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Polyhexamethyleneguanidine phosphate induces severe lung inflammation, fibrosis, and thymic atrophy

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ABSTRACT

Polyhexamethyleneguanidine phosphate (PHMG-P) has been widely used as a disinfectant because of its strong bactericidal activity and low toxicity. However, in 2011, the Korea Centers for Disease Control and Prevention and the Ministry of Health and Welfare reported that a suspicious outbreak of pulmonary disease might have originated from humidifier disinfectants. The purpose of this study was to assess the toxicity of PHMG-P following direct exposure to the lung. PHMG-P (0.3, 0.9, or 1.5 mg/kg) was instilled into the lungs of mice. The levels of proinflammatory markers and fibrotic markers were quantified in lung tissues and flow cytometry was used to evaluate T cell distribution in the thymus. Administration of PHMG-P induced proinflammatory cytokines elevation and infiltration of immune cells into the lungs. Histopathological analysis revealed a dose-dependent exacerbation of both inflammation and pulmonary fibrosis on day 14. PHMG-P also decreased the total cell number and the $CD4^+/CD8^+$ cell ratio in the thymus, with the histopathological examination indicating severe reduction of cortex and medulla. The mRNA levels of biomarkers associated with T cell development also decreased markedly. These findings suggest that exposure of lung tissue to PHMG-P leads to pulmonary inflammation and fibrosis as well as thymic atrophy.

1. Introduction

Polyhexamethyleneguanidine phosphate $((C_7H_{18}N_3O_4P)_m(C_7H_{15}N_3)_n$, CAS No. 89697-78-9) is a water-soluble chemical possessing a guanidine group. The chemical has been widely used as a biocide; in products such as humidifier disinfectants, shampoos, wet wipes, and cleaning products, because of its strong bactericidal activity and low toxicity (Aleshina et al., 2001). PHMG-P has been approved as a disinfectant for medical devices by the United States Food and Drug Administration and it is registered and sold as a disinfectant in several countries including, Japan, Australia, and China.

There are currently no published research reports regarding a deleterious effect of direct lung exposure to PHMG-P, though PHMG-P is a well-used biocide and it was used for humidifier disinfectant. Because polymers including PHMG-P have low vapor pressure and high molecular weight, inhalation toxicity of polymers has been considered to be very low. However, in 2011, the Korea Centers for Disease Control and Prevention and the Ministry of Health and Welfare reported that an outbreak of pulmonary disease with unknown cause might have originated from humidifier disinfectants whose main ingredient is PHMG-P (Press release of Korea Ministry of Health and Welfare, 2011). Thirteen pregnant women have been suffered from acute interstitial pneumonia and 4 patients of them died of rapid development of pulmonary fibrosis (Lee et al., 2012). And in an inhalation study of another biocide, polyhexamethylene biguanide, it causes severe irritation of respiratory tract, pneumonitis, bronchitis and mortality (European Chemical Agency Reports, 2011). Taken together, the inhalation toxicity study of PHMG-P is clearly needed.

The most common adverse effect of inhaled substances is lung inflammation. To remove such foreign materials from lung, a variety of immune cells, such as monocytes, neutrophils, and lymphocytes, are recruited to the site of injury and produce mediators. However, if inflammation does not resolve properly or acute lung injury is recurrent, it leads to abnormal wound healing, fibrosis (Gross and Hunninghake, 2001). Pulmonary fibrosis is

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characterized by fibroblast proliferation, transformation of fibroblasts to myofibroblasts, and excessive production of extracellular matrix (ECM) (Chaudhary et al., 2006; Giri et al., 1993; Liu et al., 2010). These changes result in distorted alveolar architecture, reduced lung volume, and impaired gas exchange. Thus, pulmonary fibrosis is a progressive, debilitating lung disease with a poor prognosis (Coker and Laurent, 1998; Horowitz and Thannickal, 2006).

Upto now, there has been no direct evidence that PHMG-P, one constituent of sterilizers, can cause pulmonary toxicity. In this study, therefore, we aimed to investigate whether PHMG-P causes lung injury and fibrosis following direct exposure to PHMG-P on the lungs. In addition, we determined the adverse effect of PHMG-P on the lymphoid organs such as thymus and spleen.

2. Materials and methods

2.1. Chemicals

PHMG-P solution was a generous gift from SK Chemicals (Seongnam, Korea). Saline was purchased from Daihan Pharmaceutical Co. (Ansan, Korea).

2.2. Animals

Seven-week-old male C57BL/7 mice were obtained from Orient Bio Inc. (Seongnam, Korea). The mice were housed in environmentally controlled animal facilities, and the animal room was maintained at 22 ± 3 °C with relative humidity of 50% ± 10%, air ventilation refreshed 10–20 times/h, and light intensity of 150–300 Lux with a 12-h light/dark cycle. Pelleted food for experimental animals (PMI Nutrition International, Richmond, USA) and UV-irradiated (Steritron SX-1; Daeyoung, Inc., Korea) and filtered (1 μm) tap water were given *ad libitum*. Mice were used after 6 days of acclimation. All experiments were approved by the Institutional Animal Care and Use Committee of Korea Institute of Toxicology.

2.3. Experimental design

The mice were randomly assigned to 4 weight-matched experimental groups by using the Path/Tox System (Version 4.2.2; Xybion Medical Systems Corporation, USA). A concentrated stock solution of PHMG-P (25%) was diluted to 6.3 μg/50 μL, 18.9 μg/50 μL, and 31.5 μg/50 μL with saline, to create equivalent doses of 0.3 mg/kg, 0.9 mg/kg, and 1.5 mg/kg, respectively, which was instilled intratracheally into the mice by using a modified automatic video instillator (Kim et al., 2010). The control group was instilled with saline via the same route. Body weight was measured prior to the first instillation, and on days 2, 4, 5, 6, 7, 8, 11, 12, and 13. The clinical signs of mice were observed every day. At day 7 and day 14 after exposure, the mice were euthanized with an overdose of isoflurane, and necropsied. The spleen, thymus, and lungs were removed and weighed. The left lungs were fixed in 10% neutral buffered formalin, and the right lungs were snap-frozen to evaluate gene expression and cytokine levels.

In order to analyze the PHMG-P-induced changes in the lymphocyte profile in the thymus, separate experiments were carried out with only 2 groups: the control group and the 0.9 mg/kg PHMG-P group; the target agent administrated via the same route as described above. The thymus was collected following euthanasia; a half of the tissue samples was used for histopathological tests and the other half for flow cytometric analysis.

2.4. Gene expression analysis

RNeasy Mini kit (Qiagen) was used to isolate total RNA from mouse lungs harvested at day 7 and day 14 after instillation. Total RNA (1 μg) was reverse-transcribed to cDNA by using the Improm-II™ Reverse Transcription System (Promega, Madison, USA). Fibronectin, monocyte chemoattractant protein 1 (MCP1), matrix metalloproteinase2 (MMP2), MMP12, GATA3, and T-bet mRNA levels were quantified using ABI StepOne (Applied Biosystems, Foster City, CA). HPRT was used as the internal control. The sequences of the primers were as follows: fibronectin : sense, 5′-CACGATGCGGGTCACTTG-3′ ; antisense, 5′-CTGCAACGTCCTCTTCATTCTTC-3′; MCP1: sense, 5′- AGGTGTCCCAAAGAAGCTGTA-3′ ; antisense, 5′-ATGTCTGGACCCATTCCTTCT-3′; MMP2: sense, 5′-CAAAGAGTTGGCAGTGCAATA-3′; antisense, 5′-GATGGTGTTCTGGTCAAGGT-3′ ; MMP12: sense, 5′-CACAACAGTGGGAGAGAAAA-3′ ; antisense, 5′-AGCTTGAATACCAGATGGGATG-3′ ; GATA3: sense, 5′-CTCGGCCATTCGTACATGGAA-3′ ; antisense, 5′- GGATACCTCTGCACCGTAGC-3′; T-bet: sense, 5′-GCCAGGGAACCGCTTATATG-3′ ; antisense, 5′-GACGATCATCTG GGTCACATTGT-3′; HPRT : sense, 5′-TTATGGACAGGACTGAAAGAC-3′ ; antisense, 5′-GCCATCTCCTGCTCGAAGTC-3′.

2.5. Cytokine measurement by ELISA

The frozen lungs were weighed and placed into phosphate-buffered saline (PBS) containing 1% Triton X-100 at a ratio of 100 mg tissue per mL. The lung tissue was homogenized using a homogenizer (IKA, Germany) and incubated at 4 °C for 30 min with shaking. The homogenates were centrifuged at 13,000 rpm, and supernatants were collected and used for quantification of CXCL1, interleukin-1β (IL-1β), interleukin-6 (IL-6), and interferon-γ (IFN-γ) (R&D Systems, Minneapolis, USA) according to the manufacture's protocol. Total protein level in the homogenates was determined using the BCA protein assay (Sigma Aldrich).

2.6. Western blot analysis

The lung tissue was homogenized in a lysis buffer (Cell Signaling) on ice and incubated at 4 °C for 30 min. The lysates were centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatants were collected, and protein concentration was determined using the BCA protein assay (Sigma Aldrich). Equal quantities of each

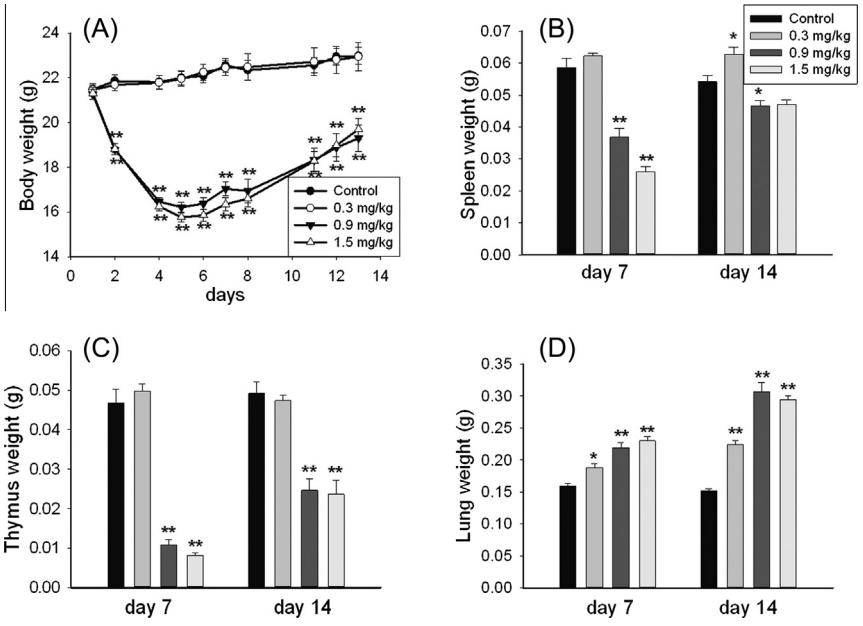

Fig. 1. Changes in body and organ weights of mice following a single instillation of PHMG-P. Mice were intratracheally instilled once with 50μL of 0.3 mg/kg, 0.9 mg/kg, or 1.5 mg/kg PHMG-P and then killed 7 days or 14 days later. The control group (Control) was treated with saline via the same route of administration. The number of mice per group was 10 at time points from day 1 through day 7, with 5 mice per group at time points from day 8 onward. (A) Body weight and organ weight, (B) the spleen, (C) the thymus, and (D) the lung. Data are expressed as mean ± SE; $^{*}p < 0.05$, $^{**}p < 0.01$ indicates significant difference from the control group.