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# Methylmercury chloride induces alveolar type II epithelial cell damage through an oxidative stress-related mitochondrial cell death pathway

Tien Hui Lu[a,1], Chun Hung Chen[b,1], Ming Jye Lee[c,d,1], Tsung Jung Ho[e,f], Yuk Man Leung[g], Dong Zong Hung[h,i], Cheng Chien Yen[j], Tsung Ying He[k], Ya Wen Chen[l,*]

[a] Department of Nutrition, College of Health Care, China Medical University, Taichung, Taiwan
[b] Department of Emergency, China Medical University Hospital, Taichung, Taiwan
[c] Department of Surgery, Chia-Yi Hospital, Taiwan
[d] Department of Health, Executive Yuan, Taiwan
[e] School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung, Taiwan
[f] Chinese Medicine Department, China Medical University Beigang Hospital, Taiwan
[g] Graduate Institute of Neural and Cognitive Sciences, College of Life Sciences, China Medical University, Taichung, Taiwan
[h] Graduate Institute of Drug Safety, College of Pharmacy, China Medical University, Taichung, Taiwan
[i] Toxicology Center, China Medical University Hospital, Taichung, Taiwan
[j] Department of Occupational Safety and Health, College of Health Care and Management, Chung Shan Medical University, Taichung, Taiwan
[k] Department of Emergency Medicine, Chia-Yi Christian Hospital, Taiwan
[l] Department of Physiology & Graduate Institute of Basic Medical Science, College of Pharmacy, China Medical University, No. 91 Hsueh-Shih Road, Taichung 40402, Taiwan

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ABSTRACT

Mercury, one of the widespread pollutants in the world, induces oxidative stress and dysfunction in many cell types. Alveolar type II epithelial cells are known to be vulnerable to oxidative stress. Alveolar type II epithelial cells produce and secrete surfactants to maintain morphological organization, biophysical functions, biochemical composition, and immunity in lung tissues. However, the precise action and mechanism of mercury on alveolar type II epithelial cell damage remains unclear. In this study, we investigate the effect and possible mechanism of methylmercury chloride (MeHgCl) on the human lung invasive carcinoma cell line (Cl1-0) and mouse lung tissue. Cl1-0 cells were exposed to MeHgCl (2.5–10 μM) for 24–72 h. The results showed a decrease in cell viability and an increase in malondialdehyde (MDA) level and ROS production at 72 h after MeHgCl exposure in a dose-dependent manner. Caspase-3 activity, sub-G1 contents and annexin-V binding were dramatically enhanced in Cl1-0 cells treated with MeHgCl. MeHgCl could also activate Bax, release cytochrome c, and cleave poly(ADP-Ribose) polymerase (PARP), and decrease surfactant proteins mRNA levels. Moreover, *in vivo* study showed that mercury contents of blood and lung tissues were significantly increased after MeHgCl treatment in mice. The MDA levels in plasma and lung tissues were also dramatically raised after MeHgCl treatment. Lung tissue sections of MeHgCl-treated mice showed pathological fibrosis as compared with vehicle control. The mRNA levels of proteins in apoptotic signaling, including p53, mdm-2, Bax, Bad, and caspase-3 were increased in mice after exposure to MeHgCl. In addition, the mRNA levels of surfactant proteins (SPs), namely, SP-A, SP-B, SP-C, and SP-D (alveolar epithelial cell functional markers) were significantly decreased. These results suggest that MeHgCl activates an oxidative stress-induced mitochondrial cell death in alveolar epithelial cells.



## 1. Introduction

Uncontrolled industrialization has resulted in an increasing proportion of the human population being exposed to agents that have the potential to cause or exacerbate diseases. For example, mercury and its congeners are extremely toxic substances, which exist in several chemical forms: elemental mercury (Hg0), divalent mercury ($Hg^{2+}$) and methylmercury (MeHgCl) (Diez, 2009). The toxic effects of mercury have been found in neurotoxicity, impaired growth and development, reproductive toxicity, liver toxicity, kidney damage, and immunotoxicity (Day et al., 2007). Among human, the major sources of exposure to mercury compounds include seafood, seeds, disinfectants, disk batteries, and dental amalgam (Diez, 2009). Because mercury, especially in MeHgCl, is often bioaccumulated and biomagnified in the food chain (Watras et al., 1998).

* Corresponding author. Tel.: +886 4 22052121x7728.
*E-mail address:* ywc@mail.cmu.edu.tw (Y.W. Chen).
[1] These authors contributed equally to this work.

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It has been reported that lung is the fastest route for mercury absorption in Nigerian dentist's attitude of amalgam safety (Udoye and Aguwa, 2008). Mercury is deposited and accumulated in the lung tissues through the bloodstream. A case study on suicide revealed that a small amount of intravenous injection of elemental mercury (1.5 mL) markedly increase the serum mercury levels, which was 172–274 μg/L. The X-ray examination showed scattered raiodence deposit in the lung, and the patient complained of breath-dependent chest pain (Eyer et al., 2006). Another case reported high mercury concentration in the blood (140 μg/L) and diffused particles were observed in the capillaries of lung (Vallant et al., 2008).

Previous studies showed that MeHgCl triggers oxidative stress, produces reactive oxygen species (ROS), promotes lipid peroxidation, and depletes antioxidant enzymes, such as superoxide dismutase and catalase (Grotto et al., 2009). For example, rat brains injected with MeHgCl had increased ROS levels in mitochondria (Myhre and Fonnum, 2001). MeHg-induced oxidative stress has been found to be related to cell dysfunction and cell death. Our previous study demonstrated that treatment of MeHgCl induced membrane lipid peroxidation in mice plasma (Chen et al., 2006a). Malondialdehyde (MDA) is an important secondary product and a biomarker of lipid peroxidation in oxidative stress (Nielsen et al., 1997).

Apoptosis was initially regarded as the predominant cell death process in MeHgCl-treated cells. MeHgCl induces apoptosis in various cell types (Chen et al., 2006a; Fujimura et al., 2009). Caspase-3 activation is generally considered to be a marker of apoptosis. A previous study has demonstrated that cell damage with the release of cytochrome c leads to apoptotic signaling including caspase-3 activation (Zhang et al., 2008). In the activation process of caspase-3, its inactive zymogen needs to undergo proteolysis to become activated p17 and p19 subunit (He et al., 2003). Bax was involved in the mitochondrial pathway of apoptosis, which is one of the death effectors of Bcl-2 family. In the early steps of apoptosis, Bax participates in mitochondrial disruption, cytochrome c (CytC) release, and caspase-3 activation (He et al., 2003). Expression of p53 results in the activation of intrinsic apoptosis pathway (Urich et al., 2009) and Bax is considered to be one of the targets of p53 (He et al., 2003). Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme which responds to genotoxic stress. PARP activates apoptosis when cells are unable to repair the damaged DNA and this process is mediated through the release of apoptotic factors from the mitochondria (Eltze et al., 2008).

It has been shown that MeHgCl (2.5–5 μM) induces cell apoptosis in human T lymphocytes (Shenker et al., 1999). Another study also showed that in the HL-7702 cell line, there was a significant increase in cell apoptotic ratio after exposure to MeHg (10–50 μM), which induced mitochondrial membrane potential disruption and increased expression of Bax, Bcl-2, CytC, and caspase-3 (Sun et al., 2009). In addition, a study conducted by Reichl et al. (2001) has demonstrated that MeHg (10 μM) increases lactatedehydrogenase (LDH) release from the alveolar epithelial cell lines. Nevertheless, the cytotoxicity and toxicological mechanism of MeHg on alveolar epithelial cells is still unclear.

In the lung, surfactant proteins (SPs) play an important role in morphological organization, biophysical functions, biochemical composition, and immunity of the lungs. SPs released from alveolar type II epithelial cells are present in four forms, SP-A, SP-B, SP-C, and SP-D, which are composed of approximately 90% lipid and 10% protein (Griese, 1999). The functions of SP-B and SP-C are to form a well-functioning system that maintains the patency of the small airways and alveoli. SP-A and SP-D are important inflammatory regulators in the lungs and they can bind to endotoxin and glycoconjugates of various viral, bacterial, and fungal pathogens. SPs release may be reduced in the progression of lung diseases, such as cystic fibrosis, acute respiratory distress syndrome and acute lung injury (Griese et al., 2004).

It has been suggested that lung epithelial cells are vulnerable to oxidative stress (Herzog et al., 2009). Altogether, in the current study, we hypothesized that oxidative stress by mercury contributes to MeHgCl-induced cell death and cell dysfunction in the lung epithelium.

## 2. Materials and methods

### 2.1.1. Drug preparation

In this study, methylmercury chloride was dissolved in distilled water in preparation for the appropriate concentrations in all experiments (Sigma, St. Louis, MO).

### 2.2. Animal

We purchased 18–25 g male ICR mice from the Animal Center of the College of Medicine, National Taiwan University. The protocols used were approved by the Institutional Animal Care and Use Committee (IACUC) and the care and use of laboratory animals were conducted in accordance with the guidelines of the Animal Research Committee of China Medical University. Mice were housed seven per cage under standard laboratory conditions at a constant temperature (23 ± 2 °C), 50 ± 20% relative humidity, given a solid diet, and 12 h of light–dark cycles. Mice were acclimatized to the laboratory conditions prior to the experiments and all experiments were carried out between 8:00 AM and 05:00 PM. Mice were weighted and randomly assigned to either MeHgCl or vehicle group. In these experimental groups, mice were gavage with 0.2 mg/kg/day of MeHgCl or saline in the MeHgCl or vehicle group, respectively. Each group contained 12 mice and was observed for daily clinical signs.

### 2.3. Cell culture

The human lung epithelial derived cell lines, Cl1-0 were kindly provided by Dr. P.-C. Yang (Department of Internal Medicine, National Taiwan University Hospital), were cultured in a humidified chamber with 5% $CO_2$ and 95% air mixture at 37 °C. The cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Gibco BRL, Life Technologies).

### 2.4. Cell viability

Cells were washed with phosphate buffered saline (PBS), re-suspended in fresh media, and cultured in 24 well plates ($2 \times 10^5$/well). Cells were then treated with MeHg (2.5–10 μM) for 24 h, 48 h or 72 h. After the indicated time of incubation, the media were aspirated and fresh media containing 30 μL of 2 mg/mL 3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyl tetrazolium bromide (MTT), were added for 4 h. After incubation, the media were pipetted out and 1 mL of dimethyl sulfoxide (Sigma, St. Louis, MO) was added to dissolve the blue formazan crystal. Following mixing, 150 μL of mixture was aliquot to the 96 wells. An enzyme-linked immunosorbent assay reader (Thermo, Multiskan Ex, Vantaa, Finland) was used for detection at an absorption band of 570 nm.

### 2.5. Lipid peroxidation assay

Cells were treated with MeHgCl (2.5–5 μM) for 72 h. After incubation, cells were first washed with PBS, and then harvested. Lung tissues and whole blood were collected after MeHgCl (0.2 mg/kg) administration. Whole blood was centrifuged at 3000 × *g* for 10 min to obtain the plasma. Cells and lung tissues were homogenized with 20 mM Tris–HCl buffer, pH 7.4 and centrifugated. The supernatant was collected and added to 3.25 volumes of diluted R1 reagent (10.3 mM *N*-methyl-2-phenylindole in acetonitrile). After mixing, the mixture was added with 0.75 volumes of 37% HCl which was then incubated at 45 °C for 60 min. An absorbance of 590 nm was measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Thermo, Multiskan Ex, Vantaa, Finland). The protein concentration was determined using the bicinchoninic acid protein assay kit with an absorption band of 570 nm (Pierce, Rockford, IL).

### 2.6. Caspase-3 activity determination

After the cells were incubated with MeHgCl for 72 h, cell lysates were incubated with 10 μM Ac-DEVD-AMC (caspase-3/CPP32 substrate) at 37 °C. The fluorescence of the cleaved substrate was measured by a spectrofluorometer (Spectramax, Molecular devices) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm (Promega Corporation, Madison, WI).

### 2.7. Annexin-V fluorescence intensity assay

Flow cytometry (FACScalibur, Becton Dickinson) analysis was performed to determine apoptosis using annexin-V fluorescein isothiocyanate (FITC) assay kit

(BioVision, USA). Cells were treated with MeHgCl (0–10 μM) for 72 h and then washed twice with PBS and stained with annexin-V-FITC for 20 min at room temperature. Subsequently, the cells were washed twice with PBS. The apoptosis level was determined by measuring fluorescence intensity of cells. Each group contained more than 10,000 individual cells.

### 2.8. Sub-G1 content determination

Cl1-0 cells were harvested and washed with PBS twice and re-suspended in 70% (v/v) ethanol, then stored at 4 °C for 24 h. Subsequently, cells were washed with PBS and stained with propidium iodide (PI; Sigma–Aldrich, USA) for 30 min in dark conditions. Then the cells were washed with PBS and cell counts were performed using flow cytometry (FACScalibur, Becton Dickinson).

### 2.9. Western blot analysis

Fifty micrograms of protein of each cell lysate was subjected to electrophoresis on 10% (w/v) SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 h in PBST (PBS, 0.05% Tween-20) containing 5% nonfat dry milk. After blocking, blots were incubated with p53, Bax, cytochrome c (Santa Cruz Biochemicals), and PARP (Oncogene Research Products) antibodies in PBS and 0.1% Tween-20 for 1 h followed by two washes (15 min each). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies. The antibody-reactive bands were revealed by enhanced chemiluminescence reagents (Amersham) and were exposed on the Kodak radiographic film.

### 2.10. Quantitative real-time PCR

Intracellular total RNA (5 μg) was extracted from Cl1-0 cells and lung tissues, either treated with or without MeHgCl, was heated to 90 °C for 5 min to remove any secondary structures, then placed rapidly on ice. Samples were reverse transcribed into cDNA using the AMV RTase (reverse transcriptase enzyme, Promega Corp. Pty Ltd.) at 42 °C. The reverse transcriptase reaction was performed as follows: RNA (5 μg) was added to a reaction buffer containing the following: dNTP (deoxynucleotide mix), 2.5 mM; RNasin (RNAase inhibitor, Promega), 40 U/μl; random hexamer primers, 100 nmol; 1× RTase buffer (supplied with RTase enzyme); AMV reverse transcriptase enzyme (RTase), 30 U; made up with nuclease-free water to a final volume of 20 μ1. The mixture was mixed and incubated at 42 °C for 60 min. Samples were then denatured at 95 °C for 10 min and placed on ice. Real-time Sybr Green primers for mouse surfactant proteins were chosen according to the previously references. SP-A, forward 5′-GACGTTTGTGTTGGAAGCCCTGG-3′ and reverse 5′-GGTACCAGTTGGTGTAGTTCACAG-3′ (Haque et al., 2007); SP-B, forward 5′-CTATCACGTCGGCCTCATC-3′ and reverse 5′-CTGGCTCTGGAAGTAG TCAAT-3′; SP-C, forward 5′-GCCTTCTCATCGTG GTTGT-3′ and reverse 5′-CCAGTATCATGCCCTTCCT-3′ (Tong et al., 2006); SP-D, forward 5′-TAT GAA TTCGCTGCATTGTTCCCTGAT-3′ and 5′-TTTCTCGAGTCAGAACTCACAGATAAC AAG-3′ (Motwani et al., 1995). Each sample (2 μL) was tested with real-time Sybr Green PCR reagent (Invitrogen, USA) with transgene-specific primers in a 25-μl reaction volume, and amplification was performed using an ABI Prism® 7900HT (PE, Applied Biosystems) real-time thermal cycler. Cycling condition was 2 min at 50 °C; 10 min at 95 °C; 40 cycles of 92 °C for 30 s; 60 °C for 1 min. Real-time fluorescence detection was performed during the 60 °C annealing/extension step of each cycle. Melt-curve analysis was performed on each primer set to ensure that no primer dimmer or nonspecific amplification was present under the optimized cycling conditions. The fold difference in mRNA expression between treatment groups was determined using the relative quantification method utilizing real-time PCR efficiencies and normalized to the housekeeping gene, β-actine, thus comparing relative $C_T$ changes between control and experimental samples. Prior to conducting statistical analyses, the fold change from the mean of the control group was calculated for each individual sample (including individual control samples to assess variability in this group).

### 2.11. Mercury contents

Mice were orally gavage with MeHgCl (0.2 mg/kg/day) for either 2 or 4 weeks. At the end of the study, the mice were anesthetized, where the whole blood and lung tissues were extracted. The levels of mercury in blood and flushed blood of the lung tissues were determined by cold vapor atomic absorption spectrophotometer.

### 2.12. Lung tissues histopathological analysis

Mice were administered with MeHgCl (0.2 mg/kg/day) for 4 weeks. At the end of the study, the animals were sacrificed for the lung, which is to be fixed in 4% paraformaldehyde–PBS overnight. After, the lung tissues were stained with hematoxylineosin (HE) and embedded in paraffin. The lung tissues pathological changes were then observed with a light microscopy and the pathological fibrosis score in the section is estimated (Long et al., 2007).

### 2.13. Statistical analysis

Data are presented as means ± SEM. The significance of difference was evaluated by Student's *t*-test. When more than one group was compared with one control, significance was evaluated according to one-way analysis of variance (ANOVA) was used for analysis, and the Duncans's post hoc test was applied to identify group differences. The *P* value less than 0.05 was considered to be significant. The statistical package SPSS, version 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

## 3. Results

### 3.1. Effects of MeHgCl on cell viability, lipid peroxidation and reactive oxygen species production in Cl1-0 cells

We used the MTT assay to test MeHgCl-induced toxicity in lung epithelial cell-derived Cl1-0 cells; cell viability was significantly decreased after 24 and 48 h of MeHg (10 μM) treatment. After 72 h of MeHgCl treatment, cell viability was dramatically reduced in a dose-dependent manner (from 2.5 to 10 μM) (Fig. 1A).

To further investigate the cytotoxic effects of MeHgCl on the reactive oxygen species production in Cl1-0 cells, we detected the cellular levels of malondialdehyde, a lipid peroxidation marker. After cells were treated with MeHgCl for 72 h, the MDA levels were dramatically enhanced (Fig. 1B). In addition, Cl1-0 cells exposure to MeHgCl (2.5, 5 and 10 μM) for 30 min, 1, 2, and 4 h, had significant increased ROS levels as observed using DCF fluorescence as an indicator of ROS formation (Fig. 1C). These data demonstrate that MeHgCl caused Cl1-0 cell death by induced oxidative stress.

### 3.2. Effects of MeHgCl-induced apoptosis in Cl1-0 cells

In a previous study, caspase-3 activation had been reported to mediate lung cell death (Pagano et al., 2005). In this study, we examined whether caspase-3 was involved in MeHgCl-induced Cl1-0 cell death. Caspase-3 activity was determined using the CaspACETM fluorometric activity assay. After the cells were treated with MeHgCl (5 μM or 10 μM) for 72 h, the caspase-3 activity was significantly increased (Fig. 2A). Therefore, it is possible to speculate that MeHgCl-induced cell death may involve enhanced caspase-3 activity.

To confirm the cytotoxicity of MeHgCl-induced Cl1-0 cells from the point view of apoptotic properties, we detected the externalization of phosphatidyl serine by annexin-V-FITC staining and sub-G1 hypodiploid cell population by flow cytometry (Chen et al., 2006a). Results showed that exposure of cells with MeHgCl (0–10 μM) for 72 h triggered the increase in both annexin-V staining and sub-G1 hypodiploid cell population in a dose-dependent manner (Fig. 2B and C). These results indicate that treatment of the Cl1-0 cells with MeHgCl induced apoptosis.

### 3.3. Effects of MeHgCl on p53, Bax and cytochrome c protein expression and cleavage of poly (ADP-Ribose) polymerase in Cl1-0 cells

p53 and Bax protein expression is involved in caspase-3-mediated apoptosis (He et al., 2003). In order to investigate the effect of MeHgCl on p53, Bax protein expression, and cytochrome c release, cells were treated with MeHgCl (2.5, 5 and 10 μM) and subsequently western blotted to analyze protein expression. After MeHg treatment, p53 and Bax expression was significantly increased at 8 and 12 h, respectively (Fig. 3A and B). Treatment of Cl1-0 cells with MeHgCl for 48 h effectively increased the cytochrome c level in the cytosol of the cells (Fig. 3C-a and -b). To further evaluate the apoptotic signaling by MeHgCl, PARP cleav-

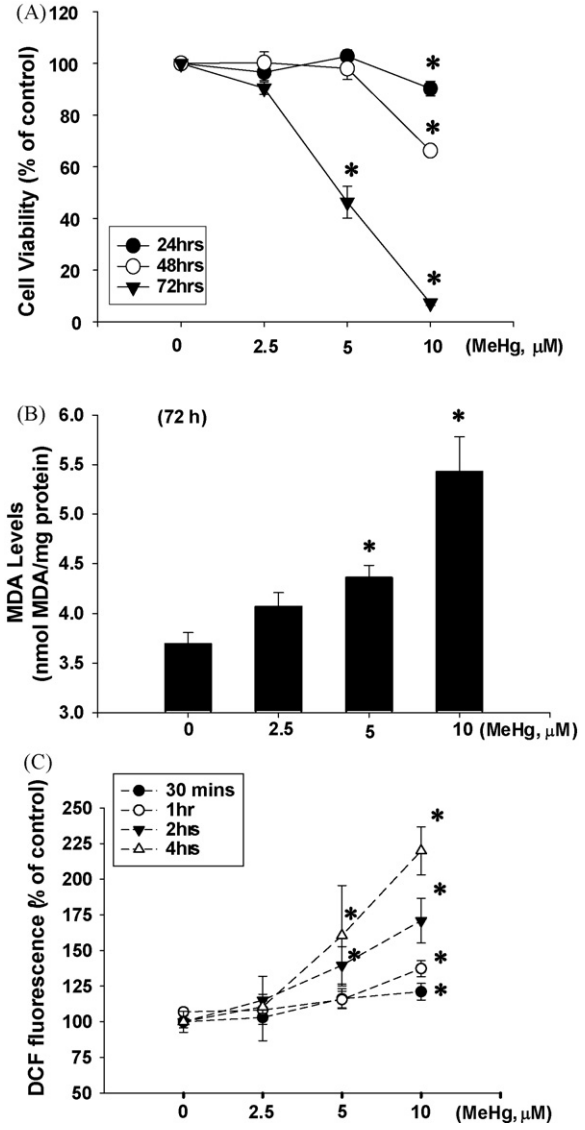


**Fig. 1.** Effects of MeHgCl on cell viability and lipid peroxidation in lung epithelial cell-derived Cl1-0 cells. (A) Cells were treated with MeHgCl (2.5–10 μM) for 24 h or 48 h or 72 h, and cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyl tetrazolium bromide) assay. (B) Cells were treated with MeHgCl (2.5–10 μM) for 72 h, and cell malondialdehyde levels were determined as described in Section 2. (C) Cells were treated with or without MeHgCl (2, 5 and 10 μM) for various time courses, and ROS was determined by flow cytometry. All data are presented as means ± SEM from four independent experiments with triplicate determinations. *$P < 0.05$ as compared with vehicle control.

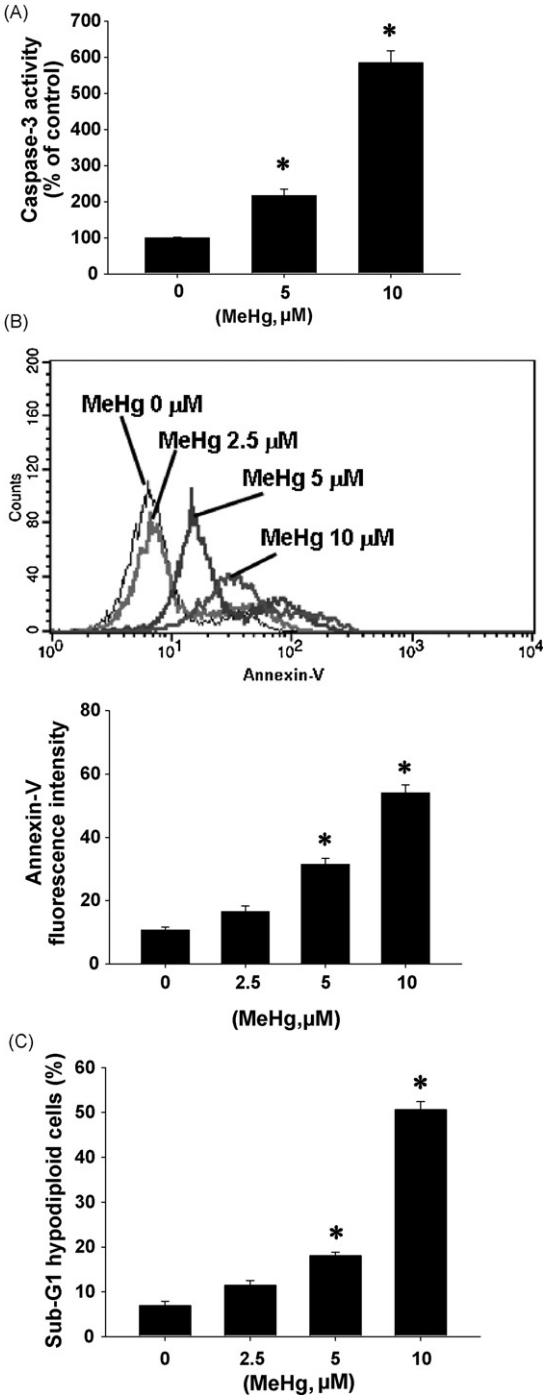


**Fig. 2.** Effects of MeHgCl on caspase-3 activity in Cl1-0 cells. (A) Cells were treated with MeHgCl (5 and 10 μM) for 72 h. Caspase-3 activity was determined as described in Section 2. (B) Cells were treated with MeHg (0, 2.5, 5 and 10 μM) for 72 h. Annexin-V-FITC fluorescence of apoptosis was determined by flow cytometry. (C) Sub-G1 hypodiploid cell content which staining with propidium iodide (PI) was analyzed by flow cytometry. All data are presented as means ± SEM for three independent experiments with triplicate determinations. *$P < 0.05$ as compared with vehicle control.

age was analyzed. After treatment of Cl1-0 cells with MeHgCl (2.5, 5 and 10 μM) for 48 h, PARP cleavage was significantly enhanced (Fig. 3D). These data suggest that MeHg induced apoptosis in Cl1-0 cells.

### 3.4. Surfactant proteins mRNA expression in MeHgCl-exposed Cl1-0 cells

To investigate the effect of MeHgCl on surfactant proteins, cells were treated with MeHgCl (2.5, 5 and 10 μM) and quantitative real-time PCR was performed to analyze mRNA expression. After MeHgCl treatment, surfactant proteins mRNA, including SP-A, SP-B, SP-C, and SP-D were significantly decreased at 72 h (Fig. 4). These results indicate that MeHgCl inhibit the lung epithelial cell function.

### 3.5. Blood mercury contents and lung tissues mercury contents in mice after MeHgCl administration

In this experiment, we investigated if oral treatment with MeHgCl would cause mercury accumulation in blood and lung tissues. Blood mercury levels in normal mice were about 1.15 ± 2.08–1.20 ± 0.37 μg/L (Table 1). But, after exposure to

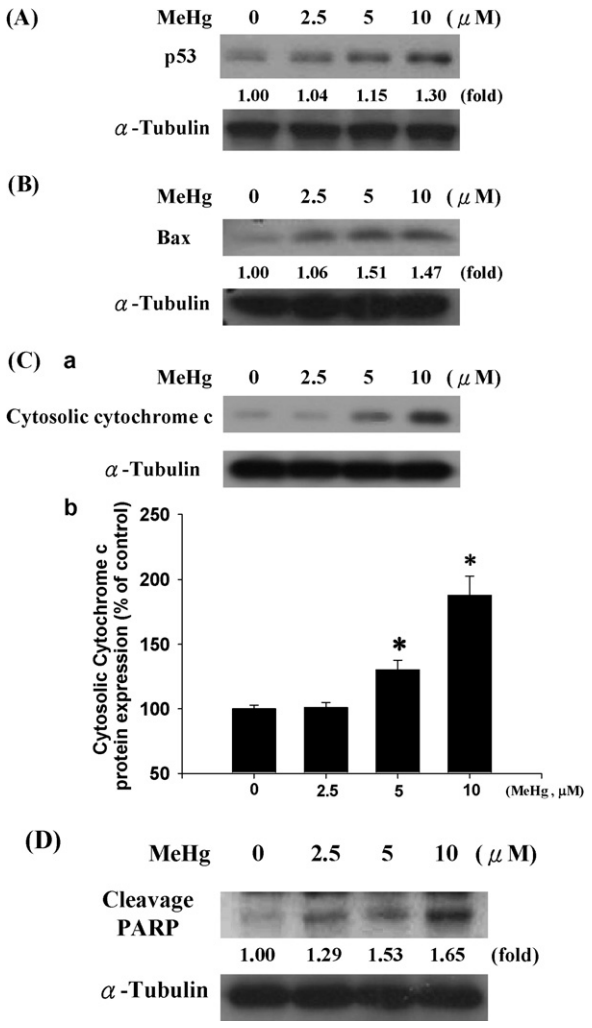


**Fig. 3.** Analysis of p53, Bax, cytochrome c expression and PARP cleavage in MeHgCl-treated Cl1-0 cells. (A) The p53 protein expression was detected by western blot in MeHgCl (2.5–10 μM)-treated cells for 8 h. (B) The Bax protein expression was detected by western blot in MeHgCl (2.5–10 μM)-treated cells for 12 h. (C) The cytosolic cytochrome c protein expression was detected by western blot in MeHg (2.5–10 μM)-treated cells for 48 h. (a) Western blot analysis and (b) is quantified of protein expression. All data are represented for three independent experiments. Results shown in (C)-(b) are $^{*}P < 0.05$ as compared with vehicle control. (D) The cleavage PARP protein expression was detected by western blot in MeHgCl (2.5–10 μM)-treated cells for 48 h. All data are represented for three independent experiments.

MeHgCl (0.2 mg/kg) for 2–4 weeks, the blood mercury concentrations were drastically elevated to 778.25 ± 156.98 μg/L. In lung tissues, mercury accumulation was significantly increased in the MeHgCl group when compared to the vehicle control (Table 1).

**Table 1**
Mercury levels in MeHg-exposed mice.

| | Week | Group | |
|---|---|---|---|
| | | Control | MeHg-0.2 mg/kg |
| Blood | 2 | 1.15 ± 0.28 | 778.25 ± 156.98* |
| | 4 | 1.20 ± 0.37 | 957.49 ± 93.18* |
| Lung | 2 | 7.63 ± 1.18 | 1340.22 ± 49.04* |
| | 4 | 9.52 ± 0.68 | 47.52 ± 516.02* |

1. Hg content of blood was expressed as μg/L of blood.
2. Hg content of lung tissue was expressed as ng/g/wet of blood.
3.Data are expressed as mean ± S.E. ($n = 8$ for each group).
* $P < 0.05$ as compared with vehicle control group.

**Table 2**
Plasma and lung tissue lipid peroxidation in MeHg-exposed mice.

| | Week | Group | |
|---|---|---|---|
| | | Control | MeHg-0.2 mg/kg |
| Plasma | 2 | 100 ± 6.57 | 164.44 ± 6.96* |
| | 4 | 100 ± 2.14 | 113.44 ± 7.41* |
| Lung | 4 | 100 ± 3.82 | 125.33 ± 5.61* |

MDA and HAE levels of plasma and lung tissues were expressed as mean ± S.E. ($n = 8$ for each group).
* $P < 0.05$ as compared with vehicle control group.

Thus, oral treatment with MeHgCl causes mercury accumulation in the blood and lung tissues.

### 3.6. Effects of MeHgCl on plasma and lung tissues lipid peroxidation and pathological changes and surfactant proteins expression in mice

To further investigate MeHgCl-induced oxidative stress *in vivo*, plasma and lung tissues were harvested after MeHgCl (0.2 mg/kg) treatment. Plasma and lung tissues MDA levels were significantly enhanced after MeHgCl administration when compared with the vehicle control (Table 2).

In the next experiment using hematoxylineosin (HE) staining, pathological sections obtained from MeHgCl-treated mice showed a widening of the alveolar septum due to deposition of extracellular matrix (Fig. 5A and B).

### 3.7. Effects of MeHgCl on p53, Bax, Bad, mdm-2, caspase-3 mRNA and surfactant proteins mRNA expression in lung tissues

To further evaluate the apoptotic signaling in lung tissues, we measured p53, Bax, Bad, mdm-2, and caspase-3 mRNA expression in the lung tissues of mice. Mice were administered with 0.2 mg/kg/day of MeHgCl for 4 weeks. We found that p53, Bax, Bad, mdm-2, and caspase-3 mRNA expressions were all significantly enhanced in the MeHgCl group when compared with the vehicle control group (Fig. 6A). These results suggest that MeHgCl induces apoptotic signaling in mice.

The surfactant proteins mRNA, including SP-A mRNA, SP-B mRNA, SP-C mRNA, and SP-D mRNA expressions were significantly reduced after MeHgCl exposure for 4 weeks in the lung tissues of mice (Fig. 6B). Thus, the results suggest that MeHgCl induces lung damage and fibrosis through apoptotic pathway.

## 4. Discussion

Lipid peroxidation has been demonstrated to mediate oxidative stress in apoptosis (Groc et al., 2001). Oxidative stress is known to influence numerous gene expressions and signal transduction pathways (Cardona, 2004). Elevation of ROS production leads to lipid peroxidation, subsequently resulting in rapid cytotoxicity in apoptosis (Canal-Raffin et al., 2008). Mitochondrial transmembrane potential disruption, release of mitochondrial death mediators and subsequent activation of caspases are involved in ROS-mediated apoptosis pathway (Chen et al., 2006a). It has been demonstrated that MeHgCl increases ROS production and lipid peroxidation and depletion of antioxidant enzymes in apoptosis pathway (Franco et al., 2009). In lung epithelial cells, mercury compounds have been shown to increase ROS levels and intracellular lactatedehydrogenase release (Han et al., 2007). However, the role of oxidative stress in the cytotoxic effects of MeHgCl on lung epithelial cells has not been clarified.

In the present study, we used the human lung epithelium-derived cell line, Cl1-0, to analyze the cell viability, malondialde-

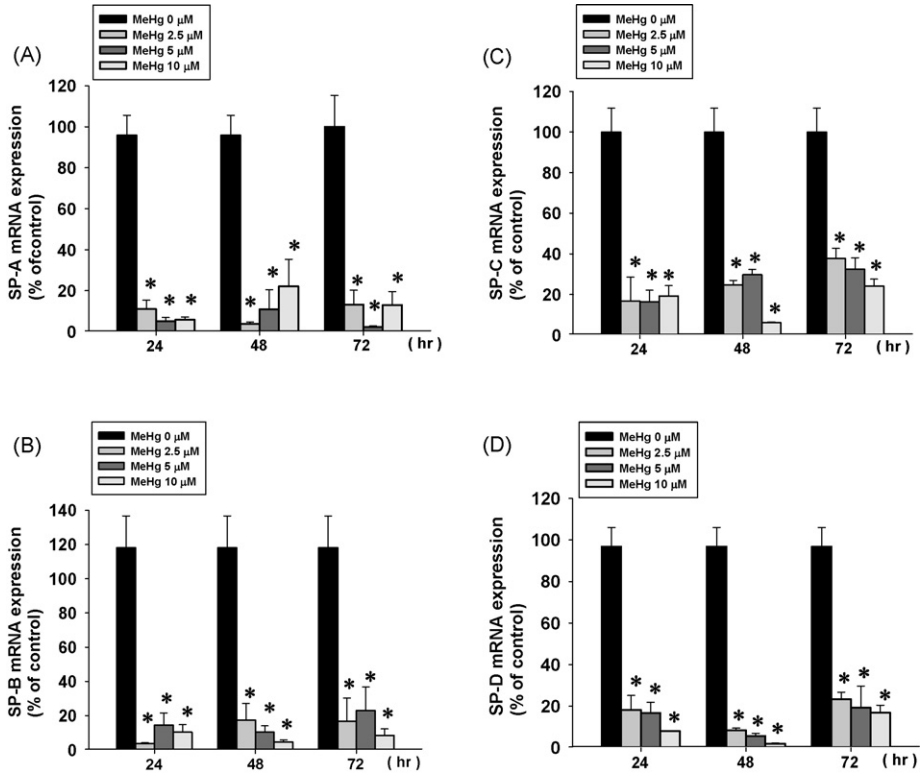


**Fig. 4.** Surfactant proteins mRNA expression in MeHgCl-treated Cl1-0 cells. Cells were administered with MeHgCl (2.5–10 μM) for 24, 48 and 72 h. Four types of surfactant protein mRNA [(A) SP-A mRNA, (B) SP-B mRNA, (C) SP-C mRNA and (D) SP-D mRNA] were determined by quantitative real-time PCR. All data are expressed as mean ± S.E. ($n = 4$ for each group). *$P < 0.05$ as compared with vehicle control group.

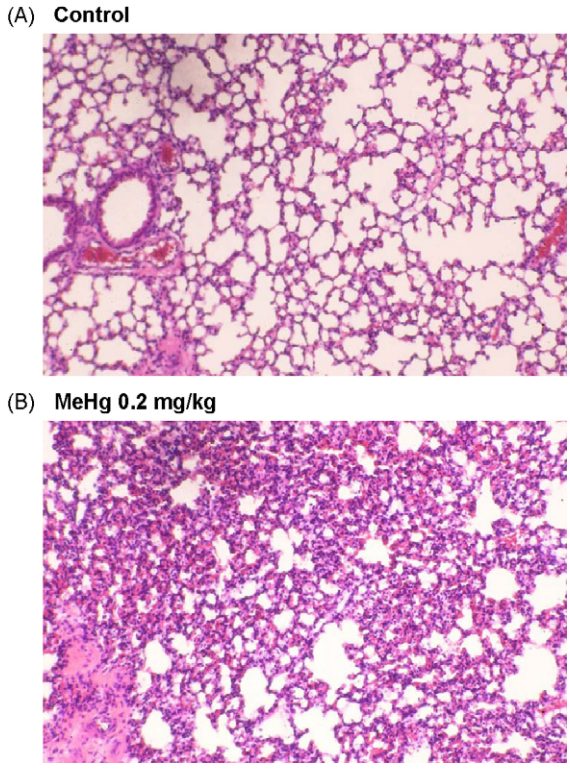


**Fig. 5.** Pathologic of features of lung tissues (HE staining, original magnification 100×) on 4 weeks after MeHgCl treatment. (A) Control group; (B) MeHgCl (0.2 mg/kg/day) group. Histopathologic assessment of pulmonary fibrosis of each group by the Ashcroft score ($n = 4$). *$P < 0.05$ significantly different from control group.

hyde levels and ROS production after exposure to MeHgCl at various concentrations (2.5, 5 and 10 μM). The results showed that MeHgCl significantly induced cell death, enhanced lipid peroxidation levels and increased ROS production. These data suggest that MeHgCl exposure increases oxidative levels, which may be involved in lung epithelial cell death and dysfunction pathways.

It has been suggested that lung epithelial cell is highly sensitive to oxidative stress (van der Toorn et al., 2009). MeHgCl-induced oxidative stress may deplete antioxidant enzymes, resulting in cell damage (Grotto et al., 2009). A previous study has demonstrated that increased ROS production caused apoptosis of lung epithelial cells. As a result an increase of lipid peroxidation, depletion of intracellular glutathione (GSH) levels and decreased superoxide dismutase (SOD) expression (Periyakaruppan et al., 2007) were observed. Indeed, oxidative stress has been implicated in lung pathogenesis, such as acute respiratory distress syndrome, asthma, lung fibrosis, and bronchopulmonary dysplasia (Kim et al., 2003). In this study, we found that MeHgCl (5 and 10 μM) significantly decreased cell viability at 72 h. At this time point, the MDA level and ROS, which are lipid peroxidation and oxidative stress markers, were increased after MeHgCl (2.5, 5 and 10 μM) exposure. These data indicate that MeHgCl is able to decrease lung epithelial cell viability through an oxidative stress-trigger pathway.

Oxidative stress was found to be the major risk factor in the lung epithelial damage (Vulimiri et al., in press). A previous study has demonstrated that oxidative stress may lead to the loss of lung epithelial cell and shedding of syndecan-1. Shedding of syndecan-1, which is responsible for wound healing and neutrophil chemotaxis, caused neutrophil recruitment, increased ROS production and increased TGF-β bioavailability (Kliment et al., 2009). Oxidative stress induced activation of the apoptotic caspase-3 enzyme, NFkappaB, and AP-1 in human bronchial epithelial cells (BEAS-2B) (Pylkkanen et al., 2009). Ueda et al. (2002) also showed that oxidative stress induced mitochondrial dam-

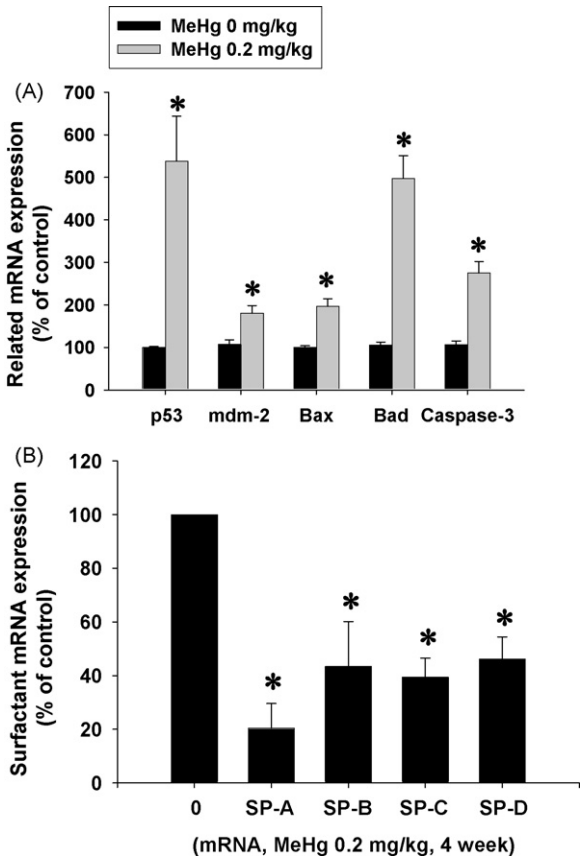


**Fig. 6.** The mRNA expression of MeHgCl-exposed mice. Mice were orally gavage with 0.2 mg/kg/day MeHgCl for 4 weeks. (A) p53, mdm-2, Bax, Bad caspase-3 mRNA, and (B) four types of surfactant protein mRNA (SP-A mRNA, SP-B mRNA, SP-C mRNA and SP-D mRNA) were determined by quantitative real-time PCR. All data are expressed as mean ± S.E. ($n = 4$ for each group). $^*P < 0.05$ as compared with vehicle control group.

age from the release of cytochrome c, activation of p53, caspases and apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase. Other reports have found that lung epithelial fibroblast cell death was also through the apoptosis pathway, involving mitochondrial transmembrane potential reduction, upregulation of Bcl-2, degradation of p53, caspase-8-dependent Bid cleavage, release of cytochrome c, activation of caspase-9 and -3, and PARP cleavage (Oh et al., 2004). There has been evidence that chemical toxicity, such as *p*-phenylenediamine was related to p53 gene expression. High or low levels of chemical toxicity influence the type of cell death, for example, apoptosis and necrosis (van Vliet et al., 2007). In mitochondria, pro-(Bax, Bid, Bad), and anti-apoptotic Bcl-2 members (Bcl-2, Bcl-XL) regulate the apoptotic pathway. The Bcl-2/Bcl-XL proteins are localized to the outer mitochondrial membrane to prevent apoptosis of cytochrome c release from mitochondria. Recently, it has been shown that activation of Bax promotes Bcl-2-independent cytochrome c release (Oh et al., 2004). We have previously demonstrated that mercury caused ROS production, disruption of mitochondrial membrane potential, release of cytochrome c from mitochondria to cytosol and activation of caspase-3, eventually leading to pancreatic islet β-cell apoptosis (Chen et al., 2006a). Lee et al. (2006) demonstrated that mercury induced death-signaling pathways via cytochrome c release to cytosol and activation of caspase-3 in tubular epithelial Madin–Darby canine kidney (MDCK) cell line. Here, we found that MeHgCl was capable of inducing cell death in lung epithelial Cl0-1 cells. MeHgCl triggered p53 and Bax protein expression, cytosolic cytochrome c increase, caspase-3 activation and an increase in PARP cleavage. In lung tissues, we also found that p53, Bax, Bad, mdm-2, and caspase-3 mRNA expressions were increased in MeHgCl administered mice. These results suggest that oxidative stress caused lung epithelial cell death through a mitochondria-caspase-3-PARP pathway.

Das et al. (1997) found that intragastric intubation with methylmercury chloride of 15 mg/kg/day for 4 days showed that the lung weight to body weight ratios were increased, minimum surface tension were greater, mean alveolar diameters were reduced and alveolar walls were thickened. It may suggest that methylmercury chloride has an effect on alveolar wall structures. But there were no evidences showing that MeHgCl-induced oxidative stress in alveolar epithelial cell caused alveolar epithelial cell death and dysfunction. On the other hand, in our animals study, we used 0.2 mg/kg/day of MeHgCl to administer mice for 2–4 weeks. This dose is lower compared to the previous study conducted by Das et al. (1997). According to our previous study, mice exposed to mercury over a 2–4-week period had elevated blood mercury levels ranging from ~45 to 60 μg/L. The increased blood mercury levels in the present study approach the human-exposed levels (Chen et al., 2006b). In our study, we found that MeHgCl (0.2 mg/kg/day) orally fed to mice caused significant blood and lung tissue mercury accumulation at 2 and 4 weeks. These results suggest that environmental mercury exposure would promote mercury accumulation into the blood and lung tissues through an oral pathway. Oxidative stress can be a risk factor that induced lung epithelial cell death and dysfunction. It has been reported that oxidative stress decreases manganese superoxide dismutase (MnSOD) activity, elevates lactate dehydrogenase in human lung carcinoma cells (ATCC line H441), which have epithelial characteristics (including lamellar bodies, surfactant protein SP-A, and SP-B) (Li et al., 2002). A previous study has shown that ROS caused type II alveolar epithelial cell apoptosis, increased MDA levels and reduced surfactant associated protein C (SP-C) mRNA expression (Fu et al., 2008). Lung epithelial cell death is frequently linked to idiopathic pulmonary fibrosis (IPF), which is a progressive lung disease of unknown cause (Lepparanta et al., in press). IPF is characterized by interstitial fibrosis followed by chronic alveolitis. In IPF, apoptosis and DNA damage in bronchiolar and alveolar epithelial cells have been demonstrated (Maeyama et al., 2001). In our experiments, we found that MeHgCl induced significant cell death and apoptosis-related signaling expression both in Cl1-0 cells and lung tissues. *In vivo*, plasma and lung tissue MDA levels and lung fibrosis were dramatically enhanced after oral exposure to MeHgCl (0.2 mg/kg/day) for 2 and 4 weeks.

Lung alveolar epithelium is composed of two specialized epithelial cell types, the squamous alveolar epithelial type I (ATI) cells, which constitute approximately 93% of the alveolar epithelial surface area, and the surfactant-producing cuboidal alveolar epithelial type II (ATII) cells (Crapo et al., 1982). Pulmonary alveolar type II (AT II) epithelial cells are responsible for surfactant synthesis and secretion. These cells are highly lipogenic with a high lipid turnover (Yang et al., 2003). Surfactant is a complex of macromolecular aggregates composed of phospholipids and surfactant proteins which are essential for lowering the surface tension of alveoli and maintaining of normal lung functions (Ikegami, 2006). The damage of type II epithelial cells may trigger a decrease in surfactant content and an increase in surface tension at the air-liquid interface; these results may induce alveolar collapse and cause acute lung injury, and may even lead to the development of the respiratory distress syndrome (RDS) (Arbibe et al., 1998). Here, we found that surfactant mRNA, including those of SP-A, SP-B, SP-C, and SP-D were significantly reduced in Cl1-0 cells and lung tissues after exposure to MeHgCl.

## 5. Conclusion

Collectively, we present evidence showing that MeHg triggered oxidative stress, increase lipid peroxidation levels, induce cell apoptosis, and decrease surfactant mRNA levels in lung epithelial cell line Cl1-0 and lung tissues. The increase in MDA levels, caspase-3 activation, p53 and Bax protein expression, and cytochrome c release from mitochondria to cytosol, were involved in MeHg-induced oxidative stress and lung epithelial cell apoptosis. In lung tissues, p53, Bax, Bad, mdm-2, and caspase-3 mRNA expressions were increased in MeHgCl administered mice. The oxidative stress may also lead to lung fibrosis and reduction in surfactant mRNA levels. These results suggest that MeHg-induced oxidative stress causes alveolar epithelial cell apoptosis and dysfunction.

## Conflict of interest

All authors declare that there are no conflicts of interest in this study.

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