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Detection of intracellular lamellar bodies as a screening marker for fibrotic lesions

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ABSTRACT

With the rapid increase in application of disinfectants worldwide as a method to block the spread of coronavirus, many new products are being introduced into the market without thorough verification of their impacts on human health and the environment. In the present study, we aimed to propose a screening marker for materials that can induce fibrotic lung disease using disinfectants, which had been demonstrated as causative materials of chronic inflammation and interstitial fibrosis. We first calculated the corresponding LC50 level based on results from cell viability test and exposed the LC50 level of disinfectants to human bronchial epithelial cells for 24 h. Formation of lamellar body-like structures, cleavage of the nuclear matrix, structural damage of mitochondria were found in the cytosol of the treated cells. We also dosed disinfectants by pharyngeal aspiration to mice to determine the LD0 level. The mice were sacrificed on Day 14 after a single dosing, and lamellar body-like structures were observed in the lung tissue of mice. Herein, we hypothesize that DNA damage and metabolic disturbance may play central roles in disinfectant-induced adverse health effects. Additionally, we propose that formation of lamellar bodies can be a screening marker for interstitial fibrosis.

1. Introduction

With industrial development, especially in the chemical industry, the number and quantity of chemical exposures in daily life have rapidly expanded. According to the CAS registry system, information of new chemical substances is updated approximately every 27 s. Furthermore, these chemicals have been proposed as the main cause of various environmental diseases. For instance, interstitial lung diseases such as asbestosis and silicosis are the most representative occupational diseases among workers, thus, the pathogenesis has been continuously studied by researchers worldwide since the 1900s. However, the pathogenesis remains unclear, and there are many limitations in identifying the causality between the health effects and environmental factors due to environmental diversities that individuals experience.

In Korea, humidifier disinfectant-related lung disease has become an important social issue since 2011, and the number of related death was reported at 1553 by July 2020 (https://www.epa.gov, n.d). Especially, QAC shows a prolonged degradation rate in the environment, since first identified in December 2019 in Wuhan, China, the coronavirus pandemic is still ongoing, and application of various types of disinfectants have rapidly increased worldwide to block the spread of coronavirus. A total of 525 products are currently registered for coronavirus by the EPA, 244 and 74 products consist of quaternary ammonium chloride (QAC) and sodium hypochlorite, respectively (https://www.epa.gov, n.d). In addition, the number of related death was reported at 1553 by July 2020 (https://www.epa.gov, n.d). Especially, QAC shows a prolonged degradation rate in the environment.

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environment, suggesting possible long-term exposure to humans and ecosystems. However, evidence for adverse health effects of inhaled disinfectants is insufficient. Therefore, a marker which can predict the harmful health effects are urgently needed for safe use of aerosol-like chemicals used for disinfectant purposes.

In a previous study, we suggested that formation of lamellar bodies may be associated with pulmonary fibrosis (Park et al., 2020b). Here, we tested possible application of lamellar body-like structures as a screening marker for disinfectants which can cause lung disease, especially interstitial fibrosis, using disinfectants which had been demonstrated (or suspected) as causative materials of chronic inflammation and interstitial fibrosis.

2. Materials and methods

2.1. Sample preparation

Methylisothiazolinone (MIT), didecyldimethyl ammonium chloride (DDAC), benzalkonium chloride (BKC), benzethonium chloride (BTC), and benzylmethylhexadecylammonium chloride (BDHAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kathon CG (Kathon, a mixture of MIT and chloro-MIT) and polyhexamethylenebiguanidine phosphate (PHMG-P) were kindly provided by Dr. Kyu-Hong Lee. All the chemicals were solubilized in deionized water (DW, 1 mg/mL).

2.2. Cell culture

BEAS-2B cells, a human bronchial epithelial cell line (CRL-9609, ATCC, Manassas, VA, USA), were maintained in DMEM/F12 containing 10% fetal bovine serum and 1% antibiotics in a 5%/95% CO₂ modified incubator.

2.3. Cell viability

Stabilized cells (60–70% of confluence) were incubated with seven disinfectants for 24 h. After adding of MTT solution (2 mg/mL, 40 μL/well, Sigma-Aldrich), the cells were further incubated for 4 h. Finally, the absorbance was quantified at 540 nm using a multi-mode microplate reader (BioTek, Winooski, VT, USA), and the relative viability of the treated cells was calculated using the absorbance value of the control cells (100%).

2.4. Determination of lethal dose (LD₅₀) level using mice

Female ICR mice (7-week-old, Orient Com., Seongnam, Korea) were maintained at our facility (23 ± 3 °C temperature, 50 ± 10% relative humidity, 12-h light/12-h dark cycles with a 150–300 lx light intensity, and 10–20 times/h ventilation) for the entire experimental period. After 1 week of acclimation, test materials were a single dosed to mice by pharyngeal aspiration (50 μL/head) under isoflurane inhalation anesthesia. The control mice were treated with autoclaved DW, and the body weight of live mice was measured weekly. At 14 days after exposure, all the alive mice were sacrificed. For histopathological evaluation, the lung tissues were fixed with 10% neutral buffered formalin and embedded with paraffin according to routine technical procedures. Then, the lung tissues were cut to a thickness of 3 μm and stained with hematoxylin and eosin (H&E). All experimental procedures were approved by the Institutional Animal Care and Committee of Kyung Hee University (KHMG-IACUC-20-024) and performed in accordance with the “Guide for the Care and Use of Laboratory Animals”, an ILAR publication.

3. Results

3.1. Cell viability

In our previous studies, when cells were exposed to 4 μg/mL of DDAC and MIT for 24 h, the cytotoxic levels were 21.8 ± 8.9% and 67.9 ± 5.1%, respectively, compared with control (100%) (Park et al., 2020b; Park and Seong, 2020). In the present study, we compared the cytotoxicity levels of seven disinfectants under the same exposure conditions (Supplementary Fig. 1). Table 1 shows the LD₅₀ level calculated arithmetically using the slope.

3.2. Transformation of intracellular structures

Transformed mitochondrial structure, cleavage of the nuclear matrix and formation of lamellar body-like structure were commonly observed in all the cells treated with the disinfectants, although the representative features were slightly different between the disinfectants (Fig. 1, Supplementary Fig. 2). Transformed mitochondrial structures were more apparent in cells exposed to MIT, PHMG-P, and Kathon, and formation of lamellar body-like structures was more obvious in cells exposed to DDAC, BKC, BDHAC, and BTC. Cleavage of the nuclear matrix was significant in all the treated cells. More importantly, the mitochondria in cells which formation of lamellar body-like structures was dramatic, seemed to be so damaged that even its shape was hard to recognize.

3.3. Acute respiratory toxicity

Based on accumulated evidence, we administered disinfectants once

| Table 1 |
| --- |
| Comparison of lethal dose. |
| PHMG-P | Kathon | MIT | DDAC | BKC | BTC | BDHAC |
| 10 μg/mL | 21.4 ± 0.0 | – | – | – | – | – |
| 5.0 μg/mL | 52.1 ± 6.7 | 5.1 ± 0.6 | 7.9 ± 3.2 | 5.8 ± 1.6 | 44.3 ± 10.9 | 7.4 ± 5.2 | 9.5 ± 3.7 |
| 2.5 μg/mL | 71.7 ± 4.8 | 5.6 ± 0.5 | 17.3 ± 10.6 | 12.5 ± 2.6 | 76.6 ± 9.0 | 36.6 ± 11.8 | 31.9 ± 4.3 |
| 1.25 μg/mL | 92.8 ± 7.8 | 5.0 ± 0.8 | 55.3 ± 8.5 | 58.0 ± 13.4 | 87.5 ± 6.4 | 68.6 ± 4.3 | 69.4 ± 6.2 |
| 0.5 μg/mL | – | 37.1 ± 10.5 | – | – | – | – |
| 0.25 μg/mL | – | 78.5 ± 7.6 | – | – | – | – |
| *Calculated LD₅₀ (μg/mL) | 5.34 | 0.42 | 1.42 | 1.47 | 4.56 | 1.98 | 1.90 |

Cytotoxicity test were independently performed four times using four wells per concentration, and all the data, excepting 1.25 μg/mL of PHMG-P, showed statistical significance compared with control. *: The value was calculated using the slope value of the corresponding section. **: The value was determined by Supplementary Fig. 3.
to mice by pharyngeal aspiration (Supplementary Fig. 3), and Table 1 and Supplementary Table 1 show the LD<sub>0</sub> value following acute respiratory exposure (concentration at which all the animals lived to 14 days after a single dose of disinfectant). Furthermore, we found formation of lamellar body-like structures in the lung tissues obtained from all the mice that lived to 14 days after a single dose of disinfectant (Supplementary Fig. 4).

4. Discussion

A growing number of patients with cancer and chronic respiratory diseases as well as an increasing aging population cause a significant burden on the national economy globally (www.who.int, 2016; Prüss-Ustün et al., 2018). Furthermore, polluted ambient air and inhaled harmful chemicals are known to be closely associated with cancer and chronic respiratory diseases (Jiang et al., 2016). Bronchial epithelial cells function as a primary defence line to protect lungs from...
environmental factors entering through inhalation (Gao et al., 2015a). Therefore, impairment of bronchial epithelial cells by inhaled insults lowers the host capacity to combat xenobiotics and worsens patient prognosis.

The COVID-19 pandemic caused significant changes in lifestyle, an increased use of disinfectants may be among them. Meanwhile, direct or indirect exposure to disinfectants through the respiratory system can cause adverse health effects as evidenced by the humidiﬁer disinfectant incident in Korea, and interstitial fibrosis known as idiopathic pulmonary fibrosis is a representative disease. In previous studies, we demonstrated the main causative substances of the humidiﬁer disinfectant event that occurred in Korea induced apoptotic cell death by impairing the cell membrane and mitochondria in BEAS-2B cells, a human bronchial epithelial cell line (Park et al., 2020a; Park and Seong, 2020; Park et al., 2020b; Park et al., 2018). We also emphasized the possible application of the critical micelle concentration of the corresponding disinfectant as a method to predict the concentration at which toxicity may be induced. In addition, we found that DDAC, a QAC, formed lamellar body-like structures in the cytosol of cells. Accumulated evidence has demonstrated that PHMG-P, Kathon and DDAC induced interstitial ﬁbrosis in the lungs of patients or animals, ultimately leading to death (Lee et al., 2019; Park et al., 2020a; Song et al., 2018). Similarly, 14 days-repeated inhalation of BKC induced damage to bronchiolar epithelium and occurrence of cell debris in the alveolar region (Choi et al., 2020), and Hoyle and Svendsen (2016) has proposed that products that contain chlorine derivatives, such as sodium hypochlorite, can cause acute lung injury, including ﬁbrosis, by generating toxic chlorine gas in the air (Hoyle and Svendsen, 2016).

Furthermore, some researchers have suggested that increased lamellar bodies in patients with interstitial ﬁbrosis may be attributed to lipid metabolic disturbance (Agudelo et al., 2020; Williams et al., 2004; Gochuico et al., 2012; Gao et al., 2015b). Herein, we compared cytotoxicity of seven disinfectants (non-QAC and QAC) under the same conditions and identiﬁed that the cytotoxicity of Kathon was the highest among disinfectants used in this study. More importantly, we observed that the disinfectants cleaved the nuclear matrix, formed lamellar body-like structures and transformed mitochondrial structures (blebbing or imbalanced ﬁssion and fusion). Similarly, 0.5% sodium hypochlorite formed lamella-body-like structures with division of the nuclear matrix under the same test condition (Supplementary Fig. 5). Meanwhile, although ﬁbrotic lesion was detected in patients which inhaled humidiﬁer disinfectants containing Kathon, the frequency was very low than in patients which inhaled products containing PHMG-P (Lee et al., 2019). The formed lamellar body number was also much smaller in cells exposed to Kathon (or a product containing Kathon) compared with cells exposed to other disinfectants (data not shown). Therefore, we hypothesize that DNA damage and metabolic disturbance may play central roles in disinfectant-induced adverse health effects. Additionally, we propose that formation of lamellar bodies can be a screening marker for interstitial ﬁbrosis. Furthermore, considering the appearance of lysosome clearly increased in cells exposed to PHMG-P, a representative causative agent of humidiﬁer disinfectant-induced pulmonary ﬁbrosis, we suggest that lysosomal dysfunction may be closely associated with development of pulmonary ﬁbrosis (Paget et al., 2019).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2021.115501.

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