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• Original Article

Eye irritation tests of polyhexamethylene guanidine phosphate (PHMG) and chloromethylisothiazolinone/methylisothiazolinone (CMIT/MIT) using a tissue model of reconstructed human cornea-like epithelium

Juyoung Park, Handule Lee, Kwangsik Park

College of Pharmacy, Dongduk Women's University, Seoul 02748, Korea

Disinfectants including polyhexamethylene guanidine phosphate (PHMG) and mixtures of chloromethylisothiazolinone/methylisothiazolinone (CMIT/MIT) have been widely used in Korea to prevent microbial growth in the humidifier water, which triggered an outbreak of serious respiratory diseases. In addition to the respiratory syndrome, disease-related symptoms including liver toxicity, asthma, and skin allergies were also found after extensive survey of people exposed to the humidifier disinfectants (HDs). In this study, eye irritation tests were performed based on the Organization for economic co-operation and development (OECD) test guidelines 492 using EpiOcular™ which is a tissue model of reconstructed human cornea-like epithelium. As results, the raw materials of PHMG (26% as active ingredient) and CMIT/MIT (1.5% as active ingredient) were classified under UN globally harmonized system of classification and labeling of chemical (GHS) category 1 or category 2. However, aqueous dilutions of raw materials such as market products of HDs that contain 0.13% of PHMG and 0.03% of CMIT/MIT or further dilutions of the market products for humidifier that contain 0.0013% of PHMG and 0.0003% of CMIT/MIT were classified under any category, which suggested absence of eye irritation at the test concentration.

Keywords: eye irritation, polyhexamethylene guanidine phosphate, chloromethylisothiazolinone, methylisothiazolinone, EpiOcular™

INTRODUCTION

In April 2011, clinicians at major hospitals in Korea reported a few cases of atypical lung injury among young pregnant women. A series of case studies revealed that the use of humidifier disinfectants (HDs) including polyhexamethylene guanidine phosphate (PHMG) was significantly associated with the development of serious lung injury [1,2,3]. The disinfectants were known to be introduced into consumer markets since 1994, and their use was rapidly increased over the years until the risk was known to the people. Based on a survey of the use of HDs, up to 30% of the entire Korean population seemed to be exposed to chemicals between 2006 and 2011 [4]. According to an online survey of the HDs exposure, the range of HDs usage was an estimated 3.5 to 4.0 million, and about 56% of the total responders were diagnosed with respiratory diseases [5]. Health conditions manifested by the responders exposed to HDs included asthma, pneumonia, bronchitis, rhinitis, atopic dermatitis, headaches, eye disease, kidney ailments, liver disease and others. According to the report, a significant dose-response relationship was observed between the duration of daily exposure and heath condition prevalence [6]. However, many symptoms have not been corroborated based on epidemiological evidence or toxicological tests except for a few cases involving lung fibrosis.

Many studies have suggested that HDs, particularly PHMG and CMIT/MIT mixtures, are associated with diffuse pulmonary fibrosis [7]. Seven-week-old male Sprague-Dawley rats exposed to PHMG aerosol particles for 3 weeks showed pulmonary inflammation and fibrosis including increased levels of inflammatory cytokines and fibronectin mRNA, as well as histopathological changes [8]. PHMG exposure induced immune cell infiltration and significant collagen deposition in

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Corresponding author: Kwangsik Park
College of Pharmacy, Dongduk Women's University, Seoul 02748, Korea
E-mail: kspark@dongduk.ac.kr

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the peri-bronchiolar and interstitial areas of lungs. It also induced fibroblast proliferation, and hyperplasia of type II epithelial cells in mice treated for 3 weeks via intratracheal instillation [9]. Intratracheal instillation of PHMG to C57BL/7 mice (1.2 mg/kg) significantly increased the number of neutrophils in the Bronchoalveolar lavage fluid (BALF). Histopathological analysis showed inflammatory cell infiltration and fibrosis in the terminal bronchioles and alveoli in the lungs of PHMG-treated mice [10]. A few epidemiological studies exploring the possible incidence of lung disease caused by CMIT/MIT also showed peripheral airway dysfunction in children. Limited data suggested that patients exposed to HDs containing only CMIT or MIT showed lung injuries clinically similar to those exposed to PHMG [11,12]

Many epidemiological and toxicological studies investigating lung diseases triggered by HDs are now underway. However, few studies have investigated the potential damage to other target organs such as central nervous system, liver, kidney, blood, heart, and skin. Eyes appear to be the direct target of HDs; however, no epidemiological survey of eye diseases or toxicological testing has ever been conducted. In this study, eye irritation tests based on the OECD Test guidelines (TG) 492 were performed using reconstructed human cornea-like epithelium EpiOcular™, to investigate the potential risk of HDs [13,14].

MATERIALS AND METHODS

Materials

Polyhexamethylene guanidine phosphate (PHMG) was provided by Dr. K. Lee of Korea institute toxicology (Daejeon, Korea). The active ingredient was known to be 26%. The stock solution was used for the preparation of HDs consumer product. CMIT/MIT, which is also known as KathonTM CG and produced by Dow Chemicals, was purchased from the supplier. The active ingredients of KathonTM CG include 5-chloro-2-methyl-4-isothiazolin-3-one (1.15%) and 2-methyl-4-isothiazolin-3-one (0.35%), present in the total aqueous mixture (1.5%) of CMIT/MIT. Magnesium nitrate and magnesium chloride salts were added as inert ingredients up to 23% by weight.

Proficiency testing for technical reliability was performed using chemicals purchased from Sigma-Aldrich (St. Louis, MO, USA). Reconstructed human Cornea-like Epithelium, EpiOcular™ OCL-200 kit, was ordered from MatTek Corporation (Ashland, MA, USA).

Preparation of test chemicals and exposure concentration

According to the supplier's protocol and OECD TG 492 liquid chemicals should be applied to the EpiOcular™ in volumes of 50 μL per tissue, and solid chemicals up to 50 mg by weight [13,14]. Sterile deionized water was used as a negative control and methyl acetate was used as the positive control. The volumes of both the negative and the positive controls were similar (50 μL). The active ingredients of HDs were tested in three different cases. In the first case, raw materials were used. The concentrations of the active ingredient in the raw materials were 26% (260,000 ppm) of PHMG and 1.5% (15,000 ppm) of CMIT/MIT, respectively. In the second case, the market products which is the diluted formulation were used and the concentration of PHMG was 1,300 ppm. That of CMIT/MIT was 300 ppm. In the last case, assuming the use of concentrations in the humidifier water, 13 ppm of PHMG and 3 ppm of CMIT/MIT mixture were used. Based on the protocol, the volume of disinfectants applied to EpiOcular™ tissue was also 50 μL.

The EpiOcular™ assay

The EpiOcular™ eye irritation test (OCL-200-EIT) was performed based on the protocol provided by the supplier, MatTek and OECD test guideline 492 [13,14]. On the day of receipt, the tissue was equilibrated to room temperature in its 24-well shipping container for about 15 min. Appropriate volume of medium was pre-warmed to 37°C and 1 mL was aliquoted into the wells of pre-labeled 6-well plates. The EpiOcular™ tissues were carefully transferred from the 24-well shipping container to the 6-well plates, and pre-incubated for 1 h in a CO_2 incubator.

The culture media comprising Dulbecco's modified eagle's medium (DMEM) were replaced with a fresh medium and the tissues were incubated for an additional 16 h. Test materials including proficiency testing chemicals were applied to the surface of the cornea tissue model at a volume of either 50 μL by liquid for 30 min or by weight of 50 mg by solid for 6 h in duplicate, respectively. Methyl acetate 50 μL was added to the positive control wells and sterile deionized water 50 μL was used in the negative control wells. The tissues were washed 3 times with 100 mL of Ca^{2+} Mg^{2+} free Dulbecco's phosphate buffered saline (D-PBS). Post-exposure immersion was performed at room temperature in culture media for 12 min in case of liquid chemicals, and for 25 min in case of solid chemicals, respectively. The tissues were transferred to fresh culture media followed by post-exposure incubation for 2 h and 18 h, using liquid and solid chemicals, respectively. The cell viability test was evaluated by MTT assay [15,16].

Classification of eye irritation

Chemicals are identified as "not requiring classification and

labeling" according to UN globally harmonized system of classification and labeling of chemical (GHS) if the mean percent tissue viability after exposure and post-exposure incubation is greater than the established cut-off value (60%) for tissue viability. In this case, no further testing by additional methods was required [13,14]. If the mean tissue viability is less than or equal to 60%, it was classified as UN GHS category 1 or 2 according to the 2015 version of the OECD TG 492 guideline. However, the "category 1 or 2" classification was changed to "no prediction can be made" in the revised and adopted version of OECD TG 492 [14]. In this case, further testing with other test methods will be required because EpiOcular™ test methods show a certain number of false positive results. The result of "no prediction can be made" would require additional information for classification purposes. Details are described in OECD TG 492 [13,14]

RESULTS

Proficiency and reliability

Prior to the routine use of EpiOcular™ tests for regulatory purposes, a laboratory should demonstrate its technical proficiency via accurate prediction of the proficiency chemicals. Fifteen chemicals were used in the proficiency test in OECD TG 492 adopted in 2015; however, a few of these chemicals were replaced in the revised version of OECD TG 492 [14]. Table 1 lists the chemical name, CAS number, physical state and viability of the proficiency chemicals. According to the protocol provided by the EpiOcular™ supplier, 5 to 10 proficiency chemicals among those listed in OECD TG, were sufficient for testing and the results of test laboratory need to be harmonized with those of OECD TG 492. In this study, five chemicals including sodium oxalate were selected and tested. The results are shown in Table 2. Results of viability and classification of the chemicals used in this study were fully harmonious with those of OECD TG 492, which guaranteed the reliability and technical proficiency in our laboratory.

EpiOcular™ test of the active ingredients in humidifier disinfectants

The active ingredients in the HDs, including both PHMG and CMIT/MIT decreased cell viability to less than 60% when applied to reconstructed cornea-like epithelium similar to the raw material concentrations. The concentration in raw material was known to be 26% in case of PHMG and 1.5% in case of CMIT/MIT, equal to 260,000 ppm and 15,000 ppm, respectively. Generally, the raw materials were diluted to obtain consumer products of HDs and the specified concentrations were

Table 1. Proficiency test substances listed in 2015 version of OECD TG 492

Classification	Chemical name	CAS No.	Physical state	Viability (%)	Classification
In vivo category 1	Methylthioglycolate	2365-48-2	liquid	10.9 ± 6.4	cat 2 / cat 1
	Tetraethylene glycol diacrylate	17831-71-9	liquid	34.9 ± 15.3	cat 2 / cat 1
	2,5-Dimethyl-2,5-hexanediol	110-03-2	solid	2.3 ± 0.2	cat 2 / cat 1
	Sodium oxalate	62-76-0	solid	29.0 ± 1.2	cat 2 / cat 1
In vivo category 2A	2,4,11,13-Tetraazatetradecane-diimidamide, N,N''-bis(4-chlorophenyl)-3,12-diimino-, di-D-gluconate (20%, aqueous)	18472-51-0	liquid	4.0 ± 1.1	cat 2 / cat 1
	1,5-Naphthalenediol	83-56-7	solid	21.0 ± 7.4	cat 2 / cat 1
In vivo category 2B	Diethyl toluamide	134-62-3	liquid	15.6 ± 6.3	cat 2 / cat 1
	2,2-Dimehtly-3-methylenebicyclo [2.2.1] heptane	79-92-5	solid	4.7 ± 1.5	cat 2 / cat 1
In vivo no category	1-Ethyl-3-methylimidazolium ethylsulphate	342573-75-5	liquid	79.9 ± 6.4	no cat
	Dipropyl disulphide	629-19-6	liquid	81.7 ± 6.4	no cat
	Piperonyl butoxide	1951-03-06	liquid	104.2 ± 4.2	no cat
	Polyethylene glycol (PEG-40) hydrogenated castor oil	61788-85-0	viscous	77.6 ± 5.4	no cat
	1-(4-Chlorophenyl)-3-(3,4-dichlorophenyl) urea	101-20-2	aolid	106.7 ± 5.3	no cat
	2,2'-Methylene-bis-(6-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)-phenol)	103597-45-1	aolid	102.7 ± 13.4	no cat
	Potassium tetrafluoroborate	14075-53-7	aolid	88.6 ± 3.3	no cat

Table 2. Results of proficiency testing for eye irritation based on the OECD TG 492

Chemical name	Cell viability (%)	Difference of viability (%)	Classification	
			Classification in OECD TG492	Results in this study
1-Ethyl-3-methylimidazolium ethylsulphate	90.3	8.0	no cat	no cat
Dipropyl disulphide	83.0	9.5	no cat	no cat
Piperonyl butoxide	102.1	5.6	no cat	no cat
2,5-Dimethyl-2,5-hexanediol	1.3	0.5	cat 2 / cat 1	cat 2 / cat 1
Sodium oxalate	14.8	2.8	cat 2 / cat 1	cat 2 / cat 1

Table 3. Results of EipOcular™ assay for the humidifier disinfectants

Substance		Viability (%) (Mean ± S.D.*)	Results of classification**
Negative control		100.0 ± 5.2	no cat
Positive control (Methyl acetate)		42.7 ± 11.5	cat 2 / cat 1
PHMG	in raw material (260,000 ppm)	40.4 ± 11.0	cat 2 / at 1
	in market product (1,300 ppm)	102.9 ± 15.9	no cat
	in humidifier (13 ppm)	106.8 ± 17.2	no cat
CMIT/MIT	in raw material (15,000 ppm)	3.7 ± 1.0	cat 2 / cat 1
	in market product (300 ppm)	105.7 ± 14.6	no cat
	in humidifier (3 ppm)	108.9 ± 10.1	no cat

*This results were obtained by performing each 3 independent runs.
** Classification was performed by OECD TG 492 adopted in 2015. In the revised version of 2018 TG, "cat 2 / cat 1" was replaced by "no prediction can be made".

1,300 ppm (PHMG) and 300 ppm (CMIT/MIT), respectively. Consumers may use the market product by 100–300-fold dilution for humidifier, based on the specification. According to the patterns of usage surveyed by Park et al., the concentration for the worst case of use appeared to be 13 ppm of PHMG and 3 ppm of CMIT/MIT [17].

The viability of methyl acetate as a positive control substance was 42.7±11.5% and that of negative control was 100.0±5.2% (Table 3), which suggests adequate performance of the test system. The viability and classification of the three different concentrations of HDs (raw materials, consumer market products and used concentration in humidifier) are also shown in Table 3. Cell viability was significantly decreased with the raw material concentrations: 40.4±11.0% with PHMG and 3.7±1.0% with CMIT/MIT. The classifications were "category 1 or 2". However, cell viability was not decreased under the concentrations specified for consumer market products and humidifier water box. They were classified under "no category", which suggests no irritation.

DISCUSSION

The Draize rabbit eye test was developed for the identification and evaluation of toxic reactions when the eyes were exposed to the test materials [18]. It was originally used to evaluate the safety of cosmetics and was further extended to evaluate the safe use of insecticides, sun screens and antiseptics [19]. In the test, changes in cornea, conjunctiva, and iris in the rabbit eye served as the endpoints of toxicity. However, the test has been criticized due to the large variation in the test results and the species differences between human and rabbit [20]. Moreover, this test inflicts severe pain on the rabbit during the test procedure. Alternative methods have been developed to reduce the suffering of animals from test chemicals [21].

Among the alternative methods, the short time exposure in vitro test was developed by OECD to identify chemicals inducing serious eye damage using cultured Statens serum institute rabbit cornea (SIRC) cells [22]. In the method, the classification depended on the cell viability at chemical concentrations of 5% and 0.05%. When cell viability was 70% and less at both test concentrations (5% and 0.05%), the chemical is classified as "category 1", which indicates serious eye damage. Another alternative method developed by OECD is based on Reconstructed human cornea-like epithelium (RhCE), which is used to identify chemicals not requiring classification or labeling for eye irritation of serious eye damage [14]. In this method, three different types of RhCE were introduced, along with their own protocols including the number of tissue replicates, treatment doses and application, exposure time and temperature, etc.

In this study, RhCE of EpiOcular™ was used for PHMG and CMIT/MIT. The standard materials for proficiency test included 15 chemicals listed in OECD TG 492 as shown in Table 1, while 5 chemicals were adequate based on the protocols of EpiOcular™ supplier, MatTek Corporation [13,23].

As shown in Table 2, the proficiency test results were in harmony with that of OECD TG 492 [13]. Although additional chemicals were listed in OECD TG 492 than in the EpiOcular™ supplier's protocol, they were adequate to guarantee the technical proficiency of laboratory testing and the reliability of data generated in this study. The classification of the chemicals tested in this study was identical to the classification suggested by OECD TG 492 [13]. In addition to the proficiency test, the reliability of EpiOcular™ test in this study was also confirmed by the positive and negative control substances, respectively. As shown in Table 3, the viability in the tissues treated with negative control was 100±5.2%, which was classified under "no category" and the viability of the tissues treated with methyl acetate as positive control was 42.7±11.5%. The viability of methyl acetate-treated tissue was less than 50%, which was the cut-off value of the positive standard. The classification of methyl acetate was "category 1 or 2" as of 2015

version and "no prediction can be made" as of 2018a version, which is harmonious with the classification provided in the OECD TG 492 [13,14]. The final classification of test substances PHMG and CMIT/MIT differed with the concentration. As shown in Table 3, only the raw material showed decreased cell viability and classified under "category 1 or 2". Both PHMG and CMIT/MIT showed similar concentration dependency, which suggested that the aqueous dilutions of the raw materials were not eye irritants, and therefore, classified under the "no category".

The exposure concentration in the water of humidifier box was an important parameter determining the risk of eye irritation for the general consumers. The actual exposure concentration for the consumer was estimated by Park et al. [17]. After surveying the concentrations of active ingredient in the commercially available products and their use pattern, the authors suggested the use of 1/100 product dilution in humidifier based on the worst case of exposure scenario. In this study, the cell viability of RhCE tissue was decreased only in tissues treated with raw materials; however, no evidence of cytotoxicity was found in tissues treated with other aqueous dilutions in market products and in the humidifier water box. The classification of "no prediction can be made" in the revised version of OECD TG 492 may suggest that the test chemicals cannot be evaluated using this test system and further testing based on other in vitro test guidelines is required [14]. The revised version of 2018a appears to replace the conclusions described in the original version of 2015 OECD TG 492, because of the highly confirmative category 1 or 2. In contrast to category 1 or 2, chemicals do not require classification or labeling in the no category.

The Draize test using albino rabbits showed that CMIT/MIT 1.5% (15,000 ppm, as active ingredients) was corrosive when tested as supplied, which is highly consistent with the EpiOcular™ test results in this study [24]. However, the aqueous dilution of CMIT/MIT at concentrations of 0.056% (560 ppm, as active ingredient) was nonirritating and 0.28% (2800 ppm) showed slight to moderate irritation [24]. The results seem to be consistent with our EpiOcular™ test. As shown in Table 3, CMIT/MIT 300 ppm was classified under "no category", suggesting lack of irritation.

In addition to eyes, CMIT/MIT induces skin irritation in rabbits and the results were similar to those of eyes [24]. As far as we know, no data pertaining to the eye or skin irritation of PHMG are available.

In summary, the raw materials of PHMG and CMIT/MIT were classified under "category 1 (serious eye damage) or category 2 (eye irritation)". The classification was replaced by "no prediction can be made" in the revised OECD TG 492, which indicates that it is not a definitive eye irritant. Further testing with other test methods will be required [14]. Aqueous dilutions of raw material found in market products or in the humidifier were classified under the "no category" in this study, which do not meet the criteria for serious eye damage or irritation.

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CONFLICT OF INTEREST

The authors have no conflicts of interest associated with this study to disclose.

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