Article

Developmental Toxicity and Thyroid Endocrine Disruption of Polyhexamethylene Guanidine Hydrochloride and Humidifier Disinfectant in Zebrafish Larvae

Suhyun Park ¹ , Hyojin Kim ² and Kyunghee Ji ^{2,*}

¹ Institute of Natural Sciences, Yongin University, Yongin 17092, Korea; park940205@gmail.com

² Department of Occupational and Environmental Health, College of Health and Welfare Science, Yongin University, Yongin 17092, Korea; khj9080a@gmail.com

* Correspondence: kyungheeji@yongin.ac.kr; Tel.: +82-31-8020-2747; Fax: +82-31-8020-2886

Abstract: Polyhexamethylene guanidine (PHMG), a major component of humidifier disinfectants (HDs), is responsible for the outbreak of pulmonary diseases in pregnant women and children in South Korea. This study aims to characterize the developmental toxicity and thyroid endocrine disruption of Vegetable Home Cleanup HD and its main component, PHMG-hydrochloride (PHMG-H), in zebrafish embryo/larvae after a 7-d exposure. Acute lethality, development, whole-body thyroid hormones, and transcription of genes related to the hypothalamus-pituitary-thyroid axis were investigated. Zebrafish embryos exposed to the actual-use concentration (0.6%) of HD exhibited significant embryo coagulation and larval mortality. The concentration of triiodothyronine (T3) was significantly high in fish exposed to 0.4 mg/L PHMG-H, accompanied by the downregulation of the *tsh β* gene. These results suggest a feedback mechanism for the regulation of increased T3 levels. Significant decrease of thyroxine (T4) concentration, increase of T3/T4 ratio, and upregulation of the *deio2* gene in fish exposed to PHMG-H suggested that there was an increase in the active T3 due to the catalysis of outer ring deiodination. Future research is required to determine the factors that contribute to the differences in toxicity between the two counter ions of PHMG.

Keywords: developmental toxicity; humidifier disinfectant; polyhexamethylene guanidine hydrochloride; thyroid endocrine disruption; zebrafish

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1. Introduction

Polyhexamethylene guanidine (PHMG), a guanidine-based cationic biocide, is an antimicrobial agent that acts on a broad spectrum of bacteria, viruses, and fungi [1]. PHMG has been widely used as an antiseptic in medicine, cotton fabric, and the food industry [2,3]. This compound has been registered as a disinfectant of medical devices by the Food and Drug Administration in the United States [4]. In South Korea, it is commonly used as a major component of household humidifier disinfectants (HDs) by mixing it with water in the humidifier reservoir in order to prevent the growth of microorganisms [5]. However, inhalation from a humidifier's aerosolizer was identified as a pulmonary toxicant after an epidemic outbreak of HD-associated lung injury [6,7].

Numerous studies have reported that HD products that contain PHMG-phosphate (PHMG-P) are associated with lung disorder, including interstitial pneumonitis and lung fibrosis [8,9]. PHMG-P induced pulmonary injuries in zebrafish, including inflammation, histological changes, and fibrosis [10]. Previous zebrafish study also confirmed that the cationic nature is considered to be one of the main causes of PHMG-P-induced cytotoxicity [11]. The adverse developmental effects of two HDs (Oxy® and Wiselect) and their major component, that is PHMG-P, are associated with thyroid endocrine disruption in zebrafish embryo/larvae [12]. PHMG-hydrochloride (PHMG-H), another derivative of PHMG, was also considered to be involved in the outbreak [9]; however, there is limited information on its properties, such as its content or toxicity in HD.

Few epidemiological, in vivo, and in vitro studies have reported on the toxicity of PHMG-H. An epidemiological study in Russia demonstrated that illegal cheap vodka (brand name “Extrasept-1”), which was composed of ethanol, diethyl phthalate, and PHMG-H, resulted in acute liver malfunction, with a severe inflammatory component causing high mortality [13]. The median lethal dose was estimated to be 600 mg/kg in SD rats, which was accompanied by signs of neurotoxicity [1]. Two-week repeated inhalation exposure to PHMG-H caused respiratory effects in rats, and the no observable adverse effect level was calculated to be below 1 mg/m³ [14]. Furthermore, PHMG-H induced oxidative stress in the lungs of F344 rats after a one-week exposure period via inhalation [15]. In human alveolar epithelial A549 cells, PHMG-H induced cellular toxicity by generating intracellular reactive oxygen species (ROS) [16]. There was significant inhibition of green algae (*Desmodesmus communis*) at a concentration of 0.005 mL/L PHMG-H; the median lethal concentration value in zebrafish (*Danio rerio*) was 0.043 mL/L PHMG-H [17]. All adult zebrafish died within 48 h of exposure to PHMG-H at concentrations of 0.05, 0.075, 0.1, and 0.125 mL/L [17]. As approximately 30% of young Korean children were exposed to HD [18] and had mental and developmental disorders [19], this reflects the importance of developmental effects and its mechanism. However, most of the toxicity studies of PHMG-H have focused on lung disease and its toxicity mechanism, and no information is available on other toxicity including developmental toxicity.

In vertebrates, the synthesis, secretion, transport, and metabolism of thyroid hormones (THs) are controlled by the hypothalamus-pituitary-thyroid (HPT) axis [20]. Thyrotropin-releasing hormone (TRH) secreted in the hypothalamus is the primary stimulator of thyroid stimulating hormone (TSH) released in the pituitary. Subsequently, TSH regulates the production of the biologically active triiodothyronine (T3) and thyroxine (T4) in the thyroid. Disruption of the HPT axis can adversely affect the functioning of the thyroid endocrine system and subsequently impact the growth and development of an organism. The effects of PHMG-P and two HDs containing this component on the thyroid endocrine system have been studied [12], but no studies have been conducted on the other derivatives PHMG-H and HD containing this component.

This study investigated the acute mortality, developmental effects, and thyroid endocrine disruption by designing a parallel study of PHMG-P and two HD products containing PHMG-P [12]. We used zebrafish as a model organism in developmental toxicity and endocrine disruption based on environmental toxicology [21]. Zebrafish eggs were exposed to Vegetable Home Cleanup HD product (hereafter Vegetable HD), and its main component, PHMG-H, for 7 d. Following the Organization for Economic Co-operation and Development (OECD) test guideline 236 with minor modification, several core endpoints for acute lethality and growth were observed. The highest concentration of PHMG-H (40 mg/L) was determined based on the PHMG-H content in Vegetable HD (6917 mg/L, [9]). To identify the underlying mechanism, the levels of THs and transcription of ten genes involved in the HPT axis were also measured.

2. Materials and Methods

2.1. Test Chemicals

Vegetable HD (General bio, Korea), which is mainly composed of PHMG-H [9,22,23], was donated by volunteers. Additionally, PHMG-H (Cas no. 57028-96-3) was purchased from Henan Tianfu Chemical (Zhengzhou, China).

2.2. Fish Maintenance and Exposures

Pairs of adult zebrafish (AB strain) were cultured in an aquarium tank under a constant photoperiod (14:10 h light/dark) and temperature (28 ± 1 °C). Zebrafish were fed twice daily with a fixed amount of newly hatched brine shrimp (*Artemia nauplii*) and mosquito larvae. Zebrafish eggs were collected from adult zebrafish pair (five female and five male fish per tank with five replicates). Fertilized eggs were sterilized with 0.3% hydrogen peroxide, one of the methods to prevent fungal and bacterial egg infection [24], and rinsed

thoroughly with dechlorinated tap water. Once these fertilized eggs reached the blastula stage (2 h post fertilization), they were exposed to Vegetable HD (0%, 0.0006%, 0.006%, 0.06%, and 0.6% [*v/v*]) and PHMG-H (0, 0.004, 0.04, 0.4, 4, and 40 mg/L) for 7 d following OECD test guideline 236 with minor modification [25]. Twenty embryos were placed in each well of a 24-well plate, and there were four replicate plates for each exposure concentration. The highest concentration of PHMG-H (40 mg/L) was based on the amount of PHMG-H contained in Vegetable HD (6920 µg/mL), as reported in previous studies [9]. During the experimental period, the dead organisms were removed as soon as possible, and the remaining solution was renewed every 24 h.

The endpoints related to the acute lethality and the retardation of development, including coagulation of embryo (24 h), non-formation of somite (24 h), absence of active heartbeat (48 h), non-detachment of the tail (48 h), and the presence of malformation (24 h~96 h) were recorded using a stereoscopic microscope. Hatchability (48 h~7 d, every 24 h), time to hatch (48 h~7 d, every 24 h), larval survival (7 d), and body length (7 d) were also observed. All fish were maintained in accordance with the animal care guidelines [26] and treated humanely.

On the 7th day of fish exposure, ten zebrafish larvae with four replicates were randomly collected for the measurement of gene transcription. Whole bodies of zebrafish larvae were preserved in 250 µL RNAlater reagent (QIAGEN, Hilden, Germany) at −80 °C until analysis. Additional sets of exposure were performed to measure THs. A total of 600 larvae (150 larvae per treatment with four replicates) were collected and stored at −80 °C until analysis.

2.3. Hormone Measurement

Whole-body concentrations of T3 and T4 were measured using an enzyme-linked immunosorbent (ELISA) assay kit for T3 (Cat number CEA453Ge, Cloud-Clone Corp., Wuhan, China) and T4 (Cat No. CEA452Ge, Cloud-Clone Corp.). The collected larvae were homogenized in phosphate-buffered saline (PBS) and centrifuged at 2000× *g* for 10 min at 4 °C. The supernatant was used for T3 and T4 analyses. The levels of THs were quantified at 450 nm using an Epoch™-Take3 microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). The T3/T4 ratio were normalized to the control and the degree of increase or decrease in treatment group was expressed as a fold difference.

2.4. Quantification of Gene Transcription

The transcriptions of ten genes (*trh*, *trhr1*, *tshβ*, *trα*, *trβ*, *tpo*, *tg*, *nis*, *deio1*, and *deio2*) related to the HPT axis and two reference genes (*β-actin* and *rpl8*) were analyzed. Whole bodies of zebrafish larvae were homogenized (ten larvae per sample with four replicates) and total RNA was isolated using the RNeasy mini kit (QIAGEN, Hilden, Germany). RNA quality (260/280 ratio >1.8) was validated using an Epoch™-Take3 microplate spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA). Then, complementary DNA (cDNA) was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Green™ PCR master mix kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The analyzed genes and primer sequences are listed in Table 1. The thermal cycling conditions were maintained at 50 °C for 1 min and 95 °C for 10 min; this was followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curve analyses were performed at the end of the amplification phase to ensure the amplification of a single product. The threshold cycle (Ct) was determined for each reaction, and the Ct values of the genes of interest were normalized to the reference genes using the $2^{-\Delta\Delta C_t}$ method [27]. The degree of induction or inhibition of the genes of interest was expressed as a fold difference, as compared to the normalized control values.

Table 1. Sequences of primers for the gene measurements.

Gene	Accession No.	Primer	Sequences (5'-3')
<i>β-actin</i>	NM_131031	Forward	TGCTGTTTCCCTCCATTG
		Reverse	TCCCATGCCAACCATCACT
<i>rpl8</i>	NM_200713	Forward	TTGTTGGTGTGTTGCTGGT
		Reverse	GGATGCTCAACAGGGTTCAT
<i>trh</i>	NM_001012365	Forward	GCTCTCTCCGTCGGTCTGTT
		Reverse	GCGAGATCCGTGCTGATGA
<i>trhr1</i>	NM_001114688	Forward	CAGTGCCATCAACCCTCTGA
		Reverse	GGCAGCGCGGAACCTTCT
<i>tshβ</i>	NM_181494	Forward	GCAAAACCCACAGTGATGAATG
		Reverse	TGCACAGGTTTGGAGCATCTC
<i>tra</i>	NM_131396	Forward	CCCCTTCCTGCACATG
		Reverse	AGCGGCGGGAACAGTTC
<i>trβ</i>	NM_131340	Forward	TGGCATGGCTAAGACTTGGT
		Reverse	TCAGCTTCCGCTTGGCTAA
<i>tpo</i>	EU267076	Forward	GTTCGGTCTGCCAGGACACT
		Reverse	TCCAAGCGCTTCAGCAGAGT
<i>tg</i>	XM_689200	Forward	GTCTCTTGAAGTGTTCGAATGACAAG
		Reverse	AAAGGCGGGCCATTAAGG
<i>nis</i>	NM_001089391	Forward	AATCAAGCCACAGGCCTGAA
		Reverse	AATGTGCAGATGAGCCCAGTT
<i>deio1</i>	BC076008	Forward	AACTTGGAGGAGAGGCTTGCT
		Reverse	AGGGCATGGAGGGTCTTCTT
<i>deio2</i>	NM_212789	Forward	CGCGAAATGGGCTTGCT
		Reverse	CCAGGCAAAATCTGCAAAGTTA

2.5. Statistical Analysis

All data are shown as mean $\pm$ standard deviation. Dunnett's one-way analysis of variance (ANOVA) was performed using the IBM SPSS Statistics version 27 (IBM Corp., New York, NY, USA) to determine the significant differences between the control and treatment groups. Before performing ANOVA, normality and homogeneity of variances were confirmed using the Kolmogorov-Smirnov and Levene's tests, respectively. A p -value ≤ 0.05 was used as the criterion for determining the statistical significance.

3. Results

3.1. Acute Lethality and Developmental Effects of Zebrafish Embryo/Larvae

The survival and developmental effects of HD and PHMG-H on fish embryos/larvae are shown in Figure 1. Coagulation was significantly increased in embryo exposed to 0.6% Vegetable HD and ≥ 4 mg/L PHMG-H, while that was significantly decreased in embryo exposed to 0.06% Vegetable HD (Figure 1A). Malformation rate was significantly increased in embryo exposed to 0.4 mg/L PHMG-H (Figure 1B), showing morphological changes such as pronounced yolk sac edema and spinal curvature. Hatchability and larval survival were significantly decreased in zebrafish that were exposed to 0.6% Vegetable HD and ≥ 4 mg/L PHMG-H (Figure 1C,D). The whole-body length of live larvae up to the 7th day of exposure was significantly decreased in fish exposed to 0.4 mg/L PHMG-H (Figure 1E).

However, no significant effects on somite formation, heartbeat, and detachment of the tail were observed in fish exposed to the Vegetable HD and PHMG-H (data not shown).

Figure 1. Cont.

Figure 1. Acute lethality and developmental effects in zebrafish embryo/larvae after exposure to Vegetable HD and PHMG-H for 7 d. (A) Coagulated embryos (%), (B) malformation rate (%), (C) hatchability (%), (D) larval survival (%), and (E) body length of larvae (mm). The values are expressed as mean $\pm$ standard deviation of four replicates. Asterisk indicates significant difference from the control group ($p < 0.05$).

3.2. Hormonal Changes in T3 and T4

There were no significant changes in the T3 and T4 levels of zebrafish that were exposed to Vegetable HD (Figure 2A,B). However, the concentration of T3 was significantly higher and that of T4 was significantly lower in fish that were exposed to 0.4 mg/L PHMG-H (Figure 2A,B). The T3 to T4 ratio (T3/T4) was significantly increased at 0.4 mg/L PHMG-H (Figure 2C).

Figure 2. Cont.

Figure 2. Concentrations of (A) triiodothyronine (T3), (B) thyroxine (T4), and (C) T3/T4 ratio in zebrafish larvae after exposure to Vegetable HD and PHMG-H for 7 d. The values are expressed as mean $\pm$ standard deviation of four replicates. Asterisk indicates significant difference from the control group ($p < 0.05$).

3.3. Transcriptional Changes in Genes Related to HPT Axis

The expression of *trhr1*, *tsh β* , *tpo*, and *tg* mRNAs was significantly downregulated in fish exposed to 0.06% Vegetable HD-treated group (Figure 3A). Moreover, the expression of *trh*, *trhr1*, *tsh β* , *tpo*, and *tg* mRNAs was significantly lower in zebrafish larvae exposed to 0.4 mg/L PHMG-H (Figure 3B). The expression of *deio2* mRNA was significantly upregulated in fish exposed to 0.06% Vegetable HD and 0.4 mg/L PHMG-H (Figure 3A,B).

Figure 3. Cont.

Figure 3. Transcription of genes related to the hypothalamus-pituitary-thyroid axis in zebrafish larvae after exposure to Vegetable HD (A) and PHMG-H (B) for 7 d. Fold change values of gene transcription are expressed as mean $\pm$ standard deviation of four replicates. Asterisk indicates significant difference from the control group ($p < 0.05$).

4. Discussion

Several types of chemicals were widely used as HDs in Korea until the end of 2011 [28]. A number of studies have been conducted on pulmonary inflammation and fibrosis as a toxic mechanism of lung disease [4,10]. However, information developmental toxicity by thyroid endocrine disruption is limited. The results of the present study demonstrated that Vegetable HD and PHMG-H affected the survival, development, production of THs, and the transcription of key genes involved in the HPT axis.

The toxicity of Vegetable HD was lower than that of Oxy® and Wiselect HDs, which were mainly composed of PHMG-P [12]. This result was based on the endpoints of coagulation of the embryo, hatchability, larval survival, body length, concentrations of THs, and gene transcription. The two counter ions of PHMG may be responsible for the observed results. A previous study also reported that people exposed to HD containing PHMG-H did not suffer from HD-associated lung disease, even though the concentration used was much higher than that of PHMG-P [29]. Differences in gene transcription related to the HPT axis between PHMG-H and PHMG-P can explain the differences in toxicity. Further research is required to determine whether the ionic interactions of PHMG are related to toxicity.

As numerous chemicals constitute a product, the toxicity of the entire product is generally greater than that of a single chemical [30]. However, in the present study, the toxicity of PHMG-H was greater than that of Vegetable HD in all observed endpoints. These results suggest that the chemical reactions of the components present in the HD product reduced the toxicity of PHMG-H. One explanation is that due to the reaction

among different chemicals, the guanidine group does not bind to the cell membrane, which is composed of neutral phospholipids [31]. Further studies are required to fully understand the important factors that determine the product toxicity.

In teleost fish, TSH is the primary stimulatory hormone for the thyroid gland. Circulating levels of T3 and T4 are regulated by negative feedback through the HPT axis by altering the release of TSH from the pituitary [32]. TRH and corticotropin-releasing hormone (CRH) are the main stimulators of TSH [18]. While there has been extensive debate on the sensitivity of molecular biomarkers, the measurement of gene transcription has been generally accepted as a sensitive biomarker for endocrine disruption [33]. The level of *tsh β* mRNA is well correlated with the production of TSH [34]. In the present study, Vegetable HD and PHMG-H significantly downregulated the *tsh β* gene and increased the concentration of T3. These results suggest that Vegetable HD and PHMG-H can modulate the concentrations of TSH, which can subsequently affect the production of THs.

The ratio of T3/T4 has been used as an indicator of TH homeostasis in the zebrafish larvae after exposure to endocrine disrupting chemicals [35]. A significantly greater T3/T4 ratio was observed in zebrafish larvae after exposure to PHMG-H, indicating that balance of THs was disrupted by this compound. Altered thyroid hormone balance could further result in adverse effects on development, growth, regulation of stress, and energy metabolism in fish [36]. T4 is converted into T3, bioactive form of TH, by deiodination [37]. Therefore, increased T3 levels are mostly due to either increase of T4 synthesis or increase of deiodinase (deio)-mediated conversion of T4 to T3 [38]. The finding of significant increase of T3/T4 ratio, based on the decrease of T4, suggests that deiodination could play a major role in the regulation of circulating T3 levels.

The changes in THs, e.g., increase of T3 and decrease of T4, are well supported by significant upregulation of *deio2* gene that were observed in the present study. In fish, three types of deio enzymes, namely deio1, deio2, and deio3, play different roles in regulating TH levels. For example, deio1 affects iodine recovery and TH removal [39], deio2 catalyzes the production of active T3 from T4 [40], and deio3 plays a role in the inactivation of enzymes [41]. Given the increase of T3 and *deio2* gene transcription, and the decline of T4 observed in this study, it can be suggested that PHMG-H can increase whole-body T3 concentration by increasing *deio2* activities.

Thyroid peroxidase (tpo) catalyzes the iodination of thyroglobulin (tg), which is stored for THs [42]. A previous study demonstrated that tpo inhibition could result in reduced production of T4 [43]. In the present study, decreased transcription of *tpo* and *tg* genes was observed in fish that were exposed to Vegetable HD and PHMG-H, suggesting the reduction of TH synthesis and inhibition of tg production. The decrease of T4 levels could either be caused by TPO inhibition, by deio induction, or by a combination of both mechanisms.

In conclusion, the results of this study demonstrate the acute mortality, developmental toxicity, and thyroid endocrine disruption of Vegetable HD and PHMG-H. Exposure to an amount that is recommended by the manufacturer of Vegetable HD may result in acute mortality. The sub-lethal concentration of PHMG-H affects the feedback regulatory circuits of THs, which delays the development of zebrafish larvae. The major reason of differences in toxicity between the two counter ions of PHMG warrant further investigation.

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