INTRODUCTION

Triclosan (TCS) as biocide (2,4,4′-trichloro-2′-hydroxy-diphenyl ether or Irgasan) is used extensively in household goods and oral hygiene products such as air freshener, mouthwash, antibacterial soaps and detergents due to antibiotic properties (Jones et al., 2000). There-fore, people may be exposed to TCS in the living envi-ronment and it is found in the urine (Calafat et al., 2008) and breast milk of human (Adolfsson-Erici et al., 2002) actually. Antibiotic mechanism of TCS is the inhibition of the enoyl-acyl carrier protein reductase (ENR) associated bacterial lipid biosynthesis (Heath et al., 1999). TCS is considered to be safe, because mammals have no function of ENR, but a previous study reported that inhaled TCS induced pulmonary toxicity in rats (Leutkemeier et al., 1974).

Recently, air freshener and deodorant are increasing-ly used for odor removal in indoor activity but they have the potential for inhalation exposure in the indoor envi-ronment. Also, spray-type household products can spread inhalable particles including nano-size into the indoors air. It is indicating that peoples may be exposed by inhalation to TCS by using the spray-type odor removal products containing TCS. Thus, TCS has the potential to cause pulmonary toxicity. However, until now, very little infor-mation is available regarding the pulmonary toxicity of TCS. Therefore, the purpose of this study was to evalu-ate pulmonary toxicity screening of TCS to provide information about potential lung damage through intratracheal instillation of TCS using SD rats.

MATERIALS AND METHODS

Chemicals and animals

TCS and reagents were obtained from the Sigma-Aldrich (St Louis, MO, USA). Stock solutions of TCS were prepared in dimethylsulfoxide (DMSO). Male Sprague-Dawley (SD, specific-pathogen-free) rats aged 7 weeks were purchased from Orient Bio Inc. (Seongnam,
Korea). All methods used in this study were approved by the Animal Care and Use Committee of Korea Institute of Toxicology (1210-0319).

Intratracheal instillation
Sixty rats were divided into three groups including control and were given single intratracheal instillation of TCS (low group: 10 μg/B.W. kg and high group: 1,000 μg/B.W. kg). These exposure doses were considered for acute exposure to low-dose or high-dose groups depending on the frequency of products use. Controls were exposed to saline with 0.5% DMSO as vehicle. Half of the animals in each group were investigated to determine bronchoalveolar lavage fluid (BALF) and the rest rats were histopathologically examined by using routine methods. Rats were sacrificed either 1 day or 2 weeks after intratracheal instillation.

BALF analysis
Animals were deeply anesthetized by isoflurane overdose and BALF was obtained BALF by repeated whole-lung lavage. The left lung was clamped off and the right lungs were lavaged five times with 3 ml of calcium and magnesium free phosphate buffer solution (PBS, pH 7.4). The samples were centrifuged at 1,500 rpm for 10 min. The cell free supernatant of the first lavage was kept separate from other samples for biochemical analyses. Recovered cells from all lavages were resuspended in PBS. Total cells (TC) in the BALF were counted with a Vi-Cell® XR analyzer (Beckman Coulter, Brea, CA, USA) and cell differentials were performed on cytospin preparations (Shandon, Pittsburgh, PA, USA) stained with Diff-Quick staining (Fisher Scientific, Swedesboro, NJ, USA). BALF cells (300/rat) were differentially counted as Polymorphonuclear leukocytes (PMN) using light microscopy. Total protein (TP) in the BALF supernatant was quantified using a BCA protein assay kit (iNtRON Biotechnology, Seoul, Korea) and the activity of lactate acid dehydrogenase (LDH) in the BALF was measured by using a QuantiChrom Lactate Dehydrogenase Kit (BioAssay Systems, Hayward, CA, USA). The level of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in BALF was estimated by commercially available enzymelinked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) and based on the manufacturer’s instructions.

Cytotoxicity
Cytotoxicity of TCS was investigated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. L2 cells (Rat lung epithelial cells, Korea cell bank, Seoul, Korea) were seeded in 96-well plates at a density of 1.0 × 10^4 cells per well, and then incubated for overnight. The cells were incubated for 4 hr and 24 hr with various concentrations of TCS. Following the incubation period, 10 μl of MTT solution (5 mg/ml in PBS) was added to each well, the supernatant was removed and then 100 μl of dimethyl sulfoxide (DMSO) was added. The absorbance was measured using a microplate reader (Tecan, Salzburg, Austria) at a wavelength of 540 nm.

Statistical analysis
A multiple variance of analysis (ANOVA) test and Student’s t-test (Graphpad Software, San Diego, CA, USA) were used to compare exposure groups with a control group. The level of significance was set at P < 0.05 and P < 0.01.

RESULTS AND DISCUSSION
The present study demonstrated that intratracheal instillation of TCS induced increase in the pulmonary toxicity marker in BALF at 1 day after exposure. However, most index levels were recovered during the convalescence period (14 days). Also, the treatment of TCS decreased cell viability with morphological change in L2 cells. Therefore, TCS may be considered to affect lung inducing the early inflammatory response as acute lung injury.

To determine the pulmonary toxicity of TCS after instillation, we investigated the inflammation response of TCS in lungs by BALF analysis. Pulmonary inflammation is accompanied by early immune response such as activation of alveolar macrophages and increase in PMN percent in BALF cells (Abraham, 2003). In the previous study, it was found that 4 days inhalation of glutaraldehyde (1% formalin solution) as biocide induced inflammatory lung effects including increase in TC count and PMNs recruitment in BALF. Our results of BALF cytology analysis showed that even if there is no significance, intratracheal instillation of TCS administration increased in TC (most alveolar macrophage, > 90%) count and percent of PMNs in BALF cells at 1 day post-exposure (Fig. 1). These results suggested that macrophages and PMNs play a major role in the protection of the lungs at an early stage by pulmonary exposure of biocide.

The LDH and TP level in BALF indicated cytotoxicity marker and injury of alveolar/capillary membrane barrier in the lungs, respectively (Warheit et al., 1991). Our results showed a significant increase in concentrations of TP in the BALF exposed to TCS compared with the con-
control for both end points in high dose group (1 day: $P$ value 0.03805 and 14 day: $P$ value 0.01842) (Fig. 2A). In the LDH analysis, there was no significant change in comparison to control group, but the LDH level of exposure groups showed a pattern of increase at the two end points (Fig. 2B). Despite the differences in material and experiment design, Ohnuma et al. (2010, 2011) reported that decyldimethylammonium chloride (DDAC) which is used as a germicide causes lung fibrosis including change of BALF cells, level of LDH and TP concentration in BALF after intratracheal instillation. However, in histopathological examination, our results were not found to be treatment-related lesions in the lungs at higher exposure dose compared with DDAC study (data not shown).

The next step was to determine the inflammatory cytokine such as TNF-α and IL-6 by using ELISA analysis. Pro-inflammatory cytokines including TNF-α and IL-6 were related to the destruction of alveolar epitheli-
um and leakage of capillaries associated with pulmonary injury (Ware, 2006). In addition, TNF-α and IL-6 released by activated alveolar macrophages were regulated by PMNs recruitment which is reaction of acute lung damage to the lungs. (Kumar and Sharma, 2010). Our results showed a transient increase in TNF-α and IL-6 secretion at the exposure groups, recovered which was after two weeks (Fig. 3). Also, these results were consistent with those of BALF cytology (TC count and PMNs percent) and LDH analysis.

As shown in Fig. 4A, the lung epithelial cells (L2) exposed to TCS for 4 hr and 24 hr showed a dose- and time-dependent decrease in cell viability. The IC_{50} values were 188.58 μM and 118.86 μM at the 4 hr and 24 hr exposure.
treatment, respectively. Also, the cell morphology changes were observed after exposure of TCS in a dose-dependent manner (Fig. 4B). These results are corresponding with previous experiments conducted with DDAC in mouse lung fibroblasts, but their cytotoxicity was approximately 15 times higher than our results of MTT assay at the 24 hr treatment. Therefore, TCS is considered a relatively having low toxicity compared to DDAC as a results on results of BALF analysis and MTT assay.

As discussed above, our results suggested that intratracheal instillation of TCS affect PMNs recruitment regulated by TNF-α and IL-6 as acute lung injury. These damages were recovered two weeks after instillation, but TP concentration remained increased, indicating that inhalation of TCS may affect lung function. In the previous study, repeated inhalation of TCS study has shown that inhaled TCS induced increase in mortality with acute inflammation and focal ulcerations in the respiratory system (Leutkemeier et al., 1974). Our study was limited to investigating effects on pulmonary toxicity of TCS due to the absence of the approach of molecular analysis to detect lung injury. Nevertheless, the present study provides basic information of pulmonary toxicity of TCS for risk assessment.

In conclusions, our pulmonary toxicity screening study demonstrated that TCS exposure by intratracheal instillation may induce acute inflammation in the lungs and affect lung function ability. Therefore, these results will be confirmed by investigation of repeated inhalation toxicity of TCS including molecular level analysis for mechanism of pulmonary toxicity.

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REFERENCES


