ATMOSPHERIC PARTICLE MATTER: PRO-INFLAMMATORY RESPONSE ON *IN VITRO* 3D NASAL EPITHELIUM

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INTRODUCTION

Particulate matter (PM) is a term used to describe the heterogeneous mixture of gases, liquids and solid particles of different origins and sizes. It is present as suspension in the air and is classified as coarse (2.5-10 µm aerodynamic diameter, PM10), fine (≤ 2.5 µm aerodynamic diameter, PM 2.5), and ultrafine (≤ 0.1 µm aerodynamic diameter) based on the size of the particles[1,2]. Large epidemiological studies suggest that fine and ultrafine PM are among the outdoor air pollutants that contribute to the onset of diseases, causing adverse effects especially those of the respiratory system. Exposure to PM 2.5 has been shown to be associated with increased risk of many human diseases, including chronic and allergic rhinitis[3].

The nasal epithelium is the first site of contact of the respiratory tract with the environment and provides significant protection to the lower respiratory tract by conditioning the inspired air[4]. It has been demonstrated that pollution weakens the first line of defense of upper airways, mainly impairing cilia structure and function, depending on contaminant concentration and duration of exposure[5]. Once these structural or clearance mechanisms fail, the epithelium itself plays a fundamental role in the activation of specific (formed by the antibodies -mainly secretory IgA and to a lesser extent IgG- and immunocompetent cells which modulate innate and adaptive responses) and non-specific (phagocytosis by cells such as neutrophils and macrophages) immune responses[6].

Compared to monolayers, 3D-reconstructed human tissues show higher biological relevance to human lung physiology and better represent the *in vivo* characteristics of the human respiratory tissues. Therefore, they offer reproducible and robust systems for studying the effects of respiratory toxicants[7,8]. Recently. FDA has mentioned that[9] human *in vitro* air-liquid-interface (ALI) airway epithelial tissue models represent a relevant tool to investigate the respiratory toxicity of inhaled substances, including tobacco smoke constituents, active ingredients, and environmental pollutants due to their realistic exposure conditions[10,11].

In this study, the effects of PM on nasal epithelium were evaluated on a commercially available 3D model of airway epithelium (Mucilair) which was produced with a pool of 14 donors (the research study was performed on 3 different batches of the same pool) to address the reproducibility issues. Interleukin-8 (IL-8) and chemokine ligand 20 (CCL-20) release have been used as inflammatory response readout parameters and human beta-defensin-2 (hBD-2) as a readout parameter for innate immunity and inflammation activation.

METHODS

Fine Atmospheric Particulate Matter characterization

A Standard Reference of fine atmospheric PM collected from an urban environment has been used (SRM-2786, Sigma-Aldrich). The particle size distribution has been determined by a laser diffractometer (Mastersizer 2000) and a small-volume sample dispersion unit (Hydro 2000 SM) through liquid suspension following manufacturer's instructions. A particle size <4 µm have been identified with a mean particle diameter of 2.8 µm.

Reconstructed human airway epithelium (MucilAir™)

MucilAir™ (Epithelix) is morphologically and functionally differentiated nasal epithelium generated from primary epithelial cells. Typical ultra-structures of the human airway epithelium, such as tight junctions, cilia, basal cells and mucous cells, are observed[12,13]. The same cells (hAEC/nasal cells derived from 14 donors of age <60 years) were used to produce MucilAir™ tissues with different cell culture ages (MP0008#2, 72 days; MP0008#3, 57 days; MP0008#4, 44 days). On each batch, one single experiment was performed using 6 biological replicates.

IL-8, CCL20 and hBD-2 release quantification

The culture media from the six tissues for each batch were used for biomarkers quantification. The quantification of the IL-8, CCL20 and hBD-2 release in the culture media have been performed on conditioned basolateral medium using ELISA. IL-8 has been quantified by Quantikine ELISA Human IL-8/CXCL8 Immunoassay kit (Bio-Techne, USA) with a sensitivity of 7.5pg/ml (detection range: 31.2-2000 pg/ml) according to manufacturer's instructions. CCL-20 has been quantified by Human CCL20/MIP-3 alpha ELISA kit (LSBIO) which quantifies proteins within the range of 16-1000 pg/ml according to manufacturer's instructions. hBD-2 has been quantified by Human DEFB4A/DEFB2 ELISA kit (LSBIO) which quantifies the protein within the range of 8-1000 pg/ml according to manufacturer's instructions. Optical densities of IL-8, CCL20 and hBD-2 were read at 450 nm with reference at 570nm using the Infinite M-200 spectrophotometer (Tecan, Austria).

Experimental design

MucilAirTM tissues were exposed to 30 µL of PM for 48h at a concentration of 300µg/mL in ultrapure water. This concentration was derived from the literature[3,14]. The results of preliminary internal studies performed on MucilAir tissues was accessed by quantification of adenylate kinase release after exposure to different amount of PM and for different exposure time (data not shown). Saline solution (30 µL, NaCl 0.9%) has been used as negative control. At the end of 48h, the supernatant was collected for IL-8, CCL-20 and hBD2 measurements. Results are expressed as compared to the negative control (NC). Statistical significance was measured by Student's t-test.

RESULTS

1. INTERLEUKIN-8 (IL-8)

As presented in **Figure 1**, significant increase in IL-8 release have been observed in all tested batches. The percentage increases of IL-8 release were 73% ($p<0.01$), 126% ($p<0.01$) and 296% ($p<0.01$) in batch MP0008#2, MP0008#3 and MP0008#4, respectively.

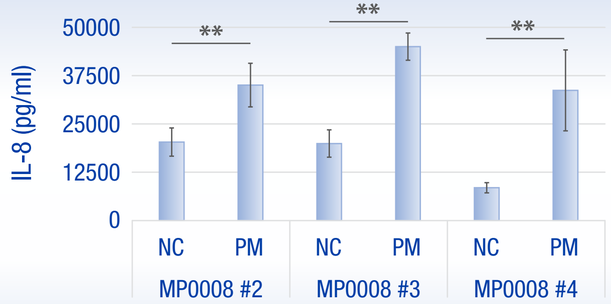

Figure 1. IL-8 release in medium after 48h of PM exposure. **p<0.01 PM vs NC. Statistical analysis by Student's t-test. PM: particulate matter; NC: negative control.

2. CHEMOKINE LIGAND-20 (CCL-20)

After 48h of PM exposure, significant increase in CCL-20 release have been observed in MP0008#2 and MP0008#3 (**Figure 2**). The percentage increases of CCL-20 release were 296% ($p<0.01$), 119% ($p<0.01$) and 3% in MP0008#2, MP0008#3 and MP0008#4, respectively.

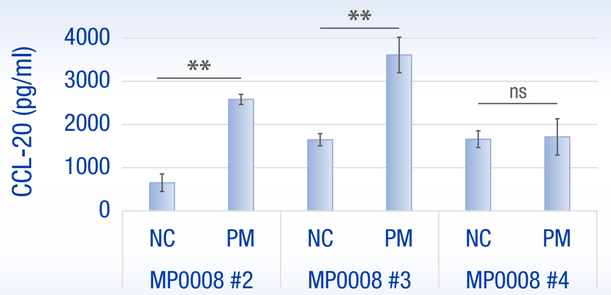

Figure 2. CCL20 release in medium after 48h of PM exposure. **p<0.01, PM vs NC. Statistical analysis by Student's t-test. PM: particulate matter; NC: negative control.

3. HUMAN BETA DEFENSIN 2 (hBD-2)

The results of hBD-2 release after 48h of PM exposure for the three tissue batches are reported in **Figure 3**. Significant increase of hBD-2 release has been observed for all three batches compared to the negative control. The percentage increases of hBD-2 release were 92% ($p<0.01$), 60% ($p<0.05$) and 124% ($p<0.01$) in MP0008#2, MP0008#3 and MP0008#4, respectively.

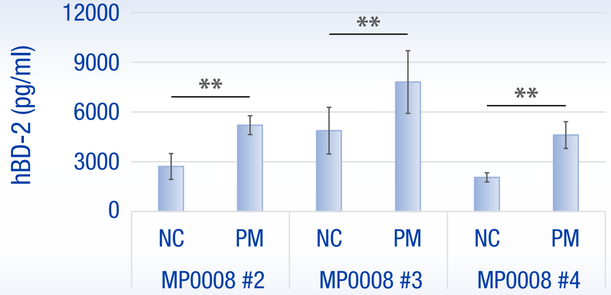

Figure 3. hBD-2 release in medium after 48h of PM exposure. **p<0.01, PM vs NC. Statistical analysis by Student's t-test. PM: particulate matter; NC: negative control.

CONCLUSIONS

The developed pre-clinical 'pollution model' recapitulates the environmental exposure to PM reproducing the inflammatory status known to be associated to PM exposure. A Standard Reference Material (SRM 2786) of PM2.5 (particle size <4 µm) was used to investigate the interaction between PM and the upper respiratory airways. PM used in this study contains trace amounts of polycyclic aromatic hydrocarbons (PAH), nitro-substituted PAHs (nitro PAH), polybrominated diphenyl ether (PBDE) congeners, hexabromocyclododecane (HBCD) isomers, polychlorinated dibenzo-p-dioxin (PCDD) and polychlorinated dibenzofuran (PCDF) congeners as characterized by a combinations of different extraction techniques, cleanup/isolation procedures, and chromatographic separation and detection techniques.

As a conclusion, the reproducible biological response recorded for IL-8 and hBD-2 and partially for CCL-20 using 3 different batches of reconstructed human nasal epithelium confirms the biological impact of PM on upper airways and further supports the importance of nasal epithelium as sentinel safeguarding from environmental and biological pollutants. Furthermore, **these results make this method a good candidate tool to consistently study the impact of pollution on the human nasal epithelium. Last but not least, the proposed approach is in compliance with the principle of the 3Rs (Reduction, Refinement and Replacement of animal experiments) as stated in Directive 2010/63/EU on the protection of animals used for scientific purposes.**

BIBLIOGRAPHY

[1] Nemmar A, Holme JA, Rosas I, et al. Biomed Res Int. 2013 Oct;2013
[2] Baldacci S, Maio S, Cerrai S, et al. Respir Med. 2015 Sep 1;109(9):1089-104
[3] Hong Z, Guo Z, Zhang R, et al. Tohoku J Exp Med. 2016;239(2):117-25
[4] Calderón-Garcidueñas L, Rodríguez-Alcaraz A, Villarreal-Calderón A, et al. Toxicol Sci. 1998 Dec 1;46(2):352-64
[5] Cho DY, Le W, Bravo DT, et al. In Int Forum Allergy Rhinol 2014 Dec (Vol. 4, No. 12, pp. 966-971)
[6] Kern RC and Decker JR. In Nasal Physiol and Pathophysiol of Nasal Dis 2013 (pp. 27-45). Springer, Berlin, Heidelberg
[7] Bhowmick R, Derakhshan T, Liang Y, et al. Tissue Eng Part A. 2018 Oct 1;24(19-20):1468-80
[8] Kabadi PK, Rodd AL, Simmons AE, et al. Part Fibre Toxicol. 2019 Dec;16(1):1-5
[9] U.S. Food and Drug Administration. Advancing New Alternative Methodol at FDA. 2021
[10] Cao X, Wang Y, Xiong R, et al. Toxicol. 2018 Nov 1;409:119-28
[11] Wang Y, Wu Q, Muskhelishvili L, et al. Toxicol In Vitro. 2019 Sep 1;59:78-86
[12] Baxter A, Thain S, Banerjee A, et al. Toxicol in Vitro. 2015 Aug 1;29(5):864-75
[13] Mercier C, Jacqueroux E, He Z, et al. Eur J Pharm Biopharm. 2019 Jun 1;139:186-96
[14] Hiraiwa K and van Eeden SF. Mediators Inflamm. 2013 Oct;2013

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