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***In-vitro* DNA and cell damage induced by 5-chloro-2-methyl-4-isothiazolin-3-one (CMIT) and suppressive effect of selected phytochemicals against CMIT toxicity**

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Abstract

Aim : The aim of the present study was to measure *in-vitro* DNA and cell damage induced by 5-chloro-2-methyl-4-isothiazolin-3-one (CMIT), one of the humidifier disinfectants, and to investigate the suppressive effect of various phytochemicals against CMIT toxicity.

Methodology : *In-vitro* comet assay was performed to determine the degree of CMIT-induced DNA damage at single cell level by measuring the olive tail moment. Upon treating CMIT on the rat lymphocytes, the inhibitory effects of Vitamin C and several phytochemicals such as berberine, curcumin and resveratrol were assessed. In addition, MTT assay was used to examine the protective effect of resveratrol on CMIT-induced cytotoxicity in cultured lung cells.

Results : In *in-vitro* comet assay, the increased olive tail moment induced by CMIT was effectively inhibited by Vitamin C, berberine, curcumin and resveratrol treatment. Especially, resveratrol showed the best suppressive effect against DNA damage by CMIT. In MTT assay, resveratrol also showed significant suppressive effect against cytotoxicity induced by CMIT in cultured lung cells.

Interpretation : Phytochemicals such as Vitamin C, berberine, curcumin and resveratrol can be utilized in the development of preventive or therapeutic compositions against the injury caused by CMIT toxicity.

Key words: Chloro methyl isothiazolinone, Comet assay, DNA damage, MTT assay, Phytochemicals

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Introduction

Isothiazolone biocide, 5-chloro-2-methyl-4-isothiazolin-3-one (CMIT), also referred as methylchloroisothiazolinone, is used in a wide range of personal care products with a broad-spectrum biocide efficacy (Nagorka *et al.*, 2015). It is also used as a preservative with antibacterial and antifungal effect, with the registered tradename Kathon CG in combination with methylisothiazolinone (MIT). The most widely used biocide of isothiazolinones is CMIT and MIT mixture (3:1), showing effective killing against most bacteria, yeast and fungi (Frenzel *et al.*, 2011).

Recently, significant peripheral airway dysfunction has been reported in children with high-level inhalation exposure to a mixture of CMIT/MIT (Cho *et al.*, 2017). CMIT/MIT mixture has been registered as a secondary inhalation toxicant by US Environmental Protection Agency (EPA). Approximately 900 victims have been reported to suffer from severe lung diseases, associated with exposure to humidifier disinfectant containing polyhexamethylene guanidine (PHMG) and oligo (2-(2-ethoxy) ethoxyethyl guanidinium chloride (PGH) as well as CMIT/MIT in Korea. In addition, inhalation toxicities together with positive findings of *in-vitro* genotoxicity studies have been reported in toxicological reports for humidifier disinfectant (Kim and Paek, 2016). As a result, CMIT/MIT mixture has been prohibited in all spray type of products in Korea, due to the potential health concerns associated with possible lung toxicity, allergy and neurotoxicity. Recently, Kim *et al.* (2017) reported positive association between CMIT/MIT exposure and death in a mouse model.

In cosmetic products, CMIT and MIT are two of the most key contact allergens. Dermal irritation occurs commonly in products with CMIT and CMIT/MIT mixtures, but not as common in products with only MIT (Maio *et al.*, 2012). CMIT is more potent than MIT in lymphocyte proliferation as a skin sensitizer (Botham *et al.*, 1991) and in binding proteins and triggering auricular lymph node cell proliferation (Potter and Hazelton, 1995). Moreover, CMIT is mutagenic in nature whereas MIT is not (Conner *et al.*, 1996). The exposure to liquid MIT showed significant neurotoxicity in cerebral cortex cells, whereas the exposure did not show notable neurotoxicity in animals through drinking water (He *et al.*, 2006; Poon *et al.*, 2011; Pelletier *et al.*, 2014). Recently, MIT toxicity has been reported to induce cell death and inflammatory response via DNA damage and nuclear envelope rupture (Park *et al.*, 2018). In addition, CMIT is not considered as a carcinogen by International Agency for Research on Cancer (IARC) (Kahrilas *et al.*, 2014), but chronic and high exposure can cause fatal symptoms.

Comet assay, also referred to as single cell gel electrophoresis assay, is one of the most sensitive and accurate techniques to measure oxidative DNA damage in eukaryotic cells. At the same time, it is also useful in testing DNA repair after direct and oxidative DNA damage (Collins, 2004). It has sensitive detection limit for low levels of DNA breaks in small number of cells, although the mechanism underlying the genotoxic and

repair activity by the inducers cannot be identified through comet assay. In addition, further follow-up studies including animal tests are needed to reduce the false positive results frequently shown in *in-vitro* genotoxicity testing (Kirkland *et al.*, 2007).

The reports on CMIT toxicity at DNA and cellular level is limited. Moreover, specific natural products that reduce CMIT toxicity have not been addressed yet, thus, there is a great need to find the agents to reduce, prevent or ameliorate CMIT toxicity. Phytochemicals, alone or in combination with others, have attracted notable attention to prevent the onset of a wide variety of chronic diseases including cardiovascular impairments, cancer and metabolic abnormalities (Upadhyay and Dixit, 2015). In this investigation, CMIT toxicity was assessed *in-vitro* in terms of DNA damage and cytotoxicity, and the suppressive effects of selected phytochemicals on CMIT toxicity was also investigated.

Materials and Methods

Preparation of lymphocytes: Male Sprague Dawley rats weighing 200~250g were purchased from the Orient Bio Co. Ltd. (Seongnam, Korea). Room temperature was maintained at $20 \pm 2^\circ\text{C}$ and the relative humidity at $60 \pm 10\%$. A 200 μl of whole blood from Sprague Dawley rats was added to 800 μl of phosphate-buffered saline (PBS) and layered onto 200 μl of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). After centrifugation at 1,500 rpm for 5 min at room temperature, the lymphocytes were collected from the buffy-coat layer and washed with PBS. To examine the protective effect of phytochemicals, a 400 μl of whole blood from Sprague Dawley rats was used. Comet assay using rat blood was conducted with the approval of Animal Research Ethics Committee at Soonchunhyang University (SCH15-0007).

CMIT treatment: 5-chloro-2-methyl-4-isothiazolin-3-one CMIT (Cayman chemical company, Michigan, USA) was diluted to 10 $\mu\text{g ml}^{-1}$ in PBS. To examine the protective effect of Vitamin C and phytochemicals against DNA damage, 1~7 $\mu\text{g ml}^{-1}$ of Vitamin C, berberine, curcumin and resveratrol was directly added to the lymphocytes which were treated for 30 min at 37°C before CMIT treatment. Vitamin C and berberine were dissolved in water and resveratrol and curcumin, which had low solubility in water, were dissolved in dimethyl sulfoxide (DMSO). All materials were diluted in PBS to ensure that the concentration of DMSO in the cells did not exceed 0.5%.

Determination of DNA damage by comet assay: Alkaline comet assay was performed according to the method of Singh *et al.* (1988) with slight modification to evaluate CMIT-induced DNA damage and protective effect of phytochemicals. The lymphocytes were mixed with 75 μl of 0.7% low-melting-point agarose (LMA) and placed onto slides coated with 1% normal-melting-point agarose (NMA) and then allowed to solidify over 30 min at 4°C . After solidification, the slides were covered with 100 μl of 0.7% LMA. When the last applied agarose had solidified, the slides were immersed in lysis solution [2.5M NaCl, 0.1 M EDTA,

0.01 M Tris, 1% Triton X-100 and 10% DMSO (pH 10.0)] for more than 2 hrs at 4°C under dark condition. To unwind DNA, the slides were placed on an electrophoresis tank containing electrophoresis buffer [10 N NaOH and 0.2 M Na₂EDTA (pH 13.0)] for 20 min at 4°C. Electrophoresis was performed at 25 V/0.3 A for 20 min at 4°C. The slides were washed with neutralizing buffer [0.4 M Tris • HCl (pH 7.5)] three times for 5 min at 4°C and fixed with crude ethanol for 5 min. The slides were then air dried for 15 min, and stored in slide box at 4°C. The entire electrophoresis process was carried out in dark.

Image analysis: The slides were stained with ethidium bromide and the fluorescence was measured using a fluorescence microscope (Leica, Wetzlar, Germany) in dark condition and viewed with a CCD camera (Hitachi, Japan). The captured image was analyzed using Komet 5.5 software (Kinetic Imaging, UK). DNA damage was quantified via comet assay, wherein the olive tail moment was calculated by the formula: (Tail. mean-Head. mean) × Tail% DNA / 100. A total of 100 lymphocytes were randomly captured and the comets were examined from each slide. All measurements were made in duplicate and performed from three independent experiments.

Cell culture: Human lung cancer cell (A549) was obtained from the American Type Culture Collection (ATCC, Rockville, USA). Cells were maintained in RPMI-1640 [with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS)] in a humidified atmosphere containing 5% CO₂ at 37°C.

MTT cell viability assay: A549 cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

(MTT) assay. The cells were cultured in 96-well plated at a density of 1×10^4 cells per well. The cells were then treated with varying concentration of CMIT for 24 hrs. The cells were washed and treated with 50 µl of MTT, after which the plates were incubated at 37°C in dark for 3 hrs. After the formation of formazan, 50 µl of DMSO was added and the absorbance was read at 570 nm using a microtiter plate reader. A549 cells were treated with 10 µg ml⁻¹ of CMIT, and then 1~7 µg ml⁻¹ of Vitamin C, curcumin, resveratrol and berberine were directly added to the A549 cells in order to assess measure the protection against CMIT-induced cytotoxicity.

Statistics: All experiments were carried out in triplicate and analyzed using SPSS package for Windows version 13 (SPSS Inc., Chicago, IL). The analytical data were presented as mean ± SD. The results from each treatment of CMIT and phytochemicals were compared with each other using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. $p < 0.05$ was considered significant.

Results and Discussion

The information on the toxicity of CMIT/MIT mixture and individual component is limited, although the toxicity and reactivity of CMIT has been reported to be more potent than MIT (Cho *et al.*, 2017). The carcinogenicity of CMIT was ruled out by IARC (Kahrilas *et al.*, 2014), but chronic and high exposure can cause a detrimental effect at DNA and cellular level. Based on the earlier findings of *in-vitro* genotoxicity for humidifier disinfectant (Kim and Paek, 2016), DNA damage via comet assay and its protection by phytochemicals were investigated in this study. The general toxicity triggered by chemicals can be detected by

Fig. 1: CMIT-induced oxidative DNA damage measured by comet assay. Values not sharing the same letter are significantly different from one another ($p < 0.05$) according to Duncan's Multiple Range Test.

Fig. 2: Suppressive effect of Vitamin C on CMIT-induced oxidative DNA damage. Values not sharing the same letter are significantly different from one another ($p < 0.05$) according to Duncan's Multiple Range Test.

enhanced levels of reactive oxygen species (ROS), pro-inflammatory cytokines and oxidative metabolic enzymes (Xiao *et al.*, 2017). ROS induces the expression of NF- κ B, a key transcription factor in the inflammatory response, as well as a variety of proteins linked with oxidative stress-associated reactions. In addition, phytochemicals with antioxidant activity, such as carotenoid (Caramujo *et al.*, 2012) and polyphenols including phenolic acids (Sevgi *et al.*, 2015), flavonoids (Manzoli *et al.*, 2015) and stilbenes (Biais *et al.*, 2017), have been extensively used to reduce toxicity triggered by toxic chemicals.

Fig. 1 shows the degree of oxidative DNA damage in lymphocytes of rats treated with 1, 3, 5 and 10 $\mu\text{g ml}^{-1}$ of CMIT. DNA damage was enhanced in proportion to the CMIT concentrations with statistical significance ($p < 0.05$). The olive tail moment was 1.1 in the control group treated with PBS, however,

the olive tail moment at 10 $\mu\text{g ml}^{-1}$ CMIT was about 7.5-fold higher than the control group. However, as shown in Fig. 2, the enhanced olive tail moment by 10 $\mu\text{g ml}^{-1}$ CMIT decreased sharply upon treatment with Vitamin C. Especially, 5 $\mu\text{g ml}^{-1}$ of Vitamin C was very close to *i.e.*, 1.0. These results show that Vitamin C might inhibit oxidative DNA damage by CMIT, probably due to quenching of superoxide and hydroxyl radicals. These results also suggest that DNA damage caused by CMIT might be linked to generation of reactive oxygen species (ROS) (Ettorre *et al.*, 2003), although CMIT's mode of action against DNA structure has not been fully elucidated yet. There are many reports that phytochemicals play pivotal roles in blunting the toxicity induced by chemicals or environmental factors. Antioxidants including resveratrol and curcumin have been effective in reducing acrylamide toxicity in cell lines and/or animal studies, via anti-oxidative, anti-apoptotic, anti-inflammatory and

Fig. 3: Suppressive effect of (A) Berberine, (B) Curcumin and (C) Resveratrol on CMIT-induced oxidative DNA damage. Values not sharing same letter are significantly different from one another ($p < 0.05$) according to Duncan's Multiple Range Test.

reduction of chemical absorption (Kahkeshani *et al.*, 2015). Also, curcumin, berberine and sulforaphane prevented the oxidative DNA damage progression by chrysotile (Ryu *et al.*, 2012), hydrofluoric acid (Seo and Lee, 2013) and nano sized TiO_2 (Ryu *et al.*, 2016; Valentini *et al.*, 2017). Four different phytochemicals from edible plants such as auraptene, nobiletin, zerumbone and ($\pm$)-13-hydroxy-10-oxo-trans-11-octadecenoic acid was effective in reversing the inflammatory action by dioxine in U937 human macrophages (Sciullo *et al.*, 2010).

The inhibitory effect of berberine on CMIT-induced DNA damage in lymphocytes is shown in Fig. 3A. The olive tail moment of CMIT alone was 9.8, however, 1, 3, 5 and 7 $\mu\text{g ml}^{-1}$ berberine treatment reduced 18%, 41%, 70% and 80% olive tail moment induced by CMIT, respectively. Especially, at 7 $\mu\text{g ml}^{-1}$ berberine, olive tail moment showed a low level similar to that of CMIT-treated control group. Berberine, a major component of shrub *Berberis vulgaris*, has been reported to have antioxidant (Kim *et al.*, 2016), anti-inflammatory (Tsang *et al.*, 2016), antibacterial (Peng *et al.*, 2015), antihypertensive (Guo *et al.*, 2015), antitumor (Goto *et al.*, 2014) and anxiolytic activity (Peng *et al.*, 2004). The results of this paper also suggest that berberine may act as an antioxidant to quench reactive oxygen species generated by CMIT treatment.

The olive tail moment of CMIT alone was 6.0, however, 3, 5 and 7 $\mu\text{g ml}^{-1}$ curcumin treatment reduced approximately 10%, 60% and 72% of olive tail moment, respectively (Fig. 3B). The olive tail moment of curcumin-treated group decreased in a dose-dependent mode. Tumeric has curcuminoid compounds such as curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Of these, curcumin plays an important role as an antioxidant (Xu *et al.*, 2018), analgesic (Chin *et al.*, 2016) and anti-inflammatory agent (Wilken *et al.*, 2011). Resveratrol is a typical phytochemical exist in many edible plants including peanuts, grapes and berries. The activities of resveratrol such as anti-oxidant, anti-cancer, antiviral and anti-inflammatory activity have been examined in a variety of *in vivo* and *in vitro* studies (Gambini *et al.*, 2015). Resveratrol, as an efficient antioxidant with free radical scavenger activity, enhanced and/or maintained the level of several antioxidant enzymes, including superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase. However, the direct scavenging activities of resveratrol are yet not clarified. Some of the gene-regulating effects of resveratrol has been reported to be mediated by the histone/protein deacetylase sirtuin 1 or by the nuclear factor-E2-related factor-2 (Xia *et al.*, 2017). As shown in Fig. 3C, DNA damaged by 10 $\mu\text{g ml}^{-1}$ CMIT showed an olive tail moment of 11.1 ± 2.16 , however, approximately 70% reduction of the olive tail moment was obtained at 1, 3, 5 $\mu\text{g ml}^{-1}$ of resveratrol. These data indicated that all doses of resveratrol used here reduced DNA damage similar to the control level. Taken together, resveratrol might be the most superior inhibitor against CMIT toxicity assessed in Comet system.

Finally, the cell viability of A549 was measured with or without resveratrol under CMIT toxicity. As shown in Fig. 4, about

Fig. 4: The protective effect of resveratrol on CMIT-induced cytotoxicity. Values not sharing same letter are significantly different from one another ($p < 0.05$) according to Duncan's Multiple Range Test.

41.3% of the cells died as compared to control at 50 $\mu\text{g ml}^{-1}$ CMIT, whereas no cytotoxicity was observed from 10 to 40 $\mu\text{g ml}^{-1}$ CMIT. Cells treated with 3 and 5 $\mu\text{g ml}^{-1}$ of resveratrol did not prevent the cell death by CMIT. However, treatment with 7 $\mu\text{g ml}^{-1}$ of resveratrol showed similar level of cell survival as that of control group. In Fig. 3C, lymphocytes treated with 3 and 5 $\mu\text{g ml}^{-1}$ resveratrol showed protective effect against 10 $\mu\text{g ml}^{-1}$ CMIT toxicity. These results suggest that Comet assay with DNA damage is more sensitive than MTT assay with cytotoxicity in measuring CMIT toxicity. Further, protective studies against cytotoxicity by CMIT are being performed in various systems with various phytochemicals.

In conclusion, Vitamin C, and curcumin, berberine and resveratrol have *in-vitro* protective effects against CMIT-induced lymphocyte DNA damage. In addition, the lowest concentration of resveratrol showed the highest protective effect against DNA damage, and inhibited CMIT cytotoxicity at cellular level. The preventive effect of selected phytochemicals against DNA damage and cytotoxicity due to CMIT may be applied for the development of anti-CMIT agent.

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