Published in final edited form as:

Exp Lung Res. 2018 ; 44(4-5): 252–261. doi:10.1080/01902148.2018.1517837.

Repeated pulmonary exposures to zinc ions enhance inflammatory responses to subsequent metal exposures

Christa Watson-Wright, Priscila Queiroz, Sylvia Rodrigues, Thomas C. Donaghey, Joseph D. Brain, and Ramon M. Molina*

Molecular and Integrative Physiological Sciences Program, Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA

Abstract

Aim of study: Metal contaminants contribute to adverse human health effects via acute and chronic exposures. Acute metal exposures followed by prolonged secondary metal exposures may elicit exaggerated inflammatory responses in certain individuals. The aim of this study is to determine whether repeated pulmonary exposures to zinc chloride (ZnCl₂) alters subsequent responses to zinc or cerium exposures.

Materials and Methods: Rats were intratracheally (IT) instilled with physiologic saline (n=24) or 0.05 mg/kg ZnCl₂ (n=16) twice weekly for 4 weeks. Four days after last dosing, the saline group was divided into 3 subgroups, each IT-instilled with either saline, ZnCl₂ or CeCl₃ (both at 0.1 mg/kg). The ZnCl₂ pre-instilled rats were divided into 2 subgroups, each instilled with 0.1 mg/kg ZnCl₂ or CeCl₃. Biomarkers of lung injury/inflammation were assessed in bronchoalveolar lavage (BAL) fluid collected 24 hours later. Oxidative stress was evaluated as total and reduced glutathione in BAL.

Results: Increases in inflammatory cells, LDH, albumin, leptin, MCP-1, IP-10, fractalkine, TNF α and RANTES were observed in rats instilled with multiple PBS and then with 0.1 mg/kg ZnCl₂ and CeCl₃. However, rats pre-exposed repeatedly to 0.05 mg/kg ZnCl₂ and then challenged with 0.1 mg/kg ZnCl₂ or CeCl₃ showed even more eosinophils, lymphocytes, and increased concentrations of hemoglobin and MIP-1 α . Significant reduction in GSH/GSSG ratios in BAL in response to all ZnCl₂ or CeCl₃ exposures indicated oxidative stress.

Conclusion: Previous exposure to zinc ions increases responsiveness to subsequent exposures to zinc and cerium ions. These findings suggest enhanced sensitization possibly due to a reduction in antioxidant defenses.

Keywords

metals; metal ions; sensitization; oxidative stress; reactive oxygen species; zinc; cerium

*Corresponding author: Ramon M. Molina, Molecular and Integrative Physiological Sciences Program, Department of Environmental Health, Harvard T. H. Chan School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA. Phone: 1-617-432-2311, Fax: 1-617-432-4710, rmolina@hsph.harvard.edu.

Financial Disclosures: The authors report no conflict of interest.

Introduction

Due to increasing technological advances, we are intentionally and/or unintentionally exposed to various chemicals on a regular basis. Such exposures may elicit allergic and non-allergic responses that are not fully understood. Current evidence suggests that certain individuals are more prone to developing intolerances to one or multiple chemicals causing multi-symptom organ and system manifestations or multiple chemical sensitivity (MCS) or toxicant-induced loss of tolerance (TILT) (1–6). Multiple chemical sensitivity is a clinical condition consisting of adverse health effects to low-dose exposures to common substances such as fragrances, household cleaners, organophosphate pesticides, fuels and metals (7, 8). MCS patients with pre-exposures to chemicals report similar patterns of multisystem symptoms and new-onset intolerances when exposed to chemicals again. These patients react to odor components especially at the brain cortical level (9). For example, an initial exposure to pesticides causes intolerance to smoke and fuels (10). Headaches, fatigue, and depression have been reported after exposures leading to reduced quality of life (10). Likewise, exposures to certain metals such as mercury have been associated with the diagnosis of MCS (8). These findings suggest that there can be environmental component to MCS where metal exposures play a critical role. Thus, understanding the causes and outcomes of primary and secondary metal exposures could alleviate symptoms and costs associated with diagnosing and treating MCS.

Although several clinical symptoms are often reported by MCS patients, defining pathologically relevant mechanisms of action and developing animal models to explore corresponding pathways of sensitization versus adaptation are major challenges. This is related to the complex relationships between chemical toxicants and inflammatory responses, which are mediated by several factors such as chemical properties, exposure parameters (i.e. route, duration, level of frequency and degree), and individual genetic susceptibility (11). Chemical allergens generally manifest helper T-cell type 2 (Th2-type) responses within the airways, which play a crucial role in respiratory sensitization development (11) and atopy (12). A variety of cytokines (IL-4, IL-5, IL-10, among others) associated with Th2 responses are produced by Th2-type cells along with generation of immunoglobulin E (IgE) antibody all of which have been implicated in a number of allergic diseases. Individuals suffering from MCS typically display significant cytokine dysregulation or enhanced nonspecific response to low-level irritants (13).

A number of host susceptibility factors such as metabolic variation (hereditary or acquired), abnormalities in hereditary oncogenes/tumor suppressor genes, immunogenic factors, neuroepithelial function, and nutritional status, have been identified as potentially influencing individual sensitivity to chemicals and MCS (14). In particular, differences in metabolic status or detoxification processes including xenobiotic pathways have been implicated in the occurrence of MCS after chemical exposures (15). With regard to metal exposures where elevated reactive oxygen species could be at play due to Fenton reactions, diminutions in glutathione and glutathione S-transferase have been noted (16, 17). Glutathione is a tripeptide antioxidant necessary for the deactivation of bio transformed xenobiotics and free metals due to its nucleophilic nature (18). The importance of glutathione has also been demonstrated in cases involving multiple chemical sensitivity,

where reduced levels of glutathione and other antioxidants in patients suffering from MCS were found (19). Moreover, glutathione depletion along with increased plasma inflammatory cytokines have been proposed as useful parameters in diagnosing MCS (20). Thus, defining the role of inflammation in MCS may contribute to a better understanding of this condition and may provide insights into potential treatments and/or prevention.

We seek to determine what distinguishes sensitization versus adaptation due to metal exposures via the respiratory tract. The aim of this study is to develop an animal model to investigate multiple-chemical sensitivity. For this purpose, we compared two groups of animals: one that was repeatedly exposed to ZnCl_2 for 4 weeks and another group that was not. We also exposed animals to a challenge dose of CeCl_3 to study the possibility of cross reactivity between zinc and cerium ions. The inflammatory response was assessed through the analysis of bronchoalveolar lavage fluid. Inflammatory cytokines and biomarkers of injury such as lactate dehydrogenase (LDH), myeloperoxidase (MPO), albumin and hemoglobin were determined. Levels of total and reduced glutathione were evaluated to examine the role of oxidative stress in chemical sensitivity.

Methods

ZnCl_2 Dose Range Exploration

All animal protocols used in this study were approved by the Harvard Medical Area Animal Care and Use Committee. To determine the relevant threshold limits to zinc chloride, 20 male 9-week old Wistar Han rats were randomly divided into 4 groups. The first group received phosphate-buffered physiological saline (PBS) as a control group. The other 3 groups received a single dose of ZnCl_2 (0.05 mg/kg, 0.1 mg/kg and 0.5 mg/kg). Each rat was weighed, anesthetized with 3-4 % vaporized isoflurane, and placed on a slanted board where it was supported by an elastic band under the upper incisors. The tracheal opening was exposed by depressing the tongue with a narrow spatula. Transillumination of the larynx was provided by a microscope lamp shining on the neck for better visualization. Saline or ZnCl_2 solution (1.5 ml/kg) was delivered to the lungs through the trachea with a sterile 22-gauge, 3-inch gavage needle with 1.25 mm diameter ball tip inserted between the vocal cords. The procedure takes 5-10 seconds. The chest was then massaged to facilitate spread of the instillate as well as to stimulate breathing during and after anesthesia. Each rat was then moved back to its cage to complete recovery from anesthesia (3-5 minutes).

Twenty-four hours later, the animals were anesthetized with 4.5% isoflurane followed by exsanguination via the abdominal aorta. The abdomen was opened, and the hemidiaphragm was cut to create a bilateral pneumothorax. The trachea was exposed and cannulated. The lungs were lavaged *in situ* 12 times with 3 ml-washes of sterile 0.9% saline (without Ca^{++} and Mg^{++}). The first two washes were pooled. Cells were separated from the supernatant in all washes ($400 \times g$ at 4°C for 10 minutes). Total and differential cell counts, and hemoglobin measurements were made from the cell pellets. The supernatant fraction of the first two washes was clarified by sedimentation at $15,000 \times g$ at 4°C for 30 minutes and was used for measurement of enzyme activity, and albumin determination. Standard spectrophotometric assays were used for LDH (released during tissue damage), MPO (an

enzyme released during neutrophil degranulation), albumin and hemoglobin (Hb), measures of air-blood barrier integrity.

Repeated Exposures

Based on exploration of various ZnCl_2 doses, 0.05 mg/kg ZnCl_2 was chosen for multiple instillations. A group of 40 male 9-week old Wistar Han rats were randomly divided into 2 groups: physiologic saline (PBS) (n=24) or ZnCl_2 (n=16). Four days after the last instillation, the PBS group of rats was divided into 3 groups: PBS (Control), ZnCl_2 (0.1 mg/kg of ZnCl_2) or CeCl_3 (0.1 mg/kg). The group pre-treated with 0.05 mg/kg ZnCl_2 was also divided into 2 groups: ZnCl_2 (0.1 mg/kg of ZnCl_2) or CeCl_3 (0.1 mg/kg). All rats were analyzed 24 hours later as described earlier.

Cytokine Analyses

The following cytokines were measured in the BAL fluid using Eve Technologies Multiplexed ELISA system (Eve Technologies, Calgary, Canada): G-CSF, Eotaxin, GM-CSF, IL-1 α , Leptin, MIP-1 α , IL-4, IL-1 β , IL-2, IL-6, EGF, IL-13, IL-10, IL-12, IFN γ , IL-5, IL-17A, IL-18, MCP-1, IP-10, GRO/KC, VEGF, Fractalkine, LIX, MIP-2, TNF α and RANTES.

Oxidative Stress Analyses

The BAL supernatants were analyzed for total glutathione levels using a chemiluminescence-based GSH-Glo Glutathione Assay (Promega, Madison, WI). BAL supernatants from the first two BAL washes were mixed with GSH-Glo Reagent 1 $\times$ (1:1) incubated at room temperature for 30 min. Then, luciferin detection reagent (100 μl /well) was added, followed by 15 min incubation. Reduced glutathione was assessed using the GSH/GSSG assay (Abcam, Cambridge, MA) using the manufacturer's instructions.

Statistical Analyses

The means of different BAL parameters such as differential cell counts, levels of MPO, LDH, albumin and Hb were analyzed with multivariate analysis of variance (MANOVA) to detect significant group variations, followed by Ryan-Einot-Gabriel-Welsh multiple range *posthoc* tests using SAS Statistical Analysis Software (SAS Institute, Cary, NC). MANOVA and post hoc analyses were performed separately on data from rats challenged with ZnCl_2 and CeCl_3 . Total glutathione and GSH/GSSG ratios were analyzed with Prism 5 (GraphPad Software, Inc., La Jolla, CA).

Results

Pulmonary Responses to Single instillation of ZnCl_2 in Rats

Dose-dependent increases in BAL neutrophil, lymphocyte and eosinophil counts (Figure 1A), LDH and albumin up to 0.1 mg/kg ZnCl_2 dose were observed (Figure 1B). There was also a dose-dependent decrease in macrophage numbers possibly due to cytotoxicity and/or increased macrophage adhesion to the alveolar epithelium as a result of cell activation. Based on these data, we chose 0.05 mg/kg dose to use for multiple instillations to explore

whether they would elicit enhanced or reduced responses to subsequent metal exposures. We observed that 0.05 mg/kg ZnCl₂ elicited minimal inflammatory response and did not affect their overall health and growth rates (Figure 2). For the final challenge dose (after multiple exposures), we chose 0.1 mg/kg. To see whether responses to another metal ion would be altered, we chose the same mass challenge dose for CeCl₃. This dose of CeCl₃ had induced pulmonary responses comparable to ZnCl₂ (21). The challenge dose of 0.1 mg/kg ZnCl₂ or CeCl₃ was chosen since it elicited modest responses that would allow us to observe either adaptive or enhanced responses when rats were pre-exposed to zinc.

Pulmonary Responses to Zinc Ions: Effects of Pre-Exposure to Low Levels of Zinc Ions

The mean weights of rats instilled repeatedly with either PBS or 0.05 mg/kg ZnCl₂ are shown in Figure 2. Their growth curves were identical indicating that multiple dosing with ZnCl₂ did not affect the development of the animals. Biomarkers of lung injury and inflammation obtained from BAL analyses are summarized in Figure 3. In response to exposure to 0.1 mg/kg ZnCl₂, rats that were previously instilled with PBS showed significantly more neutrophils, LDH, and albumin compared to PBS control. However, when rats were pre-exposed to multiple instillations of 0.05 mg/kg ZnCl₂, rats showed significantly more eosinophils, lymphocytes and Hb. MPO and albumin were not higher in the repeatedly exposed rats compared to naive animals that were exposed to zinc for the first time.

Aliquots of the BAL supernatant from the single ZnCl₂ and multiple ZnCl₂ rats were analyzed for 27 cytokines, chemokines and growth factors by EVE Technologies (Calgary, CN) using the Rat Cytokine Array / Chemokine Array 27-Plex laser bead assay. Of the 27 measured cytokines, chemokines and growth factors, there were 9 that changed significantly among the 3 groups (PBS Control, PBS→ZnCl₂, ZnCl₂→ZnCl₂) (Table 1). Specifically, MCP-1, leptin, fractalkine, IP-10 and RANTES were elevated in PBS→ZnCl₂ in comparison to the PBS control rats. Leptin, IP-10, MIP-1α and RANTES were also higher in the ZnCl₂→ZnCl₂ rats compared to PBS controls. In contrast, cytokine IL-4 and growth factors GM-CSF and G-CSF were reduced in both PBS→ZnCl₂, and ZnCl₂→ZnCl₂ groups. The significant differences between PBS→ZnCl₂ and ZnCl₂→ZnCl₂ rats was a higher MIP-1α, and lower leptin and RANTES levels in the ZnCl₂→ZnCl₂ than in PBS→ZnCl₂ rats.

Pulmonary Responses to Cerium Ions: Effects of Pre-Exposure to Low Levels of Zinc Ions

We explored if repeated low-level lung exposure of rats to ZnCl₂ would alter pulmonary responses to a different metal ion by performing BAL analyses in rats instilled with 0.1 mg/kg CeCl₃ after repeated exposures to either PBS or ZnCl₂. Similar biomarkers of lung injury and inflammation obtained from BAL analyses are summarized in Figure 3. In response to exposure to 0.1 mg/kg CeCl₃, rats that were repeatedly instilled with PBS showed significantly more eosinophils, neutrophils, LDH, and albumin compared to PBS controls. These were similar to findings with ZnCl₂ challenge. Specifically, rats pre-exposed to 0.05 mg/kg ZnCl₂ showed significantly more eosinophils, neutrophils, lymphocytes, LDH, albumin and Hb. Eosinophils and lymphocytes as well as level of Hb were also significantly higher in ZnCl₂→CeCl₃ rats than in PBS→ZnCl₂ rats. Additionally, in both

PBS→ZnCl₂ and ZnCl₂→CeCl₃ groups, MPO levels were significantly decreased compared to controls.

Of the 27 measured cytokines, chemokines and growth factors, 10 were changed significantly among the 3 groups (PBS Control, PBS→CeCl₃, ZnCl₂→CeCl₃) (Table 1). More specifically, TNFα was higher and eotaxin, GM-CSF and IL-4 levels were lower in PBS→CeCl₃ compared with PBS controls. IL-18, MCP-1, IP-10, IL-1β, MIP-1α, RANTES and TNFα were significantly elevated in ZnCl₂→CeCl₃ versus PBS control group. Interestingly, GM-CSF, eotaxin and IL-4 were reduced in both CeCl₃-instilled rats. The significant difference between the PBS→CeCl₃ and ZnCl₂→CeCl₃ groups was a higher concentration of MIP-1α in the ZnCl₂→CeCl₃ group.

Oxidative Stress: Effects of Pre-Exposure to Low Levels of Zinc Ions

To investigate the role of oxidative stress in the lung responses to ZnCl₂ and CeCl₃, we measured the levels of total and reduced glutathione in the BAL fluid (Figure 4). Although there were no significant differences in total glutathione levels (data not shown), we observed significant decreases in the GSH/GSSG ratios in all the 4 metal ions-exposed rats compared to the PBS controls.

Discussion

MCS is often characterized as an initial exposure to a substance that causes both sensitization to that substance and often cross sensitivity with other substances. The mechanisms responsible are still unclear. The diagnosis and study of MCS are challenging due to involvement of various systems and initiation by an array of different substances. Whether chronic exposures result in adaptation or sensitization is a common theme in environmental health and in diseases such as asthma. In this study, we observed that multiple exposures to ZnCl₂ caused greater responses to ZnCl₂ compared to that seen in naive animals. The same trend was observed with a challenge dose of a different substance (Ce). Thus, these data show both sensitization and cross sensitivity. Zinc and cerium are two elements belonging to different classes and have distinct atomic mass and structure. Cross sensitivity to cerium was observed despite its different physicochemical characteristics compared to zinc.

Various metal exposures have been found to result in pulmonary sensitization. Some of the most notable metals that induce hypersensitivity are beryllium, titanium, zirconium, silver, and mercury (22, 23). Importantly, these are non-essential elements unlike zinc. Each of these metals elicits an immune response in which elevated lymphocyte reactivity has been found (24). Metals for which humans have no biological requirement may be more likely to result in increased sensitivity.

In the present study evaluating the promotion of sensitization due to repeated exposure to ZnCl₂ followed by challenge exposures to either ZnCl₂, or CeCl₃, elevations in inflammatory cell counts were observed particularly of neutrophils, eosinophils and lymphocytes. In addition, lymphocytes and eosinophils were significantly different between the naive and repeatedly Zn-exposed groups. Also, a cross sensitization to cerium was

observed as evidenced by greater levels of eosinophils, lymphocytes and hemoglobin in repeatedly Zn-exposed compared to naive animals. Huang and coworkers also found similar observations in mice exposed to zinc ions. They showed higher levels of neutrophils and eosinophils at 24 h and 7 days post-exposure (25). These results show a tendency of sensitization in the chronically exposed animals.

Zinc ions and subsequent challenge exposures did not result in increased levels of all of the cytokines evaluated. Leptin, MCP-1, IP-10, fractalkine, and RANTES were increased due to challenge exposures of zinc ions after repeated doses of PBS. Conversely, rats that were exposed to ZnCl₂ repeatedly then challenged with ZnCl₂ showed decreases in leptin, MCP-1, fractalkine, and RANTES, which could be due to resolution or adaptation. Both adaptation or an augmented response to re-exposure of low-level toxins has been observed as a feature of multiple chemical sensitivity. The significant difference between naive and zinc-exposed rats in terms of chemokine response to subsequent challenge of zinc was in the modulation of macrophage inflammatory protein-1 α (MIP-1 α). Rats that were exposed to ZnCl₂ repeatedly had significantly higher levels of MIP-1 α and elevated levels of IP-10 within the BAL suggesting sensitization. Increases in MIP-1 α have been previously associated with sensitization to various types of inhaled biological and chemical toxins (26, 27).

Rats exposed for the first time to cerium showed an increase in TNF α and decreases in eotaxin, GM-CSF and IL-4. Rats that were pre-exposed to ZnCl₂ repeatedly then challenged with CeCl₃ showed increases in IL-18, MCP-1, IP-10, MIP-1 α , IL-1 β , TNF α and RANTES and decreases in eotaxin and IL-4. The significant difference between naive and zinc-exposed rats in terms of chemokine response to cerium was also in MIP-1 α . These differences in cytokine responses suggest that zinc and cerium elicit different inflammatory pathways, which may be related to the valence state of these two elements – zinc being divalent and cerium trivalent. The valence state of essential elements such as zinc and iron are well-known mediators of certain physiological processes (28) and may potentially play a role in allergies induced by metals (29). The valence status of metals may also be relevant in regard to the redox activity and antioxidant status in the lung (30). Moreover, De Luca and coworkers found that increased inflammatory cytokines and glutathione depletion along with other markers of altered redox status are defining characteristics of patients with MCS (20). Although we did not observe a change in total glutathione levels between experimental groups, a change in reduced glutathione ratios was observed indicating an alteration in redox status and oxidative stress.

In conclusion, this study contributes to a better understanding of sensitization relevant to metal induced multiple chemical sensitivity and the role that oxidative stress may play in the development of this complicated condition. Importantly, we find that previous exposures to elevated zinc concentration produce increased responses to that zinc but also to a different metal, cerium. By broadening the knowledge of this disease mechanism, plausible methods to control patient symptoms may be developed. More importantly, our findings provide evidence that zinc ion exposures may elicit multiple chemical sensitivity by promoting pulmonary inflammation and injury.

Acknowledgements:

S.R. and P.M.Q. received support from the Science Without Borders Program of Brazil administered by CAPES, the Brazilian Coordinating Office for the advancement of Higher Education. CW received support from the Harvard Yerby Fellowship. We thank Melissa Curran for editorial assistance.

Funding: This study was supported by The Hoffman Program on Chemicals and Health, Harvard T.H. Chan School of Public Health, and National Institutes of Health Grants (ES00002 and HL007118). S.R. and P.M.Q. received support from the Science Without Borders Program of Brazil administered by CAPES, the Brazilian Coordinating Office for the advancement of Higher Education. CW received support from the Harvard Yerby Fellowship Program.

References

- Andersson L, Claeson AS, Dantoft TM, Skovbjerg S, Lind N, Nordin S. Chemosensory perception, symptoms and autonomic responses during chemical exposure in multiple chemical sensitivity. *Int Arch Occup Environ Health*. 2016;89(1):79–88. [PubMed: 25917753]
- Fernandez Martin MT, Alvarez Lopez JC. Sevoflurane anaesthesia for nasal surgery in a patient with multiple chemical sensitivity. *Rev Esp Anesthesiol Reanim*. 2018;65(1):49–52. [PubMed: 28545941]
- Gibson PR, Lockaby SD, Bryant JM. Experiences of persons with multiple chemical sensitivity with mental health providers. *J Multidiscip Healthc*. 2016;9:163–72. [PubMed: 27103817]
- Loria-Kohen V, Marcos-Pasero H, de la Iglesia R, Aguilar-Aguilar E, Espinosa-Salinas I, Herranz J, et al. Multiple chemical sensitivity: Genotypic characterization, nutritional status and quality of life in 52 patients. *Med Clin (Barc)*. 2017;149(4):141–6. [PubMed: 28283271]
- Rossi S, Pitidis A. Multiple chemical sensitivity: Review of the state of the art in epidemiology, diagnosis, and future perspectives. *J Occup Environ Med*. 2018;60(2):138–46. [PubMed: 29111991]
- Viziano A, Micarelli A, Alessandrini M. Noise sensitivity and hyperacusis in patients affected by multiple chemical sensitivity. *Int Arch Occup Environ Health*. 2017;90(2):189–96. [PubMed: 27838888]
- Lago Blanco E, Puiguriguer Ferrando J, Rodriguez Enriquez M, Aguero Gento L, Salva Coll J, Piza Portell MR. Multiple chemical sensitivity: Clinical evaluation of the severity and psychopathological profile. *Med Clin (Barc)*. 2016;146(3):108–11. [PubMed: 26654553]
- Pigatto PD, Minoia C, Ronchi A, Brambilla L, Ferrucci SM, Spadari F, et al. Allergological and toxicological aspects in a multiple chemical sensitivity cohort. *Oxid Med Cell Longev*. 2013;2013:356235. [PubMed: 24367721]
- Alessandrini M, Micarelli A, Chiaravallotti A, Bruno E, Danieli R, Pierantozzi M, et al. Involvement of subcortical brain structures during olfactory stimulation in multiple chemical sensitivity. *Brain Topogr*. 2016;29(2):243–52. [PubMed: 26438099]
- Albid I, Nogue S, Izquierdo-Dominguez A, Centellas S, Bernal-Sprekelsen M, Mullol J. Multiple chemical sensitivity worsens quality of life and cognitive and sensorial features of sense of smell. *Eur Arch Otorhinolaryngol*. 2014;271(12):3203–8. [PubMed: 24687801]
- Dotson GS, Maier A, Siegel PD, Anderson SE, Green BJ, Stefaniak AB, et al. Setting occupational exposure limits for chemical allergens-understanding the challenges. *J Occup Environ Hyg*. 2015;12 Suppl 1:S82–98. [PubMed: 26583909]
- Del Prete G. Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. *Allergy*. 1992;47(5):450–5. [PubMed: 1485646]
- Dantoft TM, Skovbjerg S, Andersson L, Claeson AS, Lind N, Nordin S, et al. Inflammatory Mediator Profiling of n-butanol Exposed Upper Airways in Individuals with Multiple Chemical Sensitivity. *PLoS One*. 2015;10(11):e0143534. [PubMed: 26599866]
- Cullen MR, Redlich CA. Significance of individual sensitivity to chemicals: elucidation of host susceptibility by use of biomarkers in environmental health research. *Clin Chem*. 1995;41(12 Pt 2):1809–13. [PubMed: 7497637]
- McKeown-Eyssen G, Baines C, Cole DE, Riley N, Tyndale RF, Marshall L, et al. Case-control study of genotypes in multiple chemical sensitivity: CYP2D6, NAT1, NAT2, PON1, PON2 and MTHFR. *Int J Epidemiol*. 2004;33(5):971–8. [PubMed: 15256524]

16. Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. *Toxicology*. 2011;283(2-3):65–87. [PubMed: 21414382]
17. Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. *Curr Med Chem*. 2005;12(10):1161–208. [PubMed: 15892631]
18. Jozefczak M, Remans T, Vangronsveld J, Cuypers A. Glutathione is a key player in metal-induced oxidative stress defenses. *I J Mol Sci*. 2012;13(3):3145–75.
19. Gugliandolo A, Gangemi C, Calabrò C, Vecchio M, Di Mauro D, Renis M, et al. Assessment of glutathione peroxidase-1 polymorphisms, oxidative stress and DNA damage in sensitivity-related illnesses. *Life Sciences*. 2016;145:27–33. [PubMed: 26685757]
20. De Luca C, Scordo MG, Cesareo E, Pastore S, Mariani S, Maiani G, et al. Biological definition of multiple chemical sensitivity from redox state and cytokine profiling and not from polymorphisms of xenobiotic-metabolizing enzymes. *Toxicol Appl Pharmacol*. 2010;248(3):285–92. [PubMed: 20430047]
21. Molina RM, Konduru NV, Jimenez RJ, Pyrgiotakis G, Demokritou P, Wohlleben W, et al. Bioavailability, distribution and clearance of tracheally instilled, gavaged or injected cerium dioxide nanoparticles and ionic cerium. *ES:Nano*. 2014;1:561–73.
22. Fireman E, Shai AB, Alcalay Y, Ophir N, Kivity S, Stejskal V. Identification of metal sensitization in sarcoid-like metal-exposed patients by the MELISA® lymphocyte proliferation test - a pilot study. *J Occup Med Toxicol*. 2016;11:18. [PubMed: 27076838]
23. Kriebel D, Brain JD, Sprince NL, Kazemi H. The pulmonary toxicity of beryllium. *Am Rev Respir Dis*. 1988;137(2):464–73. [PubMed: 3277503]
24. Stejskal VD, Danersund A, Lindvall A, Hudecek R, Nordman V, Yaqob A, et al. Metal-specific lymphocytes: biomarkers of sensitivity in man. *Neuro Endocrinol Lett*. 1999;20(5):289–98. [PubMed: 11460087]
25. Huang KL, Lee YH, Chen HI, Liao HS, Chiang BL, Cheng TJ. Zinc oxide nanoparticles induce eosinophilic airway inflammation in mice. *J Hazard Mater*. 2015;297:304–12. [PubMed: 26010476]
26. Goplen N, Karim MZ, Liang Q, Gorska MM, Rozario S, Guo L, et al. Combined sensitization of mice to extracts of dust mites, ragweed and aspergillus breaks through tolerance and establishes chronic features of asthma in mice. *J Allergy Clin Immunol*. 2009;123(4):925–32.e11. [PubMed: 19348928]
27. Watson CY, Damiani F, Ram-Mohan S, Rodrigues S, de Moura Queiroz P, Donaghey TC, et al. Screening for Chemical Toxicity Using Cryopreserved Precision Cut Lung Slices. *Toxicol Sci*. 2016;150(1):225–33. [PubMed: 26719368]
28. Zhang F, Tao Y, Zhang Z, Guo X, An P, Shen Y, et al. Metalloreductase Steap3 coordinates the regulation of iron homeostasis and inflammatory responses. *Haematologica*. 2012;97(12):1826–35. [PubMed: 22689674]
29. Shallcross L, Ritchie S, Harberts E, Tammaro A, Gaitens J, Gaspari AA. Manganese oxidation state as a cause of irritant patch test reactions. *Dermatitis*. 2014;25(2):66–71. [PubMed: 24603521]
30. Cohen MD, Sisco M, Prophete C, Yoshida K, Chen LC, Zelikoff JT, et al. Effects of metal compounds with distinct physicochemical properties on iron homeostasis and antibacterial activity in the lungs: chromium and vanadium. *Inhal Toxicol*. 2010;22(2):169–78. [PubMed: 19757987]

Figure 1. Pulmonary response to a single instillation of increasing doses of ZnCl₂ in rats.
A. Analyses of bronchoalveolar lavage at 24 hours post-instillation of ZnCl₂ showed dose-dependent decrease in lavaged macrophages, and increases in neutrophils, and no change in lymphocytes and eosinophils. **B.** Biochemical parameters of injury/inflammation. We found dose-dependent increases in lactate dehydrogenase (LDH), albumin and hemoglobin but not in myeloperoxidase (MPO). MANOVA, $P < 0.05$. Data are expressed as mean $\pm$ SE ($n=5$ rats per group).

Figure 2. Body weight of rats instilled twice per week with physiologic saline (PBS) or 0.05 mg/kg ZnCl₂.

The growth rates of control (PBS, n=24) and low dose ZnCl₂ (n=16) groups of rats were nearly identical. Data are expressed as mean $\pm$ SE % of their initial body weight.

Figure 3. Pulmonary responses to challenge ZnCl₂ or CeCl₃ dosing in rats pre-treated with multiple instillations of PBS or ZnCl₂ in rats.

Cohorts of rats were instilled with PBS or 0.05 mg/kg ZnCl₂ twice weekly for 4 weeks. The volume dose was 1.5 ml/kg. Four days after the last instillation, the PBS group of rats was divided into 3 groups. One group was instilled with PBS (PBS→PBS), another group with 0.1 mg/kg of ZnCl₂ solution (PBS→ZnCl₂), and the third group with 0.1 mg/kg of CeCl₃ solution (PBS→CeCl₃). The group pre-treated with 0.05 mg/kg ZnCl₂ was divided into two groups. One group was challenged with 0.1 mg/kg ZnCl₂ (ZnCl₂→ZnCl₂), and the other

with 0.1 mg/kg of CeCl_3 ($\text{ZnCl}_2 \rightarrow \text{CeCl}_3$). All rats were analyzed 24 hours later. **A.** The total number of lavaged cells in both groups pre-exposed to zinc ($\text{ZnCl}_2 \rightarrow \text{ZnCl}_2$ and $\text{ZnCl}_2 \rightarrow \text{CeCl}_3$) were significantly increased compared to $\text{PBS} \rightarrow \text{PBS}$ control. The increased numbers were due to increased neutrophils, eosinophils and lymphocytes. Moreover, the ZnCl_2 pre-exposed groups when challenged with ZnCl_2 or CeCl_3 also had more lavaged eosinophils and lymphocytes than its corresponding PBS-instilled pair. **B.** Lactate dehydrogenase, albumin and hemoglobin levels in BAL were increased in the four groups ($\text{PBS} \rightarrow \text{ZnCl}_2$, $\text{ZnCl}_2 \rightarrow \text{ZnCl}_2$, $\text{PBS} \rightarrow \text{CeCl}_3$, $\text{ZnCl}_2 \rightarrow \text{CeCl}_3$) compared to PBS controls. In addition, both groups pre-exposed to zinc ($\text{ZnCl}_2 \rightarrow \text{ZnCl}_2$ and $\text{ZnCl}_2 \rightarrow \text{CeCl}_3$) also had more hemoglobin than their corresponding PBS-instilled pairs ($\text{PBS} \rightarrow \text{ZnCl}_2$, $\text{ZnCl}_2 \rightarrow \text{CeCl}_3$). Myeloperoxidase was not changed. Data are mean $\pm$ SE, (n=8 rats per group). MANOVA, * $P < 0.05$, vs. $\text{PBS} \rightarrow \text{PBS}$ control; § $P < 0.05$, $\text{PBS} \rightarrow \text{ZnCl}_2$ vs. $\text{ZnCl}_2 \rightarrow \text{ZnCl}_2$; # $P < 0.05$ $\text{PBS} \rightarrow \text{ZnCl}_2$ vs. $\text{ZnCl}_2 \rightarrow \text{CeCl}_3$.

Figure 4. Oxidative stress assessments after repeated exposure to zinc ions and challenge exposures to either zinc or cerium ions.

Aliquots of the BAL supernatant from each rat were analyzed for total and reduced levels of glutathione. Measurement of the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) is a useful indicator of oxidative stress in tissues. Although there was no trend in total glutathione levels (data not shown), we observed significant decreases in the GSH/GSSG ratios in all metal ion-exposed rats, suggestive of oxidative stress. Data are mean $\pm$ SE, (n=4 rats per group). *P<0.05, PBS→PBS vs. other groups, # P<0.05 PBS→ZnCl₂ vs. ZnCl₂→ZnCl₂.

Table 1.
List of cytokines, chemokines and growth factors in BAL fluid that differ by treatment.

	Repeated PBS Control	Repeated PBS→ZnCl ₂	Repeated ZnCl ₂ →ZnCl ₂	Repeated PBS→CeCl ₃	Repeated ZnCl ₂ →CeCl ₃
IL-18	154.0 ± 20.9	189.0 ± 27.02	265.4 ± 64.2	199.7 ± 24.4	237.3 ± 23.9 [*]
MCP-1	83.5 ± 11.1	1011.0 ± 270.3 [*]	491.0 ± 200.6	1263.1 ± 443.3	1857.7 ± 755.6 [*]
Leptin	70.1 ± 5.5	517.9 ± 124.2 [*]	255.3 ± 74.4 [#]	389.6 ± 142.3	513.8 ± 243.2
Fractalkine	12.4 ± 2.0	25.5 ± 3.9 [*]	21.3 ± 3.5	21.5 ± 3.3	23.4 ± 4.6
IP-10	8.6 ± 1.4	39.2 ± 5.6 [*]	50.4 ± 14.2 [*]	35.5 ± 5.6	61.1 ± 22.4 [*]
G-CSF	6.2 ± 0.2	5.4 ± 0.3	5.1 ± 0.4 [*]	5.4 ± 0.5	4.75 ± 0.5
IL-4	5.8 ± 0.4	3.9 ± 0.3 [*]	5.0 ± 0.5	4.2 ± 0.3 [*]	4.4 ± 0.4 [*]
GM-CSF	5.1 ± 1.4	1.8 ± 0.4 [*]	2.0 ± 0.2 [*]	1.6 ± 0.5 [*]	1.6 ± 0.6 [*]
IL-1β	4.4 ± 0.5	6.5 ± 1.7	5.6 ± 1.4	7.3 ± 1.3	8.4 ± 1.4 [*]
Eotaxin	3.3 ± 0.5	1.8 ± 0.1	2.1 ± 0.5	1.8 ± 0.3 [*]	1.6 ± 0.5 [*]
MIP-1α	1.8 ± 0.2	3.5 ± 0.5	15.7 ± 5.2 [#]	6.8 ± 1.3	17.2 ± 5.5 ^{*,§}
RANTES	1.0 ± 0.0	9.6 ± 2.1 [*]	3.3 ± 1.0 [#]	7.2 ± 2.9	9.7 ± 3.9 [*]
TNFrα	0.7 ± 0.3	1.3 ± 0.4	1.3 ± 0.3	1.9 ± 0.3 [*]	1.9 ± 0.3 [*]

Data are mean ± SE pg/ml from 7-8 rats per group.

^{*} P<0.05, vs. Repeated PBS control

[#] P<0.05, Repeated PBS→ZnCl₂ vs. Repeated ZnCl₂→ZnCl₂

[§] P<0.05, Repeated PBS→CeCl₃ vs. Repeated ZnCl₂→CeCl₃;