Acute pulmonary toxicity and inflammation induced by combined exposure to didecyldimethylammonium chloride and ethylene glycol in rats

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ABSTRACT — Didecyldimethylammonium chloride (DDAC), an antimicrobial agent, has been reported to induce pulmonary toxicity in animal studies. DDAC is frequently used in spray-form household products in combination with ethylene glycol (EG). The purpose of this study was to evaluate the toxic interaction between DDAC and EG in the lung. DDAC at a sub-toxic dose (100 μg/kg body weight) was mixed with a non-toxic dose of EG (100 or 200 μg/kg body weight), and was administrated to rats via intratracheal instillation. Lactate dehydrogenase activity and total protein content in the bronchoalveolar lavage fluid (BALF) were not changed by singly treated DDAC or EG, but significantly enhanced at 1 d after treatment with the mixture, with the effect dependent on the dose of EG. Total cell count in BALF was largely increased and polymorphonuclear leukocytes were predominantly recruited to the lung in rats administrated with the mixture. Inflammatory cytokines, tumor necrosis factor-alpha and interleukin-6 also appeared to be increased by the mixture of DDAC and EG (200 μg/kg body weight) at 1 d post-exposure, which might be associated with the increase in inflammatory cells in lung. BALF protein content and inflammatory cell recruitment in the lung still remained elevated at 7 d after the administration of DDAC with the higher dose of EG. These results suggest that the combination of DDAC and EG can synergistically induce pulmonary cytotoxicity and inflammation, and EG appears to amplify the harmful effects of DDAC on the lung. Therefore pulmonary exposure to these two chemicals commonly found in commercial products can be a potential hazard to human health.

Key words: Didecyldimethylammonium chloride, Ethylene glycol, Acute pulmonary toxicity, Inflammation

INTRODUCTION

Didecyldimethylammonium chloride (DDAC) is an antimicrobial agent widely used as a preservative and a sanitizer for industrial and medical purposes (Ioannou et al., 2007; Yoshimatsu and Hiyama, 2007). The long alkyl chain in its structure permeates the bacterial cell membrane and disrupts its physical and biochemical properties, leading to leakage of intracellular molecules (Wessels and Ingmer, 2013). Occupational exposure to DDAC has been reported to induce contact dermatitis (Dejobert et al., 1997; Geier et al., 2013). Occupational exposure to DDAC has been reported to induce contact dermatitis (Dejobert et al., 1997; Geier et al., 2013). Currently, various household products such as bathroom cleaners, hand soaps, cosmetics, air fresheners and deodorants contain DDAC as a preservative (Kwon et al., 2014). In particular, the usage of spray-form products containing DDAC can lead to direct exposure to the human respiratory system. The pulmonary toxicity of DDAC has been demonstrated in several studies. Intratracheal instillation of DDAC caused alveolar cell injuries, inflammation and fibrosis in the rodent lung (Ohnuma et al., 2010; Ohnuma-Koyama et al., 2013; Shim et al., 2013). Interstitial pneumonia and inflammatory cell infiltration to the lung were observed in rats that inhaled DDAC for 2 weeks (Lim and Chung, 2014). Mitochondrial/lysosomal damages and growth inhibition were also induced by...
DDAC in human alveolar cells (Kwon et al., 2014). Thus, respiratory exposure to DDAC can be a potential risk factor for pulmonary dysfunction.

Ethylene glycol (EG) is a liquid used as an anti-freezing agent, solvent, surfactant and emulsifier in diverse commercial products (NTP-CERHR, 2004). Ingestion of EG is known to promote renal injury (Porter, 2012). In contrast, inhalation of EG has been thought to be relatively safe, as healthy volunteers exposed to aerosolized EG did not show any abnormal symptoms in clinical studies (Wills et al., 1974; Carstens et al., 2003; Upadhyay et al., 2008).

It was investigated that EG (1-2% w/v) is included as a solvent in common spray-type air fresheners and deodorants that also contain DDAC (0.1-1% w/v), indicating that the two substances are likely to be inhaled together when using these products (Shim et al., 2013). Recently, we reported that DDAC and EG induced synergistic cytotoxicity in human lung epithelial (BEAS-2B) cells (Kwon et al., 2015). In that study, the combination of DDAC and non-toxic concentration of EG induced more severe cytotoxicity than DDAC alone, and the effect was dependent on the concentration of EG. This suggest the possibility that the inhalation of EG and DDAC mixture may produce unexpected effects to human respiratory organs as a result of the toxic interaction between the two chemicals. However, there is currently no toxicological information from animal study related to the pulmonary exposure of an EG and DDAC mixture.

The purpose of the present study was to evaluate the toxicity of the combination of DDAC and EG in the lung using a rat model. We hypothesized that DDAC would cause toxicity when administered in combination with EG. Therefore, DDAC was consistently administered at a sub-toxic dose, whereas the dose of EG was varied to specifically examine the toxic effects of their interaction. Animals were treated with the two chemicals via intratracheal instillation, and the acute toxic responses and recovery were monitored.

MATERIALS AND METHODS

Chemicals

DDAC was obtained from Pioneer Tech (Anhui, China) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals and treatments

Male Sprague-Dawley rats (8 weeks old) were purchased from Orient Bio Inc. (Seongnam, Korea). Animals were acclimated to temperature- (22 ± 3°C) and humidity-(50 ± 10%) controlled rooms with a 12-hr light/dark cycle for 1 week before the experiment. The animal experimentation protocol was approved by the Institutional Animal Care and Use Committee of the National Institute of Environmental Research (NIER-2014-01-01). Animals were randomly divided into six groups: control, D100, E100, E200, D100+E100, and D100+E200. DDAC and EG, dissolved in phosphate-buffered saline (PBS, pH 7.4), were intratracheally instilled to rats, and the administration volume was 1 mL/kg. Control rats (n = 10) were given PBS. Rats in D100 (n = 10), E100 (n = 10) and E200 (n = 10) groups were treated with 100 μg/kg of DDAC, 100 μg/kg of EG, and 200 μg/kg of EG, respectively. D100+E100 and D100+E200 groups were administered 100 μg/kg of DDAC in combination with 100 μg/kg and 200 μg/kg of EG, respectively. Animals were sacrificed at either 1 d (n = 5) or 7 d (n = 5) after the administration, and bronchoalveolar lavage fluid (BALF) was collected for analysis of lung cell damage and pulmonary inflammation.

BALF analysis

Animals were anesthetized by the intraperitoneal injection of 50 mg/kg tiletamine + zolazepam (Zoletil® 50, Virbac; Carros, France) and 15 mg/kg xylazine hydrochloride (Rumpun®, Bayer; Leverkusen, Germany). BALF was obtained from the lung by lavage with 5 mL of magnesium and calcium free PBS (pH 7.4) five times. The BALF samples were centrifuged (200 × g, 4°C, 10 min) and the cell free supernatant of the first lavage was used for the analysis of lactate dehydrogenase (LDH), total protein, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). LDH activity was measured using a QuantiChrom LDH Kit (BioAssay Systems; Hayward, CA, USA). Total protein content was quantified using a BCA protein assay kit (iNtRON Biotechnology; Seoul, Korea). The levels of IL-6 and TNF-α were determined using a Quantikine® enzyme-linked immunosorbent assay kit (R&D Systems; Minneapolis, MN, USA) according to the manufacturer’s instructions. Cells in all lavages were resuspended in PBS, and total cell counts in BALF were measured on Vi-Cell® XR analyzer (Beckman Coulter; Brea, CA, USA). Cell differential counts were performed on cytopsin preparations (Shandon; Pittsburgh, PA, USA), and the cells were stained with Diff-Quik (Fisher Scientific; Swedesboro, NJ, USA). Polymorphonuclear leukocytes (PMNs) were counted using light microscopy (Olympus Co.; Tokyo, Japan).

Statistical analysis

All results are expressed as the mean ± S.E. Means
of different groups were compared using two-tailed unpaired Student’s t-tests or One-way analysis of variance (ANOVA, Tukey’s multiple comparison test) by GraphPad Prism version 5.0 software (GraphPad Software Inc.; La Jolla, CA, USA).

RESULTS AND DISCUSSION

Previously, it was reported that intratracheal instillation of DDAC (150 μg/kg) induced cytotoxicity, determined by LDH activity and protein content in BALF, whereas a lower dose (15 μg/kg) did not show any visible changes in mice (Ohnuma et al., 2010). In our preliminary experimentation, 200 μg/kg of DDAC intratracheally administrated to rats promoted mild but significant elevations of LDH (P < 0.05) and protein (P < 0.05) levels in BALF compared to PBS treatment. However, lower doses (50 and 100 μg/kg) of DDAC and several doses (50, 150, 300 μg/kg) of EG were observed to be not toxic to lung cells (data not shown). Therefore, in the present study, 100 μg/kg of DDAC, expected to be insufficient to induce pulmonary injury, was administrated intratracheally to rats with a non-toxic dose of EG (100 or 200 μg/kg) in order to identify the potential toxicity produced by the combined exposure of the two chemicals to lung. The concentration of DDAC used in this study was 100 ppm due to injection volume being 1 mL/kg, which can be considered to be suitable for evaluation of its toxicity because maximally 2383 ppm of DDAC is allowed in spray-form products (USEPA, 2006). The doses of EG were selected considering the typical concentration ratios between DDAC and EG in the products.

Singly treated DDAC (100 μg/kg) or EG (100 and 200 μg/kg) did not induce any changes in body weight compared with the control rats (Fig. 1). However, DDAC (100 μg/kg) and EG (200 μg/kg) mixture significantly decreased the body weight at 1 d following the treatment, indicating the adverse effect of the mixture (Fig. 1). BALF is usually analyzed to estimate pulmonary toxicity (Gupta, 2014). LDH, a cytosolic enzyme, is released from injured bronchial/alveolar cells. Damages to airway and alveolar structures also cause leakage of vascular proteins into alveolar spaces. Thus, LDH activity and total protein content in BALF are regarded as biomarkers of pulmonary cytotoxicity and the permeability of the alveolar-capillary barrier (Sayes et al., 2007; Gupta, 2014). In the present study, DDAC or EG did not individually induce any changes in LDH activity and protein content in BALF.

![Fig. 1](image-url)  
**Fig. 1.** Body weight changes of rats after pulmonary exposure to didecyldimethylammonium chloride (DDAC) and ethylene glycol (EG) mixtures. Rats were intratracheally instilled with DDAC, EG or a mixture of the two. CON, control; D100, DDAC 100 μg/kg; E100, EG 100 μg/kg; E200, EG 200 μg/kg; D100+E100, DDAC 100 μg/kg and EG 100 μg/kg mixture; D100+E200, DDAC 100 μg/kg and EG 200 μg/kg mixture. Each value represents mean ± S.E. (n = 5). *P < 0.05 vs. CON; #P < 0.05 vs. D100; **P < 0.01 vs. E200 (Student’s t-test).
at 1 and 7 d after exposure in rats. However, animals treated with DDAC and EG mixtures showed a pattern of increase in the two biomarkers at 1 d after exposure in proportion to the dose of EG in the mixture (Figs. 2A and 2B). Especially, administration of DDAC with the higher dose of EG (200 μg/kg) significantly increased cytotoxicity and membrane permeability as shown by 1.6- and 2.8-fold elevations in LDH activity and total protein content, respectively. Moreover, protein content in the high-dose EG with DDAC exposure group was still elevated until 7 d after exposure. These results indicate that combined exposure to DDAC and EG induced acute pulmonary toxicity with the toxic responses dependent on the dose of EG, which was not observed in singly treated DDAC or EG. Furthermore, EG appears to delay the recovery from epithelial membrane damage induced by DDAC.

Inflammation is usually implicated in the pathogenesis of pulmonary diseases, and is closely associated with the lung toxicity induced by inhaled chemicals (Hiraiwa and van Eden, 2013; Gomes and Florida-James, 2014). Inflammatory responses are initiated with the phagocytosis of the foreign materials by alveolar macrophages to remove them, and the activated macrophage produces and releases inflammatory mediators to attract other immune cells such as polymorphonuclear cells (PMNs) into air spaces (Hiraiwa and van Eden, 2013; Gomes and Florida-James, 2014). PMNs, including neutrophils, eosinophils, and basophils are easily observed to be migrated to the injured and inflamed site of the lung from blood circulation for effective clearance of the air spaces by phagocytosis of exogenous substances. It was reported that macrophages are the major cell type in BALF from healthy animals, whereas PMNs are predominantly infiltrated into bronchoalveolar regions in the early phase of pulmonary inflammation (McClellan and Henderson, 1995). Thus, the increased numbers of total cells and percentage of PMNs in BALF are good indicators of the beginning of inflammatory response in lung (Gomes and Florida-James, 2014). In the present study, more than two-fold increases in total cell counts were observed in the mixture-treated groups at 1 d after administration, whereas no alterations were seen in the single chemical exposure group (Fig. 3A). Microscopic images (Fig. 3C) indicated that similar numbers of macrophages in BALF were observed in all groups, but the numbers of PMNs (indicated with arrow) were significantly increased by administration of the mixtures at 1 d. The ratios of PMNs to total cells were increased more than 300% and 500% in the groups exposed to DDAC with 100 μg/kg and

![Fig. 2](image-url). Pulmonary toxicity induced by didecyldimethylammonium chloride (DDAC) and ethylene glycol (EG) in rats. (A) Lactate dehydrogenase (LDH) activities and (B) total protein (TP) contents in bronchoalveolar lavage fluid (BALF). Rats were intratracheally treated with DDAC, EG, or a mixture of both chemicals. CON, control; D100, DDAC 100 μg/kg; E100, EG 100 μg/kg; E200, EG 200 μg/kg; D100+E100, DDAC 100 μg/kg and EG 100 μg/kg mixture; D100+E200, DDAC 100 μg/kg and EG 200 μg/kg mixture. Each value represents mean ± S.E. (n = 5). Values with different letters (a, b) are significantly different from one another, and the comparison is indicated in black and gray letters, 1 d and 7 d respectively, among the groups at the same time (one-way ANOVA followed by Tukey’s multiple comparison test, P < 0.05).
200 μg/kg of EG, respectively, at 1 d post-exposure (Fig. 3B). Total cell and PMNs counts in mixture exposure groups appeared to recover at 7 d, but still remained significantly elevated in the DDAC and 200 μg/kg of EG-treated group (Figs. 3B and 3C). These results show that the DDAC and EG mixture synergistically promoted acute inflammation in lung, and that the intensity and duration of inflammatory responses appeared to be closely associated with the dose of EG.

Inflammatory cytokines have been reported to contribute to the induction and maintenance of pulmonary inflammation (Bhatia and Moochhala, 2004; Mukhopadhyay et al, 2006; Bradley, 2008; Rincon and Irvin, 2012). TNF-α is a pro-inflammatory mediator mainly secreted by activat-
ed alveolar macrophages, and is known to induce oxidative stress-mediated toxicity in lung cells (Mukhopadhyay et al., 2006). This cytokine also promotes circulating inflammatory cells to adhere to the endothelium, and stimulates the release of chemoattractants such as IL-6 (Henderson, 2005). IL-6 is predominantly produced by macrophages and monocytes, and plays important roles in recruitment of inflammatory cells, including PMNs, into the lung (Yu et al., 2002; Gabay, 2006; Jones et al., 2006). Therefore, the concentrations of TNF-α and IL-6 in BALF are usually analyzed to determine the acute pulmonary inflammation (Sayes et al., 2007; Kwon et al., 2013). In the current study, the BALF levels of TNF-α were not changed by singly treated DDAC or EG, but those in mixture-treated rats seemed to be slightly increased even though there were no statistical differences (Fig. 4A). IL-6 levels in the mixture-administered rats showed similar increased patterns with TNF-α, but much more significant changes \((P < 0.05)\) were shown compared to the control group (Fig. 4B). The mixture of DDAC and EG (200 μg/kg) elevated the IL-6 level at about 17% compared with control group at 1 d post exposure though each chemical did not alter the cytokine level by itself. Thereafter, elevated IL-6 recovered to control level at 7 d after exposure. IL-6 promotes endothelial cells to express leukocyte adhesion molecules (intracellular adhesion molecule-1 and vascular cell adhesion molecules-1) and secrete other chemokines such as IL-8, leading to leukocyte accumulation in lung (Gabay, 2006; Barnes et al., 2011). Thus, it can be suggested that the increase in IL-6 may be responsible for the increases of PMNs in both DDAC and EG-treated rats. Although macrophages have been considered to be the major cells that produce IL-6 and TNF-α, PMNs have also been reported to serve as predominant sources of the two cytokines in lung inflammation (Xing et al., 1993, 1994). Thus, the increase in PMNs infiltration also appears to be responsible for the increases observed in inflammatory cytokine levels in BALF.

The present results show that a sub-toxic dose of DDAC can exert harmful effects on respiratory organs when exposed together with EG. The mechanism of the toxic interaction between the two substances is unclear. Synergistic bactericidal effects of DDAC and surfactants against Staphylococcus aureus were reported (Gomi et al., 2012). A sub-lethal concentration of DDAC showed bactericidal activity in the presence of low concentrations of non-ionic surfactants such as polyoxypropylene glycol,

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**Fig. 4.** Alterations in the pulmonary levels of inflammatory cytokines induced by the mixtures of didecyldimethylammonium chloride (DDAC) and ethylene glycol (EG) in rats. (A) Tumor necrosis factor alpha (TNF-α) and (B) interleukin-6 (IL-6) levels in bronchoalveolar lavage fluid (BALF). Rats were treated with DDAC, EG or a mixture of the two via intratracheal instillation. CON, control; D100, DDAC 100 μg/kg; E100, EG 100 μg/kg; E200, EG 200 μg/kg; D100+E100, DDAC 100 μg/kg and EG 100 μg/kg mixture; D100+E200, DDAC 100 μg/kg and EG 200 μg/kg mixture. Each value represents mean ± S.E. \((n = 5)\). Values with different letters \((a, b)\) are significantly different from one another, and the letter indicates the comparison among the groups at 1 d after exposure. (one-way ANOVA followed by Tukey’s multiple comparison test, \(P < 0.05\)).
and it was suggested that the surfactants allowed DDAC to more easily penetrate the membrane lipid bilayer. EG is an organic solvent that also has surfactant-like properties (NTP-CERHR, 2004). Recently, we reported that the cytotoxicity of DDAC to human bronchial epithelial (BEAS-2B) cells was significantly elevated in the presence of EG, and the effect was dependent on the concentration of EG (Kwon et al., 2015). The study suggested that EG enhanced the absorption of DDAC into cells since the intracellular concentrations of DDAC were higher in the cells treated with both DDAC and EG than those incubated with DDAC alone. Thus, in the present study, it is also possible that intratracheally treated EG facilitates the permeation of DDAC into the airway cells through the cell membranes in rats. DDAC is not only a membrane-active compound but also an intracellular toxicant that binds to DNA (Wessels and Ingmer, 2013), degrades RNA (Ioannou et al., 2007) and induces oxidative stress in cellular organelles (Kwon et al., 2014). It was also demonstrated that generation of reactive oxygen species and oxidation of glutathione induced by DDAC was amplified by co-treated EG in human bronchial cells (Kwon et al., 2015). Therefore, the enhancement of DDAC absorption into lung cells conferred by EG with the consequent generation of oxidative stress can be a potential reason for the increased lung cell damage.

In summary, intratracheal instillation of DDAC and EG mixture caused pulmonary cell damage and inflammation in rats while singly treated DDAC or EG did not induce any harmful effects. Moreover, the cytotoxic and inflammatory responses were enhanced in an EG dose-dependent manner, demonstrating that EG potentiated the toxicity of DDAC in the lung. Thus, the results of this study suggest that combined exposure to DDAC and EG can synergistically induce pulmonary toxicity. This is the first animal study to indicate that the interaction between DDAC and EG, even at sub-toxic doses, can be substantially toxic. Further studies are needed to evaluate the safety of the two chemicals in household products, and inhalation experiments to assess the pulmonary toxicity of this combination are currently underway.

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Conflict of interest----- The authors declare that there is no conflict of interest.

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Lim, C.H. and Chung, Y.H. (2014): Effects of didecylldimethylammonium chloride and ethylene glycol on human bronchial epithelial cells and inflammatory responses induced by co-treated EG in human bronchial cells (Kwon et al., 2015). Therefore, the enhancement of DDAC absorption into lung cells conferred by EG with the consequent generation of oxidative stress can be a potential reason for the increased lung cell damage.


