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Full Length Article

Analysis of genomic responses in a rat lung model treated with a humidifier sterilizer containing polyhexamethyleneguanidine phosphate

Min-Seok Kim[a,1], Seok Won Jeong[b,1], Seong-Jin Choi[a], Jin-Young Han[a,d], Sung-Hwan Kim[a], Seokjoo Yoon[c,d], Jung-Hwa Oh[c,d], Kyuhong Lee[a,d,*]

[a] Department of Inhalation Toxicology Research, Korea Institute of Toxicology, Jeonbuk 56212, Republic of Korea
[b] Department of Research and Development, Genoplan Inc., Seoul 06221, Republic of Korea
[c] Department of Predictive Toxicology, Korea Institute of Toxicology, Daejeon, 34114, Republic of Korea
[d] Department of Human and Environmental Toxicology, University of Science & Technology, Daejeon, 34113, Republic of Korea

HIGHLIGHTS

- We investigated the geno-toxicological effects of PHMG phosphate in the rat lung using a DNA microarray.
- PHMG phosphate altered expression of genes involved in the urea cycle, inflammation, and oxidative stress.
- 21 genes were induced and 4 genes were repressed in response to PHMG phosphate in a timedependent manner.

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ABSTRACT

The antimicrobial biocide polyhexamethyleneguanidine (PHMG) phosphate is the main ingredient in the commercially available humidifier disinfectant. PHMG phosphate-based humidifier disinfectants can cause pulmonary fibrosis and induce inflammatory and fibrotic responses both *in vivo* and *in vitro*. However, toxicological mechanisms including genomic alterations induced by inhalation exposure to PHMG phosphate have not been elucidated. Therefore, this study evaluated the toxicological effects of the PHMG phosphate-containing humidifier disinfectant. We used DNA microarray to identify global gene expression changes in rats treated with PHMG phosphate-containing humidifier disinfectant for 4 weeks and 10 weeks. Functional significance of differentially expressed genes (DEGs) was estimated by gene ontology (GO) analysis. Four weeks post-exposure, 320 and 392 DEGs were identified in female and male rats, respectively (>2-fold, $p < 0.05$). Ten weeks post-exposure, 1290 and 995 DEGs were identified in females and males, respectively. Of these, 119 and 556 genes overlapped between females and males at 4 weeks and 10 weeks, respectively, post-PHMG phosphate exposure. In addition, 21 genes were upregulated and 4 genes were downregulated in response to PHMG phosphate in a time-dependent manner. Thus, we predict that changes in genomic responses could be a significant molecular mechanism underlying PHMG phosphate toxicity. Further studies are required to determine the detailed mechanism of PHMG phosphate-induced pulmonary toxicity.

1. Introduction

Humidifiers are widely used in dry seasons like winter to regulate indoor humidity. Humidifier disinfectants are often used to sterilize household humidifiers in poorly ventilated rooms. In 2011, an epidemiological study in Korea has reported that more than 28 people exposed to humidifier disinfectants were died especially children and pregnant women caused by respiratory

* Corresponding author at: Department of Inhalation Toxicology Research, Korea Institute of Toxicology, Jeonbuk 56212, Republic of Korea; Human and Environment Toxicology, University of Science and Technology, Daejeon 34113, Republic of Korea.
E-mail addresses: khlee@kitox.re.kr, khleekit@gmail.com (K. Lee).
[1] Theses authors contributed equally to this study.

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fibrosis. It has been suggested that humidifier disinfectants has emerged as a causative chemical of pulmonary diseases (Korea Centers for Disease Control and Prevention, 2011). As a result, Korea Food and Drug Administration (KFDA) had prohibited displaying humidifier disinfectants on the market (Kim et al., 2012). According to the Korea Ministry of Environment survey performed in 2013 and 2014, more than 530 people were harmed by use of humidifier disinfectants, 143 of them dead. Unfortunately, the number of victims is expected to increase by further investigation of the Korean government (Park, 2016).

One of the major ingredients of humidifier disinfectants, Polyhexamethyleneguanidine (PHMG) phosphate, classified as an antimicrobial biocide of the guanidine family is the main component in commercially available products. Although the non-guanidine compounds, Methylchloroisothiazolinone (CMIT) and Methylisothiazolinone (MIT), are often mixed in several humidifier disinfectant products, PHMG phosphate is not mixed with other components.

PHMG phosphate is an odorless, colorless, and noncorrosive chemical (Kuznetsova, 2004). It is less toxic than other disinfectants (Müller and Kramer, 2005), making it an ideal disinfectant for use in hospitals and household facilities (Oulé et al., 2008). PHMG phosphate has also been used to eliminate hospital infections in Russia owing to its antiseptic properties (Tsisanova and Salomatin, 2010). However, intraperitoneal injection of PHMG phosphate causes hepatitis and acute inflammation in blood (Solodun et al., 2011). Another study has reported that PHMG phosphate causes cholestatic liver injury when admixed with ethyl alcohol (Ostapenko et al., 2011). In particular, intratracheal instillation of PHMG phosphate induces severe lung inflammation, fibrosis, and thymic atrophy in mice (Song et al., 2014). In addition, PHMG phosphate aerosol particles induce ROS generation and release inflammatory cytokines and fibronectin, leading to pulmonary inflammatory and fibrotic change (Kim et al., 2016).

However, several toxicological mechanisms such as genomic alterations caused by PHMG phosphate, especially via the inhalation route, have not been elucidated yet. Therefore, analysis of gene expression profiling using DNA microarray is necessary for the understanding of PHMG phosphate toxicology with respect to pulmonary disease.

DNA microarray technology has been widely used for toxicological assessments to evaluate large amounts of gene expression changes simultaneously. It provides a global understanding of the molecular mechanisms of toxicants at the transcriptome level. Toxicogenomics encompasses the application of genomic tools, including DNA microarray, to the field of general toxicology (Hamadeh et al., 2002). Several studies have shown the successful application of toxicogenomics, as reviewed recently (Bartosiewicz et al., 2001; Lettieri, 2006). Identification of certain biomarkers by DNA microarray would therefore be valuable for hazard characterization.

In this study, we evaluate the toxicological effects of the PHMG phosphate-based humidifier disinfectant using DNA microarray to investigate overall changes in the rat lung transcription profile and to identify biomarkers of pulmonary disease associated with PHMG phosphate exposure.

2. Materials and methods

2.1. Animals

Seven-week-old male and female Sprague-Dawley rats were purchased from Orient Bio (Seongnam, Korea). The animals were acclimated for 1 week and 2 rats per cages during exposure periods in stainless-steel wire cages (255W × 465L 107 × 200H) in stable conditions as follows: temperature, 22 ± 3 °C; relative humidity, 50 ± 20%; and lighting condition, light 12 h/dark 12 h. Pelleted food for experimental animals (PMI Nutrition International, USA) and UV-irradiated (Steritron SX-1; Daeyoung, Seoul, Korea) and filtered tap water were given *ad libitum*. Each of 20 male and female animals was allotted to one of 4 groups (each group, n = 5): Group 1, 4 week control; group 2, 10 week control; group 3, 4 week exposure; and group 4, 10 week exposure. The experimental procedures were approved by the Institutional Animal Care and Use Committee of Korea Institute of Toxicology.

2.2. Inhalation exposure

The ingredient of PHMG phosphate containing product is shown in Table 1. The target concentration (0.4 μg/L) of PHMG phosphate was set based on an epidemiological study (Korea Centers for Disease Control and Prevention, 2011).

We employed a 1500 L whole-body inhalation chamber system (HCT, Icheon, Korea). All animals were exposed to PHMG phosphate aerosols or a filtered clean air for 6 h/day, 5 days/week, lasting for either 4 weeks or 10 weeks. The PHMG phosphate aerosol was generated using an atomizer (HCT, Icheon, Korea). The aerosol was mixed with dilution air, and distributed in the chamber at a total airflow of 300 L/min to obtain the PHMG phosphate concentration approximating 0.4 μg/L. The concentration of aerosol was measured by gravimetric analysis of 25-mm glass fiber filters (Pallflex products, Putnam, CT, USA), and it was performed 3 times during each exposure day. A summary of actual exposure concentration is shown in Table 2. Determination of aerosol particles size distribution inside the chamber was performed through a scanning nano particle spectrometer (SNPS; HCT, Icheon, Korea). The average particle size of PHMG phosphate was as follows: 4 weeks, 75.27 ± 1.75 nm; 10 weeks, 98.18 ± 1.73 nm. Representative images of particle size are shown in Fig. 1A and B. Water was supplied to animals through a drip system during the 6 h exposure period, but food was not provided. The temperature and relative humidity in the chamber were maintained at 24–25 °C and 48–49%, respectively.

2.3. RNA extraction

Lungs were ground and lysed using lysis buffer containing 2-mercaptoethanol. Total RNA was extracted from rat lungs treated with either PHMG phosphate or control using RNeasy mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. RNA quality was determined with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) prior to microarray. RNA concentration was quantified using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA).

2.4. Microarray hybridization

The 3 individual RNA samples were randomly selected from each group for the microarray expression analysis. Five micrograms of purified total RNA from rat lung was hybridized to Affymetrix GeneChip Rat Genome 230 2.0 plus microarrays according to the Affymetrix GeneChip Expression Analysis Technical Manual (www.affymetrix.com). Briefly, RNA was reverse transcribed into double stranded cDNA and then transcribed into

Table 1
Ingredient of PHMG phosphate containing product.

Substance	Ratio (%)	CAS No.
25% PHMG phosphate	0.5	89697–78–9
H_2O	99.5	–