cm^2 , exposures to NPs aerosols led to higher measured pulmonary depositions. Indeed, for Al_2O_3 NPs exposures values of $0.04 \text{ } \mu\text{g/cm}^2$ and $0.13 \text{ } \mu\text{g/cm}^2$ were respectively obtained for UE and IE, while concentrations for co-exposures with HCl_g 5 ppm were respectively $0.03 \text{ } \mu\text{g/cm}^2$ and $0.18 \text{ } \mu\text{g/cm}^2$. Therefore, these experimental values are higher than theoretical values but consistent with aerosols high NPs concentrations. Even if we observed high NPs pulmonary depositions, these scenarios are relevant in a context of accidental exposures where specific workers (i.e. military personnel) could be exposed to combustion aerosols.

To quantify Al_2O_3 NPs biodistribution in lungs after exposures, aluminum concentrations were measured using ICP-OES. Obtained values were approximately four times higher in lungs after IE compared to UE. These results are consistent with exposure scenarios as IE correspond to four repetitions of UE. NPs accumulated in lungs after exposures and were still retained by lungs after 96 hours in the case of IE, suggesting absence or slow clearance mechanisms after Al_2O_3 NPs aerosols inhalation. This result corroborates with a study carried out on Sprague-Dawley rats by intratracheal instillations, which already concluded to a very slow clearance, gradual accumulation and pulmonary retention of alumina particles [39]. Moreover, aluminum concentration was significantly increased in case of co-exposure to Al_2O_3 NPs with HCl_g compared to Al_2O_3 NPs aerosols, while NPs concentration and size distribution were not modified. Therefore, we hypothesized that acidification of mixture aerosols may increase pulmonary deposition.

Pro-inflammatory properties of Al_2O_3 particles have already been reported in literature following inhalation [10, 11, 40]. To date and to our knowledge, no literature data were found concerning the potential inflammatory response induced by HCl_g alone or Al_2O_3 NPs / HCl_g inhalation exposures. Therefore, we studied pro-inflammatory biomarkers following Al_2O_3 NPs, HCl_g or Al_2O_3 NPs / HCl_g mixtures nose-only exposures. Different secretion profiles were observed for each cytokine, depending on aerosol composition and exposure scenario. No increase of immune cells or cytokines concentrations was measured in animals BALF exposed to air or HCl_g . Our results showed early inflammation triggered

by exposures to aerosols containing Al_2O_3 NPs. Significant amount of alveolar macrophages and PMN were measured in BALF only after IE with increased secretion of IL-1 β , TNF- α and GRO/KC. However, iterative co-exposures to Al_2O_3 NPs / HCl_g only induced PMN influx and secretion of IL-1 β and TNF- α . These results corroborate with histopathological observations with mainly interstitial and inflammatory lesions (localized areas composed of PMN and lymphocytes) after Al_2O_3 NPs inhalation, while Al_2O_3 NPs / HCl_g mixtures induced vascular and interstitial lesions. Therefore, IE induced more potent pro-inflammatory response compared to UE but different immune cells and cytokine profiles were observed after Al_2O_3 NPs and mixtures exposures. Increased cytokine concentrations observed in BALF corroborate with PMN influx as they have chemoattractant properties [10, 41, 42]. IL-1 β and TNF- α seem to orchestrate the pulmonary pro-inflammatory response. On the one hand, it has been described that Al_2O_3 NPs could stimulate the nuclear factor NF κ B pathway [43]. IL-1 β concentrations increases could thus be associated with this hypothesis, suggesting the inflammasome pathway involvement in the pulmonary pro-inflammatory response initiated by Al_2O_3 NPs. Indeed, the nuclear factor NF κ B can contribute to the inflammasome regulation [44] and together they can lead to IL-1 β synthesis [45]. This conjecture is reinforced by the fact that this pathway is also known to be activated by pulmonary inflammation in patients with chronic obstructive pulmonary disease [46]. On the other hand, Al_2O_3 NPs can also induce TNF- α release in BALF. This result corroborate with the study of Kim et al. which showed neutrophils, LDH, TNF- α and IL-6 increased concentrations in BALF after Al_2O_3 NPs nose-only exposure of rats for 28 days (5 days/week) [10]. TNF- α secretion after exposure to Al_2O_3 NPs may be linked to IL-1 β release. Indeed, in a context of acute pulmonary inflammation, Saperstein and colleagues demonstrated that IL-1 β contributes, in part, to TNF- α mediated chemokine release, and neutrophil recruitment to the lung, potentially associated with altered soluble TNFR1 release into the BALF [47]. Besides, in our study GRO/KC and MIP-2 secretion in BALF were only measured after Al_2O_3 NPs exposure. We hypothesized that co-exposure to Al_2O_3 NPs / HCl_g mixtures could modify kinetic of these cytokines secretions in BALF. Significant increase of MIP-2 concentrations was found in BALF only after UE to Al_2O_3 NPs. As a consequence, MIP-2 may act as an early step contributing to PMN, macrophages and lymphocytes recruitment. Pirela and colleagues also demonstrated down-regulation of GRO/KC and MIP-2 in the nasal lavage fluid after iterative whole-body inhalation exposure to printer-emitted engineered NPs in rats (containing aluminum oxide) [48]. Otherwise, IL-6 and INF- γ were not detected in BALF after UE and IE to the different aerosols of the study (respective detection limits: 13 pg/mL and 6.2 pg/mL). For IL-6 secretion, this result is contradictory with the study of Kim and colleagues as they showed that IL-6 was significantly increased in BALF after iterative nose-only inhalation (28 days; 5 days/week) of Al_2O_3 NPs by rats [10]. However, Adamcakova-Dodd and colleagues exposed male mice (C57Bl/6J) to aluminum oxide-based nanowhiskers 4h/day, 5 days/week for two or four weeks in a dynamic whole-body exposure chamber and did not found neutrophilic inflammation nor IL-6 secretion in BALF [49]. Moreover, IL-6 has been shown to be increased in some pathological conditions involving the respiratory system when there is sustainability of lesional processes [50]. INF- γ promotes pulmonary inflammation through oxidative stress induction [51] and could have been secreted earlier than 24h post-exposure to the aerosols, inducing nucleic acids oxidative damages (8-OHdG), which were still detected at that time. Therefore, we

hypothesized that aluminum oxide-based nano-objects are notably able to induce secretion of different cytokine secretion profiles in BALF depending of their shape, time of exposure and used inhalation system.

As oxidative stress is a physiological mechanism closely linked to pulmonary inflammation induced by NPs exposure, and indirectly to the increase of PMN number potentially releasing free radicals in the alveoli, this endpoint was investigated [52, 53, 54, 55]. Nucleic acids oxidation (8-OHdG were only detected *in situ* following UE to HCl_g, Al₂O₃ NPs or mixtures exposures while no increase of lipid peroxidation was detected in BALF (8-Isoprostane). Interestingly, preliminary results for DNA double-strand breaks immunofluorescent detection gave similar results: γ-H2AX was detected only after UE to the same aerosols. Absence of 8-OHdG following IE could be explained by lesions repairing systems induction after 24h which would limit their later appearance and persistence over time. The main mechanism implied in these oxidative damages repair is base excision repair [56], but other intracellular actors such as p53 protein [57] or PARP-1 enzyme [58] may also contribute to damages repair. As previously shown, NPs inhalation could also initiate increased production of enzymes such as endonuclease III homolog 1 (NTH1) or apurinic/apyrimidinic endonuclease 1 (APE1) [59], p53 [57] or poly [ADP-ribose] polymerase 1 (PARP-1) [58], involved in various repair mechanisms and consequently limit side effects on nucleic acids. Similar hypothesis could be made involving γ-H2AX itself as inductor of DNA repair mechanism. Indeed, γ-H2AX is DNA double-strand breaks marker, but it also initiates these specific lesions repair [60, 61, 62, 63]. Lack of lipid peroxidation with 8-isoprostane detection in BALF might be due to activation of the antioxidant response following exposures. Significant decreases of 8-isoprostane after IE to Al₂O₃ NPs or mixtures may also be due to changes of antioxidant balance. It would be interesting to perform glutathione peroxidase dosage in BALF in further studies to assess these antioxidant mechanisms [64].

As obtained results highlighted Al₂O₃ NPs lung retention after aerosols exposures, early pro-inflammatory and oxidative stress induction, the aerosols effect on alveolar-capillary barrier permeability was also studied. Significant elevations of LDH and total protein concentrations were measured in BALF only after IE to Al₂O₃ NPs and mixture aerosols containing HCl_g, indicating impairment of air-blood barrier permeability and cell cytolysis. These biological effects could be linked to pro-inflammatory mechanisms and PMN influx observed in lungs following IE to Al₂O₃ NPs alone or combined with HCl_g. Indeed, as previously demonstrated, PMN recruitment and pulmonary inflammation trigger alveolar-capillary barrier permeabilization [65, 66]. Air-blood barrier permeabilization could promote translocation of inhaled particles into bloodstream leading to potential systemic effects or local toxicity on other organs such as the brain, the liver, the spleen or kidneys. A study is currently performed on these organs to assess local histopathological effects induced by aerosols inhalation. Mainly vascular, interstitial and inflammatory lesions were rapidly observed after UE and IE to aerosols. Similar lesion scores were obtained after the two exposure scenarios and the addition of HCl_g to Al₂O₃ NPs aerosol did not worsen lesions, meaning that it is mainly the presence of Al₂O₃ NPs in aerosols, which induces early pulmonary histopathological modifications.

Conclusion

Consequently, we conclude that early pulmonary effects induced by Al_2O_3 NPs and/or HCl_g aerosols inhalation are inflammation, oxidative stress and alveolar-capillary barrier permeabilization. Obtained results underline that the exposure scenario mainly influence toxicological effects of mixtures. Al_2O_3 NPs presence in the aerosols also seem to play essential role driving toxicity. Indeed, despite an increased pulmonary aluminum biodistribution in case of IE to mixtures compared to Al_2O_3 NPs, similar pro-inflammatory responses were observed in the two conditions. UE induce oxidative stress resulting in nucleic oxidative damages, while IE essentially promote pulmonary inflammation. Special attention should be paid to pulmonary pro-inflammatory and oxidative stress induction as these phenomena are known to be closely linked to long-term pathologies such as pulmonary fibrosis or cancers [67, 68, 69]. Therefore, this study raises concerns towards potential long-term pulmonary toxicity of combustion aerosols, and emphasizes necessity to precise health hazard resulting from these aerosols exposures.

Methods

Test materials

Alumina nanoparticles (Al_2O_3 NPs) were provided by Sigma-Aldrich (718475) with the following physico-chemical specifications: primary size 13 nm, crystalline phase gamma/delta (γ/δ), specific surface [85-115 m^2/g], pH [4.5-5.5] and purity 99.8%. Hydrogen chloride gas (HCl_g) was provided by Air Liquide (0023159805-000010; “Saphir” quality). Initial HCl_g concentration is 500 ppm in bottles.

Animals

All *in vivo* experiments were approved by the “Comité Régional d’Ethique en Matière d’Expérimentation Animale” (CREMEAP) (C2EA-96). Healthy seven-week-old male rats (WISTAR RjHan:WI, Janvier Labs ; 250g) were housed in polycarbonate cages, in a temperature (20-24°C) and relative humidity (40-70%) controlled room, and had free access to food and water *ad libitum*. A 12h light – 12h dark cycle was respected. Animals (2-3 rats / cage) were acclimatized for at least 5 days before experiments.

Animal exposure

Two exposure scenarios were tested in this study. Unique exposures (UE) of 4h and iterative exposures (IE) of 4h a day for 4 days were performed. The 96 animals were distributed in 8 groups of 12 rats (6 rats for biochemistry analysis, 3 rats for histopathology analysis and 3 rats for aluminum quantification in lungs among each group) respectively exposed to air, HCl_g , Al_2O_3 NPs or Al_2O_3 NPs/ HCl_g for each exposure scenario. They were exposed in a nose-only inhalation tower (randomized placement on the inhalation tower) to nebulized Al_2O_3 NPs suspensions in ultrapure water (10 g/L) using a PALAS AGK 2000E (Palas) and/or to 5 ppm of HCl_g . Exposure flow was controlled using mass flow meters (TYLAN). Both compounds were mixed (Al_2O_3 NPs/ HCl_g exposures) prior entering in the inhalation tower at a 20

L/min flow rate. Samples were taken on bubblers or cellulose filters using mass flow meters to allow aerosols characterization. A neutralization system (bubblers with caustic soda and desiccant) was placed at the exposure device exit to eliminate generated aerosols. Rats were euthanized by isoflurane inhalation 24h after the last exposure and biological samples were collected for further analysis.

Monitoring and characterization of aerosols

Al₂O₃ NPs aggregates size, form and chemical composition were studied. Transmission electron microscopy (TEM) imaging on Al₂O₃ NPs bulk powder was performed using electron microscope JEOL JEM 1400 operated at 120 kV equipped with an CCD camera (Gatan Orius 1000). Aerosols samples were collected on copper grids and TEM observations were performed using a CM12 80kV microscope (Philips) equipped with a Megaview 3 camera (EMSIS) and an EDX probe. Al₂O₃ NPs crystal structure and size of crystallites were determined by X-Ray powder diffraction (XRD) using a D4 ENDEAVOR X-ray Diffractometer (Bruker). XRD patterns were acquired with the use of Cu-K α radiation ($\lambda = 1.5406\text{\AA}$) from powders directly hand-pressed on sample holders. Sioutas (SKC Inc.) cascade impactor was used to precise NPs sizes distribution and concentrations in the aerosols. Stability of Al₂O₃ NPs concentrations during exposures was assessed using inductively coupled plasma optical emission spectrometry (ICP-OES) to quantify aluminum on cellulose filters after samples collection, as recommended by Afnor standard NF-EN-ISO-11885. Analysis were performed on ICP-OES 5110 (Agilent) with ICP Expert II 7.3 software (Agilent).

HCl_g content was assessed indirectly by chloride ions (Cl⁻) dosages on bubbler samples (free fraction) and on cellulose filters (particulate fraction) using ion exchange chromatography according to Afnor standard NF-EN-1911.

Aluminum quantification in lungs

Rats (n=3) were euthanized 24h after the last exposure and perfused with PBS to remove blood from lungs. Aluminum burden was then quantified using ICP-OES. Briefly, tissue samples were digested in a mixture of concentrated nitric acid (HNO₃; Analpure® Analytika 69% for UE and Normatom VWR 67-69% for IE) and hydrofluoric acid (HF; Analpure® Analytika 48% for UE and Normatom VWR 47-51% for IE) using a microwave oven (Mars Xpress CEM for UE and Ethos One Milestone for IE). After cooling to a room temperature, boric acid solution was added to neutralize digests (H₃BO₄; Ultra-pure Chem Lab 99.9% for UE and Suprapur® Merck 99.9999% for IE) and samples were diluted in ultrapure water (final volume of 50 mL). Aluminum quantification was then performed using ICP-OES 5110 (Agilent, for UE, LLOQ = 0.25 $\mu\text{g/g}$ of lung) or ICP-OES Optima 8300 (Perkin Emler, for IE, LLOQ = 1.25 $\mu\text{g/g}$ of lung). Software used to realize spectra analysis were ICP Expert II 7.3 (Agilent, for UE) or WinLab32 ICP v5.5 (Perkin Emler, for IE).

Clinical symptoms and body weight

Daily monitoring of clinical symptoms was performed throughout the experiment. Respiratory, behavioral and dermal visible changes were watched. Type, date of occurrence and symptoms severity were individually recorded. Rats were weighted at the arrival, before and after each 4h exposure and before euthanasia.

Bronchoalveolar lavage fluid (BALF) analysis

Rats (n=6) were euthanized 24h after the last exposure and bronchoalveolar lavages were performed with PBS. A first bronchoalveolar lavage was performed using 5 mL of PBS. Two other lavages were then performed with 10 mL of PBS to collect more cells. Collected BALF were centrifuged at 350 g (4°C) for 10 min.

Cell pellets of the three lavages were suspended in HBSS and immune cells were counted with Cellometer auto2000 (Ozyme, France) using propidium iodide and orange acridine. BALF cytospins were prepared by methanol fixation and May-Grünwald Giemsa staining (MGG) to allow immune cells differential count (macrophages, lymphocytes, polymorphonuclear neutrophils (PMN), polymorphonuclear eosinophils (PME) and polymorphonuclear basophils (PMB)).

Supernatants recovered from the first lavage were aliquoted and stored at -80°C until biochemical analysis. BALF lactate dehydrogenases (LDH) and total proteins contents were assessed using commercial kits (respectively 74048 and 74230, Siemens Healthineers). Absorbances were measured at respectively 340/410 nm for LDH and 596/694 nm for total proteins on ADVIA 1800 clinical chemistry automaton (Siemens Healthineers). BALF pro-inflammatory cytokines were quantified by ELISA assay. IL-1 β , TNF- α and IL-6 concentrations (respective LLOQ (Lower Limit of Quantification) = 6 pg/mL ; 16 pg/mL ; 13 pg/mL) were measured using Bio-Plex Pro™ Rat kit (64178837, Bio Rad). GRO/KC, INF- γ et MIP-2 concentrations (respective LLOQ¹ = 19.7 pg/mL ; 6.2 pg/mL ; 11.3 pg/mL) were measured using Milliplex MAP Rat kit (RECYTMAG-65K, Merck). Measurements were performed on Bio-Plex® MAGPIX™ System (Bio Rad). 8-isoprostane (8-iso PGF₂ α) levels in BALF were quantified using ELISA assay (ADI-900-010, Enzo, LLOQ¹ = 6.1 pg/mL). Measurements were performed on Multiskan Go plate reader (Thermo Scientific) at 405 nm.

Lungs histopathology

After euthanasia, rats (n=3) were perfused with PBS then with 4% formalin. Injections of 4% formalin were also performed directly inside the lungs to inflate them. After 48h incubation in 4% formalin, lungs were transferred to PBS and included in paraffin. 5 μ m slices were cut from paraffin blocks with Autocut 2045 (Leica) and stained with hematoxylin, phloxine and saffron (HPS staining). Lungs histopathology study was carried out on cranial lobes of right lungs. Samples were observed under DMI6000B light microscope (Leica) with a DFC 450 C color camera (Leica).

After staining, microscopy slides were sent to Novotec society who performed anatomo-histopathological reading and lesions scoring. Localization (alveolar, vascular or bronchial) and severity of pulmonary

lesions resulting from exposures was assessed (Table 3) and lesion scores were calculated individually. Lesion scores means for each experimental group are presented in arbitrary units (A.U.).

Table 3 – Novotec scoring and corresponding pulmonary lesions.

<i>Lesion score</i>	<i>Observed lesions</i>
<i>0</i>	<i>No lesion or physiological lesions</i>
<i>1</i>	<i>Vascular lesions</i>
<i>2</i>	<i>Limited to moderate vascular and alveolar lesions, with or without bronchial lesion</i>
<i>3</i>	<i>Significant vascular and alveolar lesions, with or without bronchial lesion</i>

8-OHdG immunofluorescence labeling in situ on rat lung

To perform lungs immunofluorescence labeling, 5 µm lungs slices were cut from paraffin blocks with Autocut 2045 (Leica). After toluene dewaxing, citrate buffer (pH 6 ; 10 mM ; C9999, Sigma-Aldrich) incubation enable antigen unmasking. To reduce lung tissue autofluorescence [70], samples were stained with Sudan black (0.3% ; 30 min ; 199664, Sigma-Aldrich). Cells were then permeabilized with Triton X100 (0.025% ; 10 min ; X100, Sigma-Aldrich) and aspecific sites saturation was performed using bovine serum albumin (BSA ; 6% ; 1h ; A2153, Sigma-Aldrich). Lung slices were then incubated with primary antibody overnight at 4°C (Table 4), before shorter incubation with secondary antibody (2h, Table 4). Finally, slides were mounted using Vectashield® Hardset containing DAPI (H-1500 ,Vector). Lung sections without primary antibody incubation were used as negative controls to ensure specificity of observed labeling.

Table 4 – Summary of antibodies used for immunofluorescence application

Primary antibody				Secondary antibody			
Epitope	Species	Supplier (Reference)	Dilution used	Epitope	Species	Supplier (Reference)	Dilution used
8-OHdG	Mouse	Abcam (ab48508)	1/400	Mouse IgG	Goat	Life Technologies (A11029)	1/500

Statistical analysis

Results are presented as means $\pm$ standard deviations (SD) for each experimental group. Statistical analysis was performed using GraphPad Prism 7 software. Within the same exposure scenario, comparison with control group (air) or other experimental groups means was performed using one-way ANOVA variance tests and Tukey's post-tests. To compare exposure scenarios for the same aerosol two-way ANOVA variance tests and Sidak's post-tests were implemented. Statistical analysis was performed using an α risk of 0.05 and p-value was used to assess differences significance. P-values were represented on figures using stars or circles (* or α p-value < 0.05, ** or $\alpha\alpha$ p-value < 0.01, *** or $\alpha\alpha\alpha$ p-value < 0.001, **** or $\alpha\alpha\alpha\alpha$ p-value < 0.0001).

Declarations

Ethics approval and consent to participate

In vivo experiments were approved by the "Comité Régional d'Ethique en Matière d'Expérimentation Animale" (CREMEAP) (C2EA-96).

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author's contribution

The manuscript was written by ABg, SDk and SF. ABg, SDk and SF were involved in the project conception, experiments performance, data analysis and results interpretation. DS performed aluminum quantification in lungs by ICP-OES and took part in lungs histopathology analysis. ABg, NG, SDa, SR and AF carried out histological preparations and immunostaining. ABg and NG also participate in the BALF cell counts. FF, CF and AS were involved in BALF LDH, total proteins and pro-inflammatory cytokines dosages. EP and GL performed animals nose-only exposures and aerosols monitoring and characterization. EF was involved in Al₂O₃ NPs crystal structure determination. All authors approved the final manuscript and contributed critical revisions to its intellectual content.

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Figures

Figure 1

Bulk powder and aerosols alumina nanoparticles physico-chemical characterization. Transmission electron microscopy (TEM) images of Al₂O₃ NPs in bulk powder (A) and generated aerosol (C) (respective scale bars = 200 nm and 500 nm). Crystalline phases (γ and δ) identification using X-ray diffraction (XRD) on bulk Al₂O₃ NPs (B). NPs elementary chemical composition (in %) using energy-dispersive X-ray (EDX) spectroscopy on Al₂O₃ NPs aggregates retrieved in the generated aerosol (D).

Table E summarizes Al2O3 NPs representative particles size distribution in the generated aerosols measuring using Sioutas cascade impactor (mg/m3 for each size class in µm).

Figure 2

Pulmonary aluminum deposition after nose-only exposures. Rats were exposed to air, HCl₉ 5 ppm, Al₂O₃ 13 nm or to the mixture Al₂O₃ 13 nm + HCl₉ 5 ppm. Clear blue points symbolize unique exposures (UE) and dark blue points symbolize iterative exposures (IE) to each aerosol. Aluminum quantities (µg/g of lung) were measured in lungs 24h after the last exposure using inductively coupled plasma optical emission spectrometry (ICP-OES). One-way ANOVA and Tukey's post-test (n=3, ** p-value < 0.01, **** p-value < 0.0001, α risk = 0.05) were performed to compare each experimental condition to the control within the same exposure scenario and Two-way ANOVA and Sidak's post-test (n=3, αααα p-value < 0.001, ααααα p-value < 0.0001, α risk = 0.05) were performed to compare the two exposure scenarios for the same aerosol.

Figure 3

Total proteins, LDH and immune cells populations in bronchoalveolar lavage fluids (BALF). Rats were exposed to air, HClg 5 ppm, Al₂O₃ 13 nm or to the mixture Al₂O₃ 13 nm + HClg 5 ppm. Clear blue points symbolize unique exposures (UE) and dark blue points symbolize iterative exposures (IE) to each aerosol. Total proteins (g/L) (A) and lactate dehydrogenases (LDH, U/L) (B) concentrations were measured in rats BALF 24h after the last exposure. Total cells, polymorphonuclear neutrophils (PMN), macrophages and

lymphocytes (cells x106) were counted on BALF cytopins (repectively C, D, E and F). One-way ANOVA and Tukey's post-test (n=3, * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001, α risk = 0.05) were performed to compare each experimental condition to the control within the same exposure scenario and Two-way ANOVA and Sidak's post-test (n=3, α p-value < 0.05, $\alpha\alpha$ p-value < 0.01, $\alpha\alpha\alpha$ p-value < 0.001, $\alpha\alpha\alpha\alpha$ p-value < 0.0001, α risk = 0.05) were performed to compare the two exposure scenarios for the same aerosol.

Figure 4

Aerosols pro-inflammatory potential on rat lungs after nose-only inhalations. Rats were exposed to air, HClg 5 ppm, Al₂O₃ 13 nm or to the mixture Al₂O₃ 13 nm + HClg 5 ppm. Clear blue points symbolize unique exposures (UE) and dark blue points symbolize iterative exposures (IE) to each aerosol. Pro-inflammatory cytokines concentrations (pg/mL) of TNF- α (A), IL-1 β (B), GRO/KC (C) and MIP-2 (D) were measured in rats BALF 24h after the last exposure by ELISA multiplex. One-way ANOVA and Tukey's post-test (n=3, * p-value < 0.05, ** p-value < 0.01, **** p-value < 0.0001, α risk = 0.05) were performed to compare each experimental condition to the control within the same exposure scenario and Two-way ANOVA and Sidak's post-test (n=3, **** p-value < 0.0001, α risk = 0.05) were performed to compare the two exposure scenarios for the same aerosol.

Figure 5

Pulmonary histopathological lesions observations following inhalation exposures. Rats lung parenchyma stained in HPS were observed in optical microscopy for histopathological analysis. Images show respectively lung parenchyma 24h after unique exposure (UE) to air (A), HCl_g 5 ppm (C), Al₂O₃ γ/δ-13 nm NPs (E) and Al₂O₃ γ/δ-13 nm NPs + HCl_g 5 ppm (G) and 24h after iterative exposures (IE) to air (B), HCl_g 5 ppm (D), Al₂O₃ γ/δ-13 nm NPs (F) and Al₂O₃ γ/δ-13 nm NPs + HCl_g 5 ppm (H). Scale bar = 200 μm.

Pictures legends: a = alveoli ; b = bronchioles or bronchi ; v = blood vessel ; c = vascular congestion ; blue arrows = vascular edema ; red arrows = bronchial (pre)-exfoliation ; i = inflammatory area ; black squares = velamentous epithelium and black circles = interalveolar septum edema.

Figure 6

Pulmonary histopathological lesions scoring. Rats were exposed to air, HClg 5 ppm, Al₂O₃ 13 nm or to the mixture Al₂O₃ 13 nm + HClg 5 ppm. Scoring was performed on HPS stained lungs slices of three rats of each group. Lesion scores (mean ± SD, n=3) were estimated for each experimental aerosol and exposure scenario based on the presence and the gravity of vascular, alveolar and bronchial lesions observed in rats lungs 24h after the last exposure. Empty and striped bars represent respectively unique exposures (UE) and iterative exposures (IE) results.

Figure 7

Oxidative stress induction by experimental aerosols inhalation exposures. Immunofluorescence was performed to detect 8-OHdG (8-hydroxy-2'-deoxyguanosine, green fluorescence) on rat lungs slices 24h after the last exposure. DNA was stained by DAPI, which emits in blue fluorescence. Pictures represent obtained results following unique exposures (UE) to air (A), HCl_g 5 ppm (B), Al₂O₃ γ/δ-13 nm NPs (C) and Al₂O₃ γ/δ-13 nm NPs + HCl_g 5 ppm (D). Scale bars = 50 μm. Pictures legends: a = alveoli ; b =

bronchioles or bronchi. Red blood cells could also emit in green fluorescence, but red arrows and red circles point out specific 8-OHdG staining. 8-Isoprostane concentrations (pg/mL) were measured in rats BALF 24h after the last exposure (E). Clear blue points symbolize unique exposures (UE) and dark blue points symbolize iterative exposures (IE) to each aerosol. One-way ANOVA and Tukey's post-test (n=3, * p-value < 0.05, ** p-value < 0.01, **** p-value < 0.0001, α risk = 0.05) were performed to compare each experimental condition to the control within the same exposure scenario and Two-way ANOVA and Sidak's post-test (n=3, α risk = 0.05) were performed to compare the two exposure scenarios for the same aerosol.