EUROPEAN RESPIRATORY *journal*

FLAGSHIP SCIENTIFIC JOURNAL OF ERS

Early View

Research letter

Ozone exposure leads to changes in airway permeability, microbiota and metabolome: a randomised, double-blind, crossover trial

Yue Niu, Renjie Chen, Cuiping Wang, Weidong Wang, Jing Jiang, Weidong Wu, Jing Cai, Zhuohui Zhao, Xiaohui Xu, Haidong Kan

Please cite this article as: Niu Y, Chen R, Wang C, *et al*. Ozone exposure leads to changes in airway permeability, microbiota and metabolome: a randomised, double-blind, crossover trial. *Eur Respir J* 2020; in press (https://doi.org/10.1183/13993003.00165-2020).

This manuscript has recently been accepted for publication in the *European Respiratory Journal*. It is published here in its accepted form prior to copyediting and typesetting by our production team. After these production processes are complete and the authors have approved the resulting proofs, the article will move to the latest issue of the ERJ online.

Ozone exposure leads to changes in airway permeability, microbiota and metabolome: a randomised, double-blind, crossover trial

Yue Niu [1,†], Renjie Chen [1,†], Cuiping Wang[1], Weidong Wang[1], Jing Jiang[2], Weidong Wu[2], Jing Cai[1], Zhuohui Zhao[1], Xiaohui Xu[3], Haidong Kan[1,4,*]

[†] These authors contributed equally to this work.

[1] School of Public Health, Key Lab of Public Health Safety of the Ministry of Education and NHC Key Lab of Health Technology Assessment, Fudan University, Shanghai 200032, China;

[2] Department of Occupational and Environmental Health, School of Public Health, Xinxiang Medical University, Henan 453003, China;

[3] Department of Epidemiology & Biostatistics, Texas A&M School of Public Health, College Station, Texas 77843, United States;

[4] Children's Hospital of Fudan University, National Center for Children's Health, Shanghai 201102, China.

***Correspondence and requests for reprints should be addressed to** Dr. Haidong Kan, P.O. Box 249, 130 Dong-An Road, Shanghai 200032, China. Tel/fax: +86 (21) 5423 7908. E-mail: kanh@fudan.edu.cn.

Take-home message:

Ozone inhalation could lead to depleted diversity of respiratory bacterial community, imbalanced proportion between commensal and pathogenic bacteria, and elevated levels of glucose and its metabolites in respiratory tract.

To the Editor:

The associations between atmospheric ozone pollution and increased risks of respiratory diseases have been well established [1, 2], but the underlying biological mechanisms are yet fully ascertained. Lung epithelial cells may be injured by inhaled ozone, but the results were not fully consistent [3, 4]. Furthermore, respiratory microbiota and metabolic homeostasis were deemed as key factors in maintaining human respiratory health, and any disturbances in this balance have the potential to increase the susceptibility to respiratory infectious diseases [5]. However, few human studies have investigated the potential effects of ozone inhalation on respiratory microbiota and metabolome.

To address these gaps, we conducted a randomised, double-blind, crossover, controlled exposure trial in 30 healthy young adults to explore the respiratory effects of short-term ozone exposure. The sample size was calculated using an equivalence test, and was comparable to that in previous controlled exposure studies [4, 6-8]. Each subject was exposed sequentially to both filtered air and 200 ppb ozone for continuous 2 hours in random order. A washout period of at least 2 weeks was determined according to previous controlled exposure trials [8, 9]. Lung function was measured according to the recommended methods [10]. As a well-established biomarker of lung epithelial injury, serum Clara cell protein (CC16) was measured using enzyme-linked immunosorbent assays. Respiratory microbiota was analyzed with the nasal secretion using 16S rRNA amplicon sequencing. Airway metabolome was analyzed with the exhaled breath condensate (EBC) using gas

chromatography-time of flight-mass spectrometry (GC-TOF-MS).

We applied linear mixed-effect (LME) models to estimate the acute effects of ozone exposure on lung function and serum CC16. For microbiota analysis, we firstly evaluated alpha-diversity by calculating abundance-based coverage estimator (ACE), Simpson, and Shannon; then, we assessed beta-diversity by calculating weighted Unifrac distance and visualizing it via principal coordinate analysis; lastly, we identified taxa characterizing the differences between ozone and filtered air groups using a linear discriminant effect size analysis, and a taxon with a linear discriminant analysis score > 4 was considered significantly different between two groups. For metabolomics analyses, we firstly conducted an orthogonal partial least squares discriminant analysis and used the variance importance in the projection (VIP) scores to define contributions of each metabolite to the overall between-group difference; then, for metabolites with a VIP score > 1, we used LME models to evaluate the differences between groups. All tests were two-sided and a p-value < 0.05 was considered statistically significant. The study protocol was registered at ClinicalTrials.gov (NCT03697174), and all participants provided written informed consent at enrollment.

Ozone concentrations in chamber were very close to the target values with an average of 201.0 ± 1.6 ppb in the ozone group and 8.0 ± 2.6 ppb in the filtered air group. The levels of fine particulate matter, nitrogen dioxide, temperature and relative humidity were quite similar between the two groups. Relative to the filtered air group, exposure to ozone resulted in significant declines in lung function at lag 2 h, and the

decrements became more prominent at lag 15 h in the next morning. At lag 15 h, there were decrements of 3.70% [95% confidence interval (CI): 0.58%, 6.82%] in forced vital capacity and 3.14% (95%CI: 0.02%, 6.30%) in forced expiratory volume in 1 second. The findings were consistent with previous chamber studies that revealed delayed impairment of lung function after acute ozone exposure [6, 7].

Impaired lung function was always accompanied by increased epithelial permeability, which can be indicated by elevated serum levels of CC16 [4]. Similarly, we found serum CC16 increased by 41.93 % (95% CI: 31.96%, 51.89%) 2 hours after exposure to ozone compared with filtered air, and then the effect attenuated considerably but remained significant at lag 15 h. Elevated serum CC16 levels at lag 2 h was significantly correlated with decrements in both FVC and FEV_1 at lag 15 h, suggesting a potential temporal pattern — from ozone exposure, increased epithelial permeability to impaired lung function.

Notably, we presented, to our knowledge for the first time, the human-based evidence that short-term ozone exposure could significantly decrease the diversity of nasal bacterial community. As shown in Figure 1a, ACE, Simpson and Shannon were significantly lower in the ozone group than in the filtered air group, suggesting distinct reductions in both nasal bacterial community richness and evenness after ozone inhalation. Meanwhile, we observed a clear separation between the ozone groups and the filtered air group in the Figure 1b ($p = 0.007$), indicating an evident difference in nasal bacterial community between groups. Some *in vitro* studies also demonstrated the anti-microbial potential of ozone [11]. Diminished diversity of bacterial community

in upper respiratory tract have been associated with several respiratory infectious diseases in clinical studies [12, 13]. In addition, we found the composition of the nasal bacterial community was altered by ozone inhalation. Specifically, we observed significant decrements in the relative abundance of 2 phyla *Actinobacteria* and *Firmicutes* (see Figure 1c), also known as harmless commensal bacteria in nasal capacity. The deceases in the two commensal bacteria may result in diminished resistance against the colonization of foreign pathogens, and thus increase risks of bacterial or viral infection in the respiratory system [5]. In contrast, at the family level, we observed an relative enrichment of *Moraxellaceae* and *Pseudomonadaceae* following ozone exposure (see Figure 1c), which were often involved in the development of pneumonia and other infectious diseases [12]. Therefore, it is reasonable to assume that the imbalance between commensal and pathogenic bacteria in upper respiratory tract induced by ozone exposure may lead to individual vulnerability to respiratory infection.

The colonization and proliferation of bacteria in the respiratory tract may also be influenced by some substances in airway surface liquid. In this metabolomic analysis with EBC, we found that glucose was significantly elevated after an inhalation of ozone relative to filtered air with a fold change (FC) of 1.59, accompanied by significantly increased D-glyceric acid (FC=1.27) and lactic acid (FC=1.15). The alteration of airway glucose metabolism by ozone was in accordance with a previous study [8]. Increased glucose in EBC possibly resulted from an enhanced leakage of glucose from the blood into the airway through impaired lung epithelial tight junctions,

which could be supported by an elevation of serum CC16. Higher airway glucose may potentially enhance the proliferation of some specific bacteria that take glucose as carbon source by providing nutrient [14, 15]. This kind of imbalanced overgrowth may lead to perturbation in bacterial community composition, and ultimately increase the risk of respiratory infection.

This randomised, double-blind, crossover trial has the advantage of allowing for causal inference on the associations between ozone and adverse respiratory outcomes. However, this study has several limitations. First, the sample size is relatively small, adding statistical uncertainty to our results. Second, we only enrolled healthy college students as the subjects to better control behavior risk factors, but this strategy might restrict the generalizability of our findings to vulnerable populations. Third, some differential metabolites in EBC might have been missed because we only perform GC-TOF-MS analyses due to the limited amount of EBC. Fourth, the microbiota and metabolome were analysed with samples collected immediately after exposure, which did not allow for an exploration of temporal relationships.

To conclude, this randomised, double-blind, crossover, controlled exposure trial revealed that an acute inhalation of ozone could impair lung function and increase airway permeability. Our results further provided a novel mechanistic insight that ozone inhalation may increase susceptibility to respiratory infection through disturbing microbiota and glucose homeostasis in the respiratory tract.

Acknowledgements: The study was supported by the National Natural Science Foundation of China (Grant IDs: 91843302, 91643205 and 91743111).

References:

1. Medina-Ramon M, Zanobetti A, Schwartz J. The effect of ozone and PM10 on hospital admissions for pneumonia and chronic obstructive pulmonary disease: a national multicity study. *Am J Epidemiol* 2006: 163(6): 579-588.

2. Malig BJ, Pearson DL, Chang YB, Broadwin R, Basu R, Green RS, Ostro B. A Time-Stratified Case-Crossover Study of Ambient Ozone Exposure and Emergency Department Visits for Specific Respiratory Diagnoses in California (2005-2008). *Environ Health Perspect* 2016: 124(6): 745-753.

3. Lagerkvist BJ, Bernard A, Blomberg A, Bergstrom E, Forsberg B, Holmstrom K, Karp K, Lundstrom NG, Segerstedt B, Svensson M, Nordberg G. Pulmonary epithelial integrity in children: relationship to ambient ozone exposure and swimming pool attendance. *Environ Health Perspect* 2004: 112(17): 1768-1771.

4. Blomberg A, Mudway I, Svensson M, Hagenbjork-Gustafsson A, Thomasson L, Helleday R, Dumont X, Forsberg B, Nordberg G, Bernard A. Clara cell protein as a biomarker for ozone-induced lung injury in humans. *Eur Respir J* 2003: 22(6): 883-888.

5. Man WH, de Steenhuijsen Piters WA, Bogaert D. The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat Rev Microbiol* 2017: 15(5): 259-270.

6. Adams WC. Comparison of Chamber and Face Mask 6.6-Hour Exposure to 0.08 ppm Ozone Via Square-Wave and Triangular Profiles on Pulmonary Responses. *Inhal Toxicol* 2003: 15(3): 265-281.

7. Adams WC. Comparison of chamber 6.6-h exposures to 0.04-0.08 PPM ozone via square-wave and triangular profiles on pulmonary responses. *Inhal Toxicol* 2006: 18(2): 127-136.

8. Cheng W, Duncan KE, Ghio AJ, Ward-Caviness C, Karoly ED, Diaz-Sanchez D, Conolly RB, Devlin RB. Changes in Metabolites Present in Lung-Lining Fluid Following Exposure of Humans to Ozone. *Toxicol Sci* 2018: 163(2): 430-439.

9. Arjomandi M, Balmes JR, Frampton MW, Bromberg P, Rich DQ, Stark P, Alexis NE, Costantini M, Hollenbeck-Pringle D, Dagincourt N, Hazucha MJ. Respiratory Responses to Ozone Exposure. MOSES (The Multicenter Ozone Study in Older Subjects). *Am J Respir Crit Care Med* 2018: 197(10): 1319-1327.

10. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, Jensen R, Johnson DC, MacIntyre N, McKay R, Navajas D, Pedersen OF, Pellegrino R, Viegi G, Wanger J, Force AET. Standardisation of spirometry. *Eur Respir J* 2005: 26(2): 319-338.

11. Mülle P, Guggenheim B, Schmidlin PR. Efficacy of gasiform ozone and photodynamic therapy on a multispecies oral biofilm in vitro. *Eur J Oral Sci* 2007: 115(1): 77-80.

12. Santee CA, Nagalingam NA, Faruqi AA, DeMuri GP, Gern JE, Wald ER, Lynch SV. Nasopharyngeal microbiota composition of children is related to the frequency of

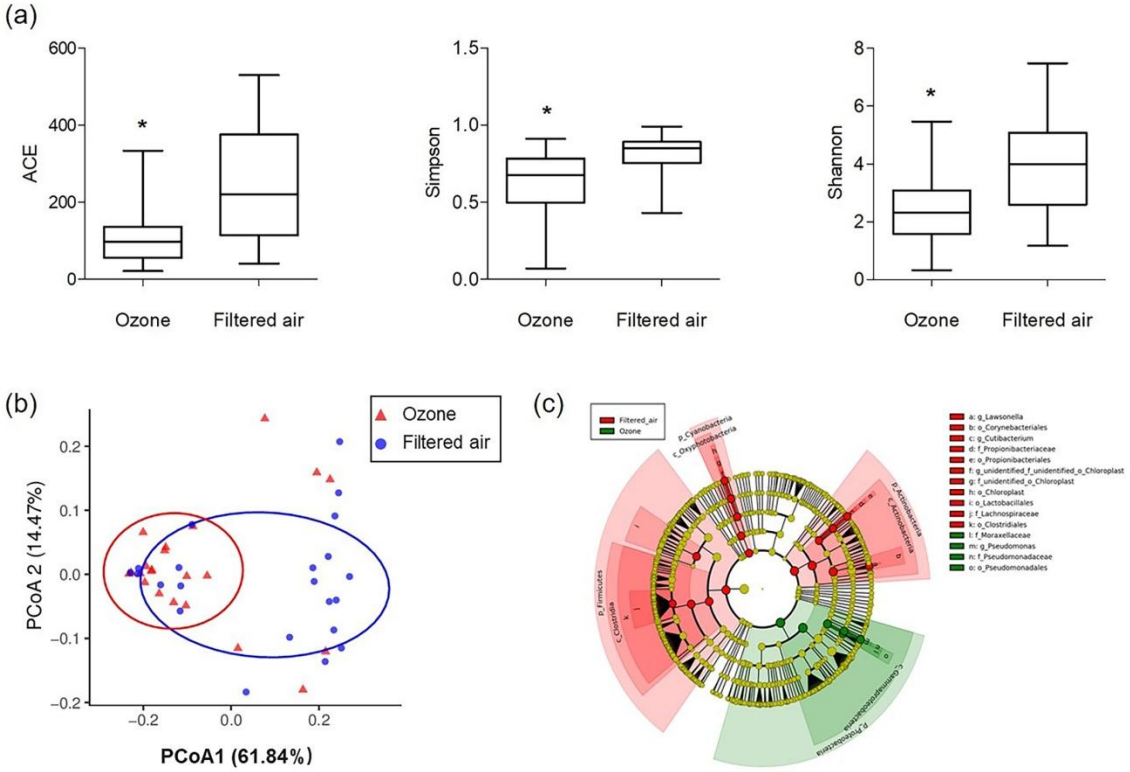
upper respiratory infection and acute sinusitis. *Microbiome* 2016: 4(1): 34.

13. Choi EB, Hong SW, Kim DK, Jeon SG, Kim KR, Cho SH, Gho YS, Jee YK, Kim YK. Decreased diversity of nasal microbiota and their secreted extracellular vesicles in patients with chronic rhinosinusitis based on a metagenomic analysis. *Allergy* 2014: 69(4): 517-526.

14. Mallia P, Webber J, Gill SK, Trujillo-Torralbo MB, Calderazzo MA, Finney L, Bakhsoliani E, Farne H, Singanayagam A, Footitt J, Hewitt R, Kebadze T, Aniscenko J, Padmanaban V, Molyneaux PL, Adcock IM, Barnes PJ, Ito K, Elkin SL, Kon OM, Cookson WO, Moffat MF, Johnston SL, Tregoning JS. Role of airway glucose in bacterial infections in patients with chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 2018: 142(3): 815-823 e816.

15. Baker EH, Baines DL. Airway Glucose Homeostasis: A New Target in the Prevention and Treatment of Pulmonary Infection. *Chest* 2018: 153(2): 507-514.

Figure 1 Changes in the diversity and composition of nasal bacterial community comparing the ozone group to the filtered air group. (a) Alpha-diversity metrics of nasal bacterial community in the two groups, including ACE, Simpson, and Shannon. *$p < 0.05$ versus filtered air group. (b) Beta-diversity of nasal bacterial community in the two groups. The cycles refer to the 95% confidence ellipses. (c) The cladogram of nasal bacterial community from a linear discriminant effect size analysis. The red area represents significantly enriched taxa in the filtered air group; the green area represents significantly enriched taxa in the ozone group; and the yellow area represents no differences in taxa between the two groups. The central point represents the root of the tree (bacteria), and each ring represents the next lower taxonomic level (from phylum to genus). The diameter of each circle represents the relative abundance of the taxon. Abbreviations: ACE, abundance-based coverage estimator; PCoA, principal coordinate analysis.


(a)
ACE
600
400
200
0
*
Ozone
Filtered air
Simpson
1.5
1.0
0.5
0.0
*
Ozone
Filtered air
Shannon
8
6
4
2
0
*
Ozone
Filtered air
(b)
Ozone
Filtered air
PCoA 2 (14.47%)
0.2
0.1
0.0
−0.1
−0.2
−0.2
0.0
0.2
PCoA1 (61.84%)
(c)
Filtered_air
Ozone
a: g_Lawsonella
b: o_Corynebacteriales
c: g_Cutibacterium
d: f_Propionibacteriaceae
e: o_Propionibacteriales
f: g_unidentified_f_unidentified_o_Chloroplast
g: f_unidentified_o_Chloroplast
h: o_Chloroplast
i: o_Lactobacillales
j: f_Lachnospiraceae
k: o_Clostridiales
l: f_Moraxellaceae
m: g_Pseudomonas
n: f_Pseudomonadaceae
o: o_Pseudomonadales