Journal of Hazardous Materials 221–222 (2012) 256–263

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Enhancement between environmental tobacco smoke and arsenic on emphysema-like lesions in mice

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ARTICLE INFO

Article history:
Received 22 November 2011
Received in revised form 13 April 2012
Accepted 17 April 2012
Available online 25 April 2012

Keywords:
Chronic obstructive pulmonary diseases
Arsenite
Environmental tobacco smoking
Plasma 8-oxodeoxyguanosine

ABSTRACT

Chronic obstructive pulmonary diseases (COPD) have been the major cause of mortality worldwide. Early identification of populations at risk allows us to prevent the occurrence and to reduce the cost of health care. In human studies, exposure to environmental tobacco smoke (ETS) and arsenic respectively increased the risk of chronic lung diseases, including COPD. We suspected that ETS and arsenic might enhance the risk of COPD. In our present study, we evaluated this hypothesis in mice and tried to identify early biomarkers for chemicals-induced lung lesions. Mice inhaled ETS and/or administrated arsenite *via* gavage for 4 weeks. At the end of experiment, exposure to ETS or arsenite alone failed to cause lung lesions or inflammation. However, co-exposure to ETS and arsenite significantly induced emphysema-like lesions, characterized with enlarged alveolar spaces and destruction of alveolar structure, although inflammation was not observed. Furthermore, co-exposure to ETS and arsenite significantly increased plasma 8-oxodeoxyguanosine (8-OHdG) levels. Our results indicated that co-exposure to ETS and arsenite induced emphysematous lesions, and plasma 8-OHdG might serve as an early biomarker for co-exposure of ETS and arsenite. With information about ETS and arsenic exposure in human populations, plasma 8-OHdG will help us to identify individuals at risk.

1. Introduction

Exposure to many chemicals, either occupationally or in the environment, has been associated with the development of chronic lung diseases, such as chronic obstructive pulmonary diseases (COPD), asthma, and lung cancer [1,2]. For example, cigarette smoking increased the risk of COPD and lung cancer [3,4]. However, recent epidemiological studies showed that at least of quarter of COPD patients were non-smokers in many countries [1,5,6]. It is likely that exposure to other chemicals may associate with the development of lung diseases in nonsmokers.

More recently, Salvi and Barnes [1] pointed out that exposure to environmental factors, including indoor air pollution, outdoor air pollution and occupational exposure to different chemicals, are risk factors for the development of lung diseases in nonsmokers. Environmental tobacco smoke (ETS) is a mixture of mainstream and side-stream smoke. Tobacco smoke contained hundreds of compounds [7]. Basically, compounds in tobacco smoke and ETS are qualitatively similar, but quantitatively different [8,9]. Some studies showed that cotinine, a biomarker for tobacco smoking, was detectable in serum and urine from most non-smokers [10]. It appears that ETS exposure is pretty common in general populations and considered as hazardous for nonsmokers [11]. Some studies showed that ETS exposure increased the risk of COPD [12–14]. Furthermore, Slowik et al. [15] recently reported that exposure to secondhand smoke increased the markers for elastin degradation in human populations. However, the causal relationship between ETS exposure and COPD was not established in animal studies [3].

Arsenic (As) exposure is also a risk factor for lung diseases. Epidemiological studies in Chile, Bangladesh and the West Bengal region of India show that chronic exposure to As *via* drinking water is correlated with increased incidence of chronic cough, chronic bronchitis, shortness of breath and obstructive or restrictive lung diseases [16–19]. However, very few studies investigated As-induced lung lesions, except lung cancer, in animals. Recently, Singh et al. [20] reported that oral administration of sodium arsenite for 180 days induced degenerative changes in bronchiolar epithelium with emphysema in mice. It appears that the lung is one of the target organs for As exposure *via* drinking water.

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http://dx.doi.org/10.1016/j.jhazmat.2012.04.042

The general public tends to simultaneously expose to variety of environmental toxicants. Sometimes, co-exposure to different toxicants enhanced the risk of adverse effects. For example, As enhanced metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in mice, which might increase the cancer risk [21]. More recently, we reported that co-treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin and NNK increased the incidence of lung tumors in mice [22]. ETS and As are common environmental toxicants and have been associated with nonmalignant lung diseases in human populations. In our present study, we tested our hypothesis in mice that co-exposure to ETS and arsenite might increase lung lesions. The routes of ETS and arsenite exposure in mice were respectively inhalation and gavage administration, which mimicked the routes of exposure in humans. We also hope to identify early biomarkers for their interaction in lung diseases.

2. Materials and methods

2.1. Animals

A total of 48 6-week-old male ICR mice were used in this study. All animals were purchased from BioLASCO (Taiwan) and housed at the Animal Facility Center at the National Health Research Institutes in Taiwan in accordance to standard and approved protocols at the facility (23 ± 1 °C, 39–43% relative humidity; water and food were available *ad libitum*).

2.2. Exposure to As and/or ETS

Animals were divided into four groups (12 mice for each group) randomly: control group (Con), inhalation with fresh air plus gavage with 100 μL saline; ETS group (ETS), inhalation with ETS plus gavage with saline; As group (As), inhalation with fresh air plus gavage with sodium arsenite ($NaAsO_2$, 10 mg/kg body weight), and ETS-As group: inhalation with ETS plus gavage with sodium arsenite. Mice were exposed to fresh air or ETS in a whole-body exposure chamber (SCIREQ Scientific Respiratory Equipment Inc., Montreal, Canada) 2 h per day, 5 days per week for 4 weeks. Oral administration of vehicle or As were daily performed, 5 days per week for 4 weeks.

ETS was generated with conditioned 3R4F cigarettes. (The Tobacco and Health Research Institute, University of Kentucky, KT, USA.) Each cigarette was burn in a smoking chamber in inhalation exposure system (SCIREQ Scientific Respiratory Equipment Inc., Montreal, Canada) with a total of 10–11 puffs, each puff in a 1-min period. In each minute, mainstream cigarette smoke was collected in a 35 mL puff of 2 s duration, and following a 840 mL fresh airflow of 48 s duration. Side-stream cigarette smoke was collected in a 210 mL of 12 s duration from smoking chamber. Mainstream and side-stream cigarette smoke was premixed in a conditioning chamber to achieve the ratio, 15/85 of main-stream/side-stream smoke, which mimics actual ETS [23], before transporting to the exposure chamber. Concentration of carbon monoxide (CO) and total suspended particulates (TSPs) were monitored in the exposure chamber. The concentration of CO was 323 ± 75 ppm, whereas the TSPs were 63.0 ± 4.5 mg/m^3. These features were comparable to other previous studies [24,25].

2.3. Bronchoalveolar lavage fluid (BALF) preparation

Animals were sacrificed *via* isoflurane inhalation to ensure no undue suffering. At necropsy, whole lung was dissected out surgically and was lavagated and inflated with 1 mL saline for 1 min, and then collected flushed out BALF. The recovered amount of lavagate was recorded and saved in individually labeled bottles. To assess the inflammatory response in lung tissues induced by treatments, the total and cell numbers and cell types in the BAL fluids from the animals were determined with a cell counter (Coulter, Inc., Miami, FL, USA). The bronchoalveolar lavage (BAL) fluid was first cytospined and collected at $450 \times g$ for 15 min using a Shandon Cytospin 4 (Thermo Scientific, Waltham, MA, USA). The cytospin smear was then prepared and Liu's staining (Tonyar Biotech, Taoyuan, Taiwan) was performed for distinguish different cell types. Cells in the BAL fluids were classified into two main categories: epithelial cells and leukocytes (including macrophages, neutrophils and lymphocytes). Data presented represent the mean of two scorings by two independent certified medical technologists.

2.4. Histostaining: hematoxylin/eosin (H&E) and elastin van Gieson (EVG) staining

After lavagation, left lung lobe of each mouse was carefully dissected and fixed in 10% PBS-buffered formaldehyde for histopathological examination without treatment. Other lung tissue samples were collected for Ribonucleic acid (RNA)/Deoxyribonucleic acid (DNA) extraction. Formalin-fixed and paraffin-embedded lung tissues were sectioned at 3-μm thickness, and stained with H&E for histological examinations and quantification of mean linear intercept on alveolar sacs. EVG stain was also performed to confirm elastin breakage on alveolar walls using an Elastin stain kit (Sigma, St. Louis, MO, USA).

2.5. Quantification of mean linear intercept (MLI) on alveolar sacs

MLI were measured using MetaMorph Offline software (Molecular Devices Corp., Sunnyvale, CA, USA). Ten black and white pictures for each mouse lung were taken under 20× magnification. Each picture was auto-threshold to define individual alveolar sac. Morphology filters on MetaMorph software were set to measure the diameter of all defined alveolar sacs on each picture, and gave a mean diameter of alveolar sacs (diameter is twice of the MLI). Ten pictures from the same mouse lung were analyzed by MetaMorph and gave the MLI values of this mouse. The increase in MLI represented enlargement of alveolar sacs.

2.6. Total protein concentration and lactate dehydrogenase (LDH) activity in the BALF

Measurement of total protein concentration in the BALF supernatant was performed by Bradford assay (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. The activity of LDH was spectrophotometrically assayed using the CytoTox96 Non-Radioactive Cytotoxicity assay (Promega Corporation, Madison, WI, USA) at 490 nm in the presence of lactate.

2.7. Quantitative real-time reverse transcription-polymerase (RT-PCR) chain reaction assay

Tissues soaked in RNA later solution (Ambion Inc., Austin, TX, USA) were incubated at 4 °C overnight and then transferred to a −80 °C environment until RNA purification. Tissues were ground with a tissue homogenizer (MM301, Retsch Technology GmbH, Haan, Germany) in TRIZOL reagent (Life Technologies, Rockville, MD, USA), and then total RNA was purified *via* chloroform extraction. Synthesis of complementary DNA (cDNA) was performed using the High-Capacity cDNA Archive kit (P/N4322171, Applied Biosystems, Foster City, CA, USA) with 3.0 μg total RNA. We reviewed literatures and ordered a custom-made PCR array, which consisted of primers for genes associated with emphysema and COPD (Applied Biosystems, Foster City, CA, USA). Eight representative lung cDNA samples were selected from control and ETS-As co-exposed groups for PCR array analysis. Differentially expressed

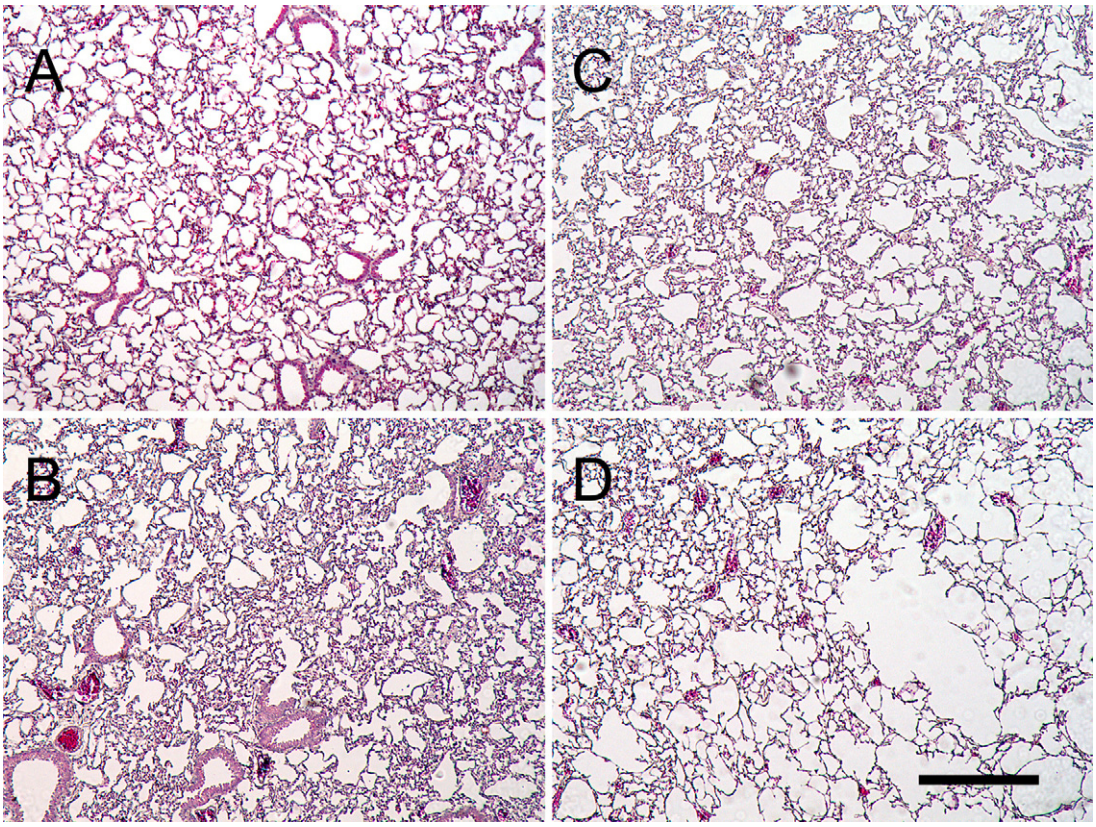

Fig. 1. Effects of ETS and/or As exposure on lung morphology. (A) Control, (B) ETS, (C) As, (D) ETS-As groups. Scale bar, 200 μm.

genes were further confirmed with quantitative real-time RT-PCR in cDNA from four treated groups using the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and analyzed on StepOnePlus™ PCR Systems (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The primers and probes for *interleukin-6* (*IL-6*), *chemokine* (*C—C motif*) *ligand 3* (CCL3), *chemokine* (*C—C motif*) *ligand 17* (CCL17) *chemokine* (*C—X—C motif*) *ligand 22* (*CXCL22*), and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) were included in the Assay-on-Demand Gene Expression Assay Mix (Applied Biosystems, Foster City, CA, USA). The PCR program was 95 °C for 10 min followed by 40 cycles of 60 °C for 1 min with 95 °C for 15 s. Quantitative values were obtained from the threshold cycle (C_T) number. The target gene expression level was normalized to *GAPDH* messenger RNA (mRNA) expression in each sample. The relative mRNA levels of the target gene = $2^{-\Delta Ct}$, $\Delta Ct = Ct_{\text{target gene}} - Ct_{GAPDH}$.

2.8. Quantification of 8-oxodeoxyguanosine (8-OH dG) in plasma and 8-oxo-deoxyguanosine (8-oxodG) in lung tissues

The plasma was collected and filtered using a 0.45 μm nylon membrane, and 0.25 mL plasma was added into a Millipore Amicon Ultra-0.5, 3 K centrifuge tube, spiked with 50 μL of the $^{15}N_5$-8-OHdG internal standard (1 ng/mL) and 200 μL of deionized water, then centrifuged at 14,000 × *g* for 60 min at 4 °C. The analytes were dried by nitrogen. Finally, 50 μL of 5% methanol (v/v) solvent with 0.1% formic acid was added and the solution was mixed by vortexing for subsequent liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.

DNA was isolated from lung tissue and digested according to the method of Peterson and Hecht [26]. DNA was then spiked with $^{15}N_5$-8-OHdG internal standard (1 ng/mL) for quantization of 8-OHdG by LC–MS/MS using a Inertsil 5 μm, ODS-80A, 150 mm × 4.6 mm at a flow rate of 1 mL/min. The mobile phase (solvent A) was 5% acetonitrile (ACN) (v/v) with 0.1% formic acid (FA), solvent B was 95% ACN (v/v) with 0.1% FA, and each was delivered at a flow rate of 1 mL/min The sample eluted from the high-performance liquid chromatography (HPLC) system was introduced into a Turbo V™ source using an electrospray ionization (ESI) probe installed on an API 4000™ triple-quadrupole mass spectrometer (AB SCIEX, Canada), operated in positive mode with a needle voltage of 5.5 kV. Nitrogen gas was used as the nebulizing, heating, curtain and collision gas, and the heater gas temperature was set at 500 °C. Data acquisition and quantitative processing were accomplished using Analyst1.4.2™ software (AB SCIEX, Canada). The optimized source parameters multiple reaction monitoring mode (MRM) transition pairs of 8-OHdG and $^{15}N_5$-8-OHdG were set as *m*/*z* 284 → 168 and *m*/*z* 289 → 173 for the quantitative pair and *m*/*z* 284 → 140 and *m*/*z* 289 → 145 for the qualitative pair, respectively.

2.9. Statistical analysis

Microarray data between two groups were compared using Student's *t*-test. Data between four groups were compared using the one-way analysis of variance (ANOVA) (SPSS, version 19.0). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Bronchoalveolar lavage and histopathology

In BALF, total cell numbers, protein concentrations and LDH activities were no significant difference among four groups (data not shown). Exposure to As or ETS alone had no similar effects (Fig. 1B and C). However, histopathological results showed that the alveolar spaces in the lung of ETS-As co-exposed mice were

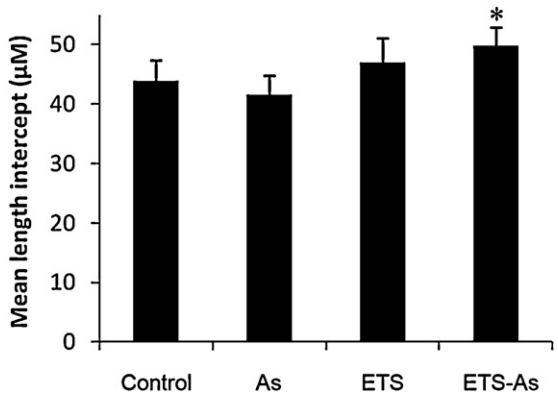

Fig. 2. Effect of ETS and/or As exposure on the alveoli spaces. The alveoli space in mouse lung was quantified by measuring MLI in H&E stained lung slides with the computer software Metamorph. MLI was determined from 10 pictures of each mouse lung. Data were presented as the mean ± SD for eight mice per group. *$P < 0.05$, compared with control group when analyzed with one-way ANOVA.

enlarged (Fig. 1D). This morphological change was further quantified with the computer program MetaMorph. The MLI of alveolar space was significantly increased in ETS-As co-exposed mice (Fig. 2). This alveolar enlargement was further confirmed as emphysema-like lesions by EVG staining (Fig. 3). The EVG stain demonstrated that alveolar walls were broken in the lung of ETS-As co-exposed mice (Fig. 3B). There was no lung inflammation over the bronchiolar and interstitial areas, which correlated with negative results by BAL analysis. The histopathology and EVG stain indicated that the destruction of alveolar structure, *i.e.*, emphysema-like lesions, occurred in mouse lungs with ETS-As co-exposure.

3.2. Pulmonary inflammatory and remodeling related gene expression on ETS-As exposed mouse lungs

Histopathological examination showed that no inflammation occurred in the lung following As and/or ETS exposure (Fig. 1). With gene microarray analysis, we identified mRNA levels of four cytokines/chemokines (*IL-6*, *CCL-3*, *CCL-17* and *CCL-22*) were significantly reduced in the lung of ETS-As co-exposed mice by Student's *t*-test analysis (Table 1). These reductions were subsequently confirmed with the real-time RT-PCR method. The mRNA levels of *IL-6* in the lungs of all treated mice and those of *CCL-3* and *CCL-22* in those of ETS-As co-exposed mice were also significantly reduced (Fig. 4). However, the reduction in CCL-17 was not significant when data of four groups were analyzed with one-way ANOVA. Thus, the enhanced effect between ETS and As co-exposure only occurred for the reduction of *CCL-3* and *CCL-22* expression.

Table 1
Pulmonary inflammatory and remodeling related gene expression on ETS-As exposed mouse lung.

Factors	Folds[a]	*P* value[b]
Proteinase/inhibitors		
MMP-2	0.85	0.16
MMP-9	0.98	0.96
MMP-10	0.68	0.15
TIMP-1	1.01	0.94
TIMP-2	0.99	0.95
Cytokines		
TNF-a	0.71	0.15
TGF-b	0.95	0.85
IL-1b	0.94	0.86
IL-6	0.46	0.01
IL-10	0.90	0.75
IL-13	3.36	0.96
IL-18	1.11	0.40
Chemokines/receptors		
CCL-1	1.18	0.60
CCL-2, MCP-1	0.6	0.23
CCL-3, MIP-1a	0.65	>0.01
CCL-4, MIP-1b	0.65	0.19
CCL-5	0.73	0.22
CCL-6, MRP-1	1.02	0.84
CCL-7, MCP-3	0.68	0.35
CCL-17, Tarc	0.52	>0.01
CCL-22, MDC	0.43	0.03
CCL-20, MIP-3a	0.72	0.26
CXCL-1, KC	0.52	0.13
CXCL-2, MIP-2	0.88	0.66
CXCL-10, IP-10	0.76	0.49
CCR-5	0.75	0.11
CCR-6	0.83	0.21
Others		
Igf-2	0.83	0.43
Nrf-2	0.88	0.51
FSP-1, S100A4	0.96	0.85

Gene expression profile was determined by real-time PCR by using customized PCR array.
[a] Fold changes on gene expression level in comparison of control group.
[b] *P* value was analyzed by Student's *t*-test.

3.3. Increased plasma 8-OHdG in mice with ETS-As co-exposure

Oxidative stress is believed to involve in the pathogenesis of emphysema [27,28]. In order to evaluate the oxidative stress induced by As and/or ETS, 8-oxo-dG and 8-OHdG were respectively quantified in the lung and in the plasma. Although 8-oxo-dG

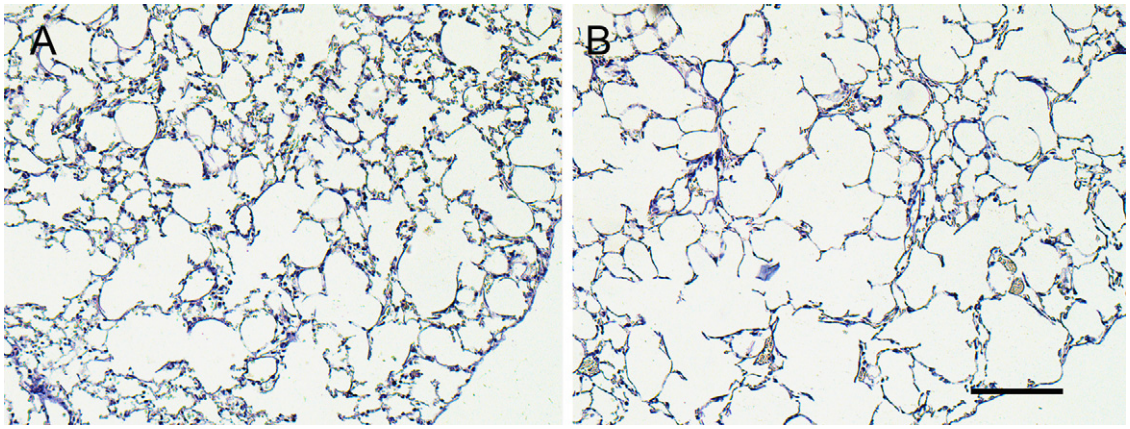

Fig. 3. Effect of ETS and/or As exposure on the integrity of elastin on the alveolar walls. The integrity of elastin on the alveolar walls was visualized with EVG staining. (A) Control and (B) ETS-As groups. Scale bar 200 μm.

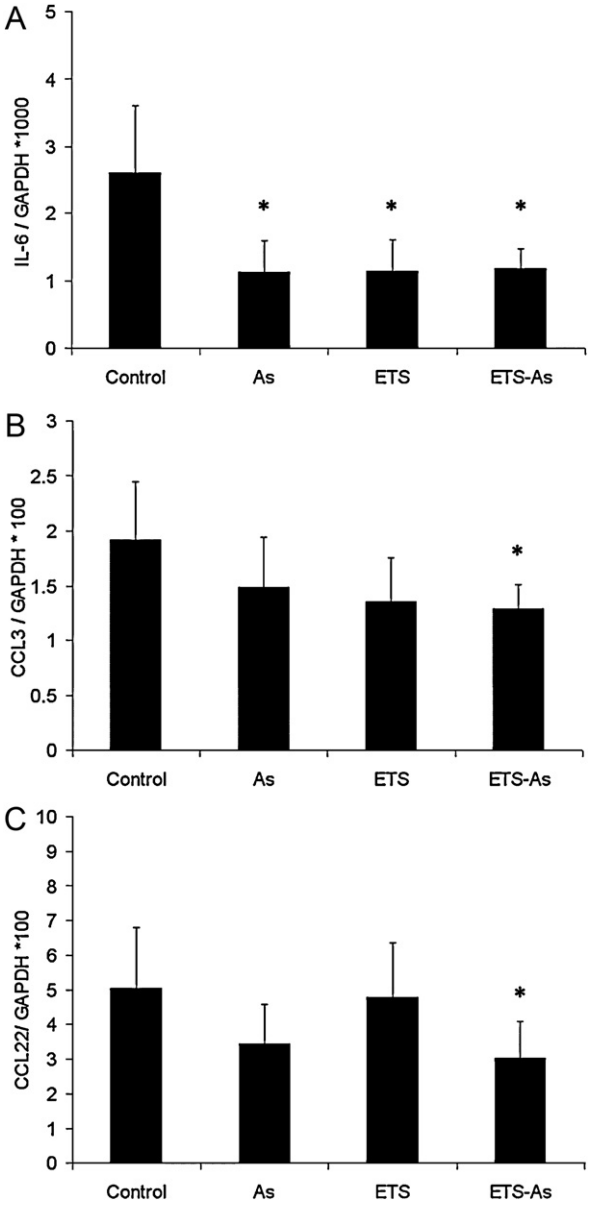

Fig. 4. Effect of ETS and/or As exposure on mRNA levels of cytokines in mouse lungs. The relative mRNA levels in lungs were determined with the real-time RT-PCR method. Data were presented as the mean ± SD for 10–12 mice per group. $^{*}P < 0.05$, compared with control group when analyzed with one-way ANOVA.

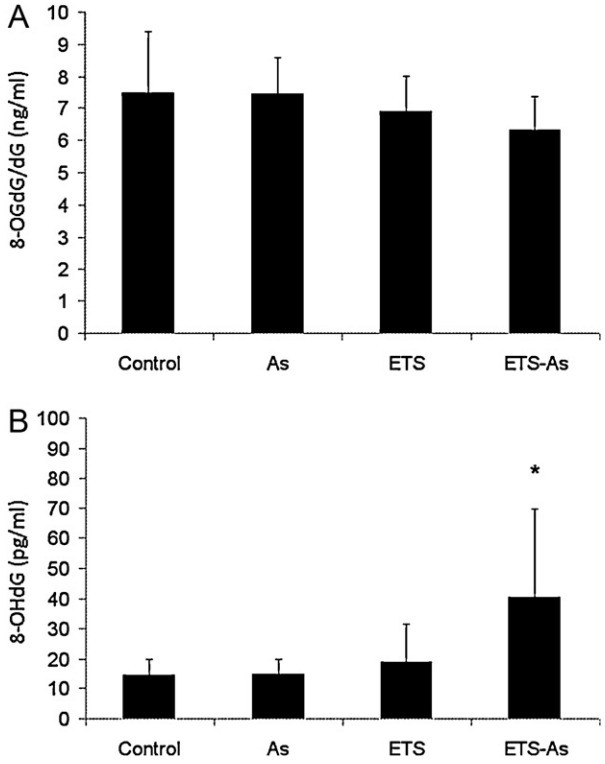

Fig. 5. Effect of ETS and/or As exposure on the accumulation of 8-oxo-dG in the lung and of 8-OHdG in plasma. (A) 8-oxo-dG levels in the lung. Data were the mean ± SD for 4–6 mice per group; (B) 8-OHdG levels in plasma. Data were presented as the mean ± SD for 11–12 mice per group. $^{*}P = 0.008$, compared with control group when analyzed with one-way ANOVA.

levels were not increased in the lungs (Fig. 5A) of all treated mice, 8-OHdG levels in plasma were significantly increased to 40.60 ± 28.93 pg/mL in ETS-As co-exposed mice in compared with 14.64 ± 5.19 pg/mL in mice of control group. Statistical analysis between control and ETS-As co-exposed groups was performed by one-way ANOVA, giving a significant *P* value of 0.008 (Fig. 5B). 8-OHdG in plasma was the product of 8-oxodG repaired and removed from tissue DNA. The elevation of plasma 8-OHdG signified that oxidative DNA damages were increased by the co-exposure to ETS and As.

4. Discussion

COPD, characterized by progressive airflow obstruction and destruction of lung parenchyma, is a major cause of mortality worldwide [29]. Exposure to environmental factors is considered as one of the major risk factors for COPD [1]. Some epidemiological studies showed that exposure to ETS and As respectively increased the prevalence of respiratory symptoms [13,18]. In our present study, we demonstrated that co-exposure to ETS and As induced emphysema-like lesions in mice within 4 weeks. Emphysema is a clinical phenotype of COPD, and characterized with destruction of alveolar walls with enlargement of airspaces distal to terminal bronchioles [30]. Because cigarette smoke caused low-grade chronic inflammation with an imbalance between proteolytic and antiproteolytic enzymes in the lung, inflammation was considered as the key mechanism for emphysema [30]. Particularly neutrophil infiltration during inflammation progress increases secretion of neutrophil elastase and relapse of oxidants is considered as a key factor causes elastin breakage [31]. However in present study, the emphysematous lesions observed in the co-exposure of As and ETS mouse lungs showed no inflammatory cell infiltration including neutrophils and alveolar macrophages.

Except neutrophil infiltration, increased alveolar macrophages are also considered as an important characteristic in CODP patients [32], the expression of pro-inflammatory cytokine/chemokines: *IL-6*, *CCL-3* and *CCL-22* which are mainly secreted and produced by activated alveolar macrophages [33] were also determined in this study for clarifying the inflammation status under As and/or ETS exposure. Our present results showed that exposure to ETS and/or As reduced *IL-6* mRNA levels. Furthermore, co-exposure to ETS-As reduced mRNA levels of *CCL-3* and *CCL-22* in the lung. Gaschler et al. [34] had a similar finding that cigarette smoke attenuated mRNA levels of some cytokines, including *IL-6* and *CCL-3*, on alveolar macrophages in mice [34]. Kent et al. [32] also observed similar results in human macrophages obtained from COPD patients that exposure to cigarette smoke extract increased *IL-8* mRNA levels, but decreased mRNA levels of *IL-6* and *CCL-3*. These *in vivo* and *in vitro* studies, at least partly, support our data and provide the

evidences that cigarette smoke has an anti-inflammatory effect in macrophages *via* repression of innate immunity. In contrast to effects of cigarette smoke on macrophages, effects of As on macrophages were rarely reported. A study by Bourdonnay et al. [33] showed that arsenic trioxide reduced mRNA levels of *CCL-22* in human macrophages. In our present study, co-exposure to ETS and As reduced mRNA levels of *CCL-3* and *CCL-22* in mouse lungs, whereas both ETS and As reduced mRNA levels of *IL-6*. Some *in vitro* studies indicated that exposure to low doses of arsenite mediated nuclear factor Kappa B (NFKappaB) and activator protein-1 (AP-1)/cAMP response element (CRE) binding protein pathways to suppress inflammatory cytokine expression, *e.g.*, *IL-6* [35–37]. Low dose treatment of arsenite was suggested to stabilize inhibitor of kappaB alpha (IKappaB alpha) and therefore inhibited NFKappaB activation on downstream *IL-6* expression in human colon adenocarcinoma cells [37]. In addition, long term exposure to arsenite also demonstrated a suppressive role in AP-1 and NFKappaB transactivity on human fibroblast cells, particularly after reactive oxygen species (ROS) insulted by H_2O_2 [38]. Here the 4-weeks exposure of arsenite probably suppressed NFKappaB/AP-1 pathways in a similar manner and leaded to reduction of inflammatory cytokine/chemokine levels. However, the exact molecular mechanism requires further clarification. Taken together, ETS and As co-exposure might suppress pro-inflammatory reaction in lung tissues when emphysematous lesions occurred in mouse lungs.

Since many inflammation reactions such as immune cell infiltration and expression of pro-inflammatory factors were not observed in present study, several alternative hypotheses have been proposed to explain the observed emphysema formation under As and ETS co-exposure, such as oxidative stress, apoptosis and senescence [39,40]. Oxidative stress generates reactive oxygen species that inactivate the antiproteolytic enzymes such as α1-antitrypsin (elastase inhibitor) in lung and subsequently leads to increased destruction of elastic tissues as in emphysema [36]. Induced oxidative stress also further enhances the elastin break by modulating protein structure and results in elastin degradation [41,42]. For example, Umeda et al. demonstrated that aorta elastin degradation can be progressed by formation of dihydrooxopyridine cross-links and isooxodesmosine by ROS [42]. Some oxidants were reported to damage extracellular matrix [43,44], but currently no significant evidence to verify the elastin degradation by direct exposure of ROS. An alternative concept is that the presence of ROS enhances the elastolysis by elastases [41]. Recent *in vivo* study also suggested that imbalance redox status enhanced susceptibility to elastase-induced emphysema development in rodent [45]. As exposure has been shown to increase the production of intracellular ROS leading to activation of the stress-related signaling pathways, cell death and apoptosis by mediating stress-related signaling pathways include NFkappaB and AP-1 [46,47]. Some possibilities were suggested: First, arsenite activated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase on the plasma membrane of cells, which catalyze the one-electron reduction of O_2 to superoxide, and its activation leaded to increases in superoxide and other ROS [48,49]. Second, arsenite inhibited the antioxidative systems, such as disturbance of intracellular glutathione status and depleting thioredoxin levels, which disturbed the homeostasis of intracellular redox status [50,51]. In regard of As and ETS co-exposure, alteration of redox status by depleting glutathione (GSH) is also reported [52]. Together the exposure of As with ETS might induce emphysema formation by producing ROS and resulted in elastin degradation.

8-oxodG is a ROS-induced DNA modification, and is generated by the repair of 8-oxodG in cellular DNA [53]. The 8-oxodG levels in leukocytes or tissues have been associated with or increased by cigarette smoking and As exposure in human or animal studies [54–58]. More recently, Thaiparambil et al. [54] demonstrated that exposure to side-stream cigarette smoke increased 8-oxodG levels in the lung of A/J mice. Hays et al. [52] showed that dual exposure of arsenic and cigarette smoke at environmentally relevant levels acted synergistically to cause DNA damage in the hamster lung. Chiang et al. [59] have investigated the relationship between the levels of total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and 8-OHdG in the plasma of humans exposed to ETS. Thus, 8-OHdG and 8-oxodG have been used as biomarkers for oxidative stress in animal and human studies. In our present study, we failed to observe an increase of 8-oxodG in lung tissue or of 8-OHdG in plasma after exposure to either ETS or As for 4 weeks in mice. It appears that the doses of ETS and As were too low to induce oxidative stress in our present study. Although co-exposure to ETS and As failed to increase 8-oxodG levels in lung tissues, the co-exposure increased plasma 8-OHdG levels. It suggested that co-exposure to ETS and As increased oxidative stress in mice. But 8-oxodG was quickly repaired and became free 8-OHdG, which was released into the circulation. Thus, the elevation of plasma 8-OHdG represented the very early stage of oxidative stress.

Although exposure to ETS and As respectively associated with respiratory symptoms in human populations, it usually takes a long and persistent exposure to ETS or As to induce lung lesions in animal and human studies. In animal studies, it usually took more than 3 months with 6 h per day exposure to induce emphysema by inhalation of cigarette smoke [60,61]. Here, mice inhaled ETS only 2 h per day for 4 weeks. Thus, it is not surprising that no lung lesion was observed after ETS exposure in our present study. Similarly, a long time exposure may be needed for As to induce lung lesions [20]. With the interaction between ETS and As, the lung lesions were observed at 4th weeks in our present study. The difference between animals and humans is always a concern and the interaction between ETS and As shall be verified in further epidemiological studies.

Here we demonstrated that co-exposure to ETS and As caused emphysema-like lesions and elevated plasma 8-OHdG without inflammation reactions and neutrophil infiltration in the lung. Expression of pro-inflammatory cytokines/chemokines in the lung was not elevated after co-exposure to ETS and As. But the relationship between immune repression and emphysema-like lesions remained to be clarified in the future. Furthermore, our result demonstrated that mice exposed to As and ETS showed significantly higher plasma 8-OHdG levels in compared with mice in control group (one-way ANOVA, $P < 0.01$). The increased plasma 8-OHdG levels suggested induced oxidative stress due to co-exposure of As and ETS. This induced oxidative stress might respond to pulmonary elastin degradation and consequent emphysema formation. However, more investigation is needed to clarify the relationship between degradation of pulmonary elastin fiber and induced oxidative stress. In conclusion, co-exposure of ETS and As induced emphysema formation, and shall be considered as a risk factor for development of COPD.

Acknowledgments

This study was supported by Grant EO-99-PP-02 from the Division of Environmental Health and Occupation Medicine, National Health Research Institutes, Taiwan, ROC. The scientific content of this manuscript does not necessarily signify the views and policies of the DEHOM/NHRI or condemn, endorse or recommend for use anything presented on the issue discussed here.

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