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Sub-chronic inhalation of reclaimed water-induced fibrotic lesion in a mouse model

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ABSTRACT

When reclaimed water is used as municipal miscellaneous water, acute exposure of the generated aerosol with high levels of endotoxins can cause severe inflammation in the lungs. However, the potential risks of long-term inhalation of reclaimed water remains unclear. To identify the adverse effects of sub-chronic reclaimed water inhalation and explain the underlying mechanisms, a mouse model of 12-week sub-chronic exposure was established, and wastewater before a membrane bioreactor (MBR, positive control) and the MBR effluent (reclaimed water, which met the quality standard of urban use and was currently used for landscape irrigation) were tested in this study. The exposure dose was set to approach the real working scenarios. Lung lavage and histology were analyzed. Obvious epithelial cell apoptosis in the bronchi was observed, along with the accumulation of myofibroblasts and the collagen deposition both in main bronchi and terminal bronchioles. All these symptoms were persistent after 4 weeks of recovery. Inflammation and induced bronchus-associated lymphoid tissues (iBALT) were also observed but diminished after recovery indicating inflammation may not be the direct cause of the symptom. Furthermore, two fibrogenic cytokines (TNF-α and TGF-β) were constantly high in the lung during the study. They might be the biomarkers of lung damage after the inhalation of reclaimed water. Adaptive immune responses were also detected as elevated levels of IgG and IgA, but not for IgE. Inhalation of reclaimed water causes sustained fibrotic lesions in the lungs, which suggests potential health risks during urban application where aerosols generated.

1. Introduction

The urban use of reclaimed water from municipal wastewater significantly reduces the stress of water resource shortages. However, the risk studies related to the application of reclaimed water fail to keep up with the prevalence of reclaimed water around the world. Most of these efforts focused on the risk of pathogens and carcinogenic compounds in water, such as enterovirus, trihalomethanes (THMs), and heavy metals (Debroux et al., 2012; Oesterholt et al., 2007; Hamilton et al., 2006). It is mainly because the accidental ingestion was hypothesized as the major exposure route in the process of application. However, when reclaimed water is used for urban miscellaneous water such as that used for car washing or landscape irrigation, the water is nebulized and the resulting aerosol can expose to the operators or passersby mainly through inhalation. However, few studies concerned the health risks generated by inhalation of reclaimed water (Lozano, 2013; Barker et al., 2017), and the key risk source was identified to be carcinogenic compounds such as volatile organic compounds (VOCs; Aina and Ahmab, 2007; Wang et al., 2013). Therefore, because the lung and associated respiratory tract is the primary organ that contacts the exogenous contaminants, the potential risk might relate to those components in water that directly or indirectly cause lung injury. Recently, endotoxins in reclaimed water were found to induce acute lung inflammation, which may result in lung damage in long-term exposure (Zhang et al., 2016; Xue et al., 2016).

Endotoxins, also known as lipopolysaccharides (LPS), are large molecules in the outer membrane of gram-negative bacteria, and elicit strong immune responses in mammals (Liebers et al., 2008). The cell-bound endotoxins are released to the water as free endotoxins when the bacteria die or are broken. The endotoxin levels in

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Abbreviations

BALF	Bronchoalveolar lavage fluid
Ig	Immunoglobulin
IL	Interleukin
MBR	Membrane bioreactor
PMN	Polymorphonuclear cell
SMA	Smooth muscle actin
TOC	Total organic carbon
TN	Total nitrogen
TNF-α	Tumor necrosis factor-α
TGF-β	Transforming growth factor-β

the reclaimed water ranged from 3 to 19700 EU/mL, and the median value was 422 EU/mL (Huang et al., 2013). Acute inhalation exposure of endotoxins in mice could induce tumor necrosis factor (TNF)-α and interleukin (IL-6) in the lung and cause polymorphonuclear cell-dominated inflammation (Zhang et al., 2016; Xue et al., 2016). The cytokine responses reached maximal levels before 1 h, and the polymorphonuclear neutrophil (PMN) percentage in lavage peaked at 3 h after exposure. All the responses fell back to the baseline level within 24 h. Thus, the acute pulmonary inflammation is not a persistent impairment caused by reclaimed water inhalation.

However, mild but persistent inflammation in the lung has proven to be closely related with other diseases, such as chronic obstructive pulmonary disease (COPD), which is a major cause of morbidity and mortality in the US (Lee et al., 2009). Inflammation biomarkers, such as C-reactive protein (CRP), fibrinogen, and leukocyte counts (WBC) in individuals were found to be significantly higher in COPD patients compared with those of the control group (Fattouh and Alkady, 2014). Airway inflammation injuries resulting from tobacco and other environmental exposures was associated with increased risk of lung cancer (Hecht, 2008). In addition to the direct effects of deregulated inflammation on airway epithelium remodeling, chronic inflammation indirectly promotes apoptosis resistance and angiogenesis and was ultimately capable of inducing a pro-tumor microenvironment (Raghuwanshi et al., 2008; Montuenga and Pio, 2007).

These observations prompt us to further question the consequences of sub-chronic exposure under an environmental exposure scenario, which is more severe due to the modest but successive stimulation through inhalation. Previous studies have found the inflammation-related profound damage caused by chronic exposure of pure lipopolysaccharide solution, with a relatively high dose, and more persistent effects were also observed, such as pulmonary dysfunction, sub-epithelial fibrin deposition, airflow obstruction, and emphysema-like changes in mouse lungs (Savov et al., 2003; Brass et al., 2008; Lai et al., 2012). Also the geometric mean of ambient air endotoxin levels was 0.3 EU/m^3 in the urban region of Denmark (Madsen, 2006) and the median value of air endotoxin levels in PM10 was lower than 0.3 EU/m^3 in a Japanese study (Yoda et al., 2017), while an air with 50% moisture from a 100 EU/mL reclaimed water at 1 atm and 25 °C can have 1155 EU/m^3 endotoxin by calculation. Together with the fact that LPS is abundant in reclaimed water, it is reasonable to assume that chronic reclaimed water exposure is more hazardous, and irreversible effects were also expected.

This study was the first attempt to test the possible adverse effects that resulted from sub-chronic inhalation exposure of reclaimed water in mice, which provides important information for further risk assessment of water reuse when aerosol is generated.

2. Materials and methods

2.1. Animals

All the animals used in this study were Imprinting Control Region (ICR) male mice (8–9 weeks old) purchased from the Vital River Company (Beijing, China) and kept in the Center of Biomedical Analysis of Tsinghua University. The innate immune systems in adult males and females are the same, and they have the same responses to endotoxins. Since this study aimed to identify the possible injury caused by the inhalation of reclaimed water, using one gender is enough and can reduce the animal number under the ethical consideration. Animal maintenance and experimental procedures were conducted in strict accordance with the Institutional Animal Care and Use Committee (IACUC) of Tsinghua University, which is a branch of the Beijing Animal Care and Use Committee. The light/dark cycle was 12/12. The mice were allowed to take food and water freely. 5 mice per group were kept in one cage. All experiments were reviewed and approved by the IACUC (permit number 13-LY1), and all efforts were made to minimize the suffering of test animals.

2.2. Water samples

Two representative water samples, the influent and effluent of the MBR process of a reclaimed water plant, were collected to illustrate the high dose and low dose exposure. The water samples were collected from the sampling valves in plastic buckets, the volume of which are about 3 L. Before the sampling, the buckets were pre-washed by the water and then filled fully with the water sample. The bucket was shipped to the lab and stored at 4 °C for 5 days of exposure. To sampling every 5 exposure days was based on the factors that the storage time of real reclaimed water in the regulation tank or distribution system could be several days depending on the application activities, and the endotoxin activities did not change in the lab conditions for two weeks. The influent water is contacted by the workers in the plant. It has high endotoxin levels and was also considered as a positive control. The effluent is used to irrigate a group of green areas. There are hundreds of sprayers in the green areas, but some regions need to be manually irrigated with pipes. The exposed person is the gardener, who is responsible for adjusting the sprayers and manual irrigation. Five water indexes were measured. Because the acute exposure caused severe inflammation, and only macromolecules and microorganisms can trigger the immune response (Xue et al., 2016), thus the total heterotrophic bacteria, total number of microbes and endotoxin activity were measured. Total organic carbon (TOC) and total nitrogen (TN) were also checked to know whether the water was properly treated. The total heterotrophic bacteria was determined by standard plate counting as previously described (Xue et al., 2016), and the total number of microbes were counted by fluorescent staining (4′,6-diamidino-2-phenylindole (DAPI)), the TOC and TN were determined by a total organic carbon analyzer (Shimadzu, Kyoto, Japan). The endotoxin levels were measured by the Standard Limulus Amebocyte Lysate (LAL) assay kits according to the manufacturer's instruction (Xiamen Houshiji Ltd, China). The major water quality parameters can be found in the supplementary materials (Figure S1).

2.3. Inhalation exposure operation and parameter determination

Animals were exposed 2 h per day 5 days per week to imitate the common situation of a gardener. The reclaimed water is also qualified for other urban miscellaneous water consumption according to a Chinese standard (GB/T 18920-2002). Thus, the daily exposure time was relatively low, if a carwash scenario was