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Oxidative Stress Levels of Kunming Mice Following Short-Term Exposure to Volatile Organic Compounds (VOCs) Mixture

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Abstract: Volatile Organic Compounds (VOCs) are one of the main substances causing multiple chemical sensitivity reactions in human indoors. In order to investigate the effect of short-term of VOCs mixture on oxidative stress responses in mice, researchers exposed male Kunming mice to filtered air (0) and four kinds of VOCs mixture treated air. The concentrations of VOCs were following about: Form Aldehyde (FA), Benzene, Toluene and Xylene (BTX) 1.0 + 1.1 + 2.0 + 2.0, 3.0 + 3.3 + 6.0 + 6.0, 5.0 + 5.5 + 10.0 + 10.0 and 10.0 + 11.0 + 20.0 + 20.0 mg m^{-3}, respectively which corresponded to 10, 30, 50 and 100 times of indoor air quality standard in China for 2 h day^{-1} for 5 days/week for 2 weeks in the whole body exposure chamber. One day following the last VOCs mixture exposure researchers collected liver, serum, lung and Bronchia Alveolus Lavage Fluid (BALF) from each mouse and examined LDH in BALF, ROS in lung, antioxidant enzyme activities, ROS, lipid and protein peroxidation and Reactive Nitrogen Species (RNS) in liver, DNA damage marker in serum. LDH release in BALF indicated cell impairment in the alveolar area. There was a positive correlation between LDH release in BALF and ROS production in lung ($r = 0.636$, $p<0.001$). The oxidative stress and injury in the liver occurred as VOCs mixture appeared to trigger a cascade of reactions such as lipid and protein peroxidation. Total antioxidation capacity and activities of antioxidative enzymes other than CAT were significantly decreased and nitric oxide was increased in liver in mice exposure to VOCs. The 8-OHdg in serum exposure to a high dose of VOCs was significantly higher than control. These results showed that inhalation of short-term VOCs mixture influence oxidative stress of the mice so as to induce a variety of oxidative products. ROS, GSH, GSH-Px, T-AOC, iNOS in liver could be used as sensitive biological makers of oxidative damage under short-term VOCs mixture condition. These findings also provide the occupational exposure limit concentrations for VOCs with valuable reference.

Key words: VOCs mixture, oxidative stress, RNS, 8-OHdg, biological maker, DNA

INTRODUCTION

Indoor levels of chemical pollutants may be >5 times and occasionally 100 times higher than those outdoors (Kotzias, 2005; USEPA, 2007). Moreover, modern person spend >8% of their time indoors in particularly for sensitive individuals like young children and old person as well as sick people (Fischader *et al*., 2008). Therefore, indoor exposure is found to be more important than outdoor exposure. Multiple Chemical Sensitivity (MCS) and Sick Building Soffice are characterized by non-specific complaints such as mental fatigue, headache, stuffy nose and sore throat (Skov and Valbjorn, 1987; Malkin *et al*., 1998). Important indoor environmental chemicals are Volatile Organic Compounds (VOCs). These are organic chemicals which are evaporated into the atmosphere at room temperature. Possible sources for VOCs exposure include paints, adhesives, building materials, cosmetics, furnishings, dry cleaned clothes, cleaning agents, carpets and tobacco smoke (Guo *et al*., 2000; Guo and Murray, 2000, 2001). Formaldehyde (FA), benzene, toluene and xylene are typical VOCs indoors (Saito *et al*., 2011; Kim *et al*., 2011) and their harmful effects have been reported. Inhalation exposure to air pollutants is more significant pathway than other exposure pathways. Hence, inhalation exposure gains the attention of indoor air quality researchers. However, most of the hazardous effects induced by inhaling VOCs have been investigated under single VOCs.

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An imbalance between Reactive Oxygen Species (ROS) and antioxidant defenses in favor of the former has been described as oxidative stress. Antioxidant defenses including antioxidants and related enzymes which widely present in animal tissue have been recognized as affecting numerous biological functions including aging, hepatic and renal diseases, cancer and detoxification mechanisms (Jang *et al*., 1998; Qujeq *et al*., 2004). Therefore, ROS are important mediators of cellular injury, play a putative causing in oxidative stress and also can contribute to a variety of diseases and may be present in situations of toxicity (Halliwell, 1997; Kadiiska and Mason, 2000). ROS-initiated oxidative stress can be regulated by cell defense mechanisms including Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxide (GSH-Px) and Glutathione (GSH). ROS may induce lipids, proteins oxidative and DNA damage. MDA and carbonyl are markers of lipids and protein oxidative stress (Cederbegr *et al*., 2001; Cakatay *et al*., 2003). One major pathway of ROS-induced DNA damage involves a reaction at the C-8 position of 2-deoxyguanosine to form 8-hydroxy-2-deoxyguanosine (8-OHdg). As such, 8OHdg is considered to be a marker of DNA oxidative stress (Loft *et al*., 1993; Pilger and Rudiger, 2006). Nitric Oxide (NO) and the oxidative metabolite, peroxynitrite ($ONOO^-$) are referred to as Reactive Nitrogen Species (RNS). NO is a free radical, synthesized from L-Arginine by three kinds of NO Synthase (NOS) and can be involved in diverse processes such as modification of neuronal function, dilution of blood vessels, disruption of host defenses and inflammatory responses (Brune *et al*., 1998; Gordge, 1998; MacMicking *et al*., 1997). NO may be scavenged via a variety of ROS to form a range of RNS species (Reynaert *et al*., 2005).

Oxidative stress of animal exposed to VOCs has attracted extensive studies in recent years. The inhalation of FA and xylene induced the anti-oxidant system and lipid peroxidation in the renal and liver of rat (Kum *et al*., 2007a, b). FA causes a disruption of the physiological balance between oxidant and antioxidant enzymes (Lino-dos-Santos-Franco *et al*., 2011) to increase the level of protein oxidative, DNA damage (Sul *et al*., 2007; Speit *et al*., 2009) and NO in the tissues of rat (Mukaddes *et al*., 2006; Songur *et al*., 2008). Toluene influenced significantly antioxidant enzymes activity in rat tissue, blood (Stajkovic *et al*., 2009; Coskun *et al*., 2007) and oxidative stress of exposure worker (Kim *et al*., 2011). Occupational exposure to benzene also caused oxidative stress in gasoline filling workers (Uzma *et al*., 2010). Despite the studies evaluating the oxidative stress effects of VOCs have been reported, there was no detailed experimental evidence showing oxidative damage of VOCs mixture exposure. In the present study, researchers exposed Kunming mice to 4 different concentrations of VOCs mixture gas and investigated the levels of oxidative stress in Kunming mice. The aim of the present study was to find out the mechanism and products of oxidative damage and biological sensitive markers for short-term VOCs mixture. Moreover to perfect the occupational exposure limit concentrations for VOCs, the study can also provide the valuable reference.

MATERIALS AND METHODS

Animals: About 5 weeks old male (18-20 g) Kunming mice were purchased from Experimental Animal Care Center of Dalian Medical University. The mice were maintained under the following laboratory conditions of temperature: 22±2°C, humidity: 50±10% and 12 h/12 h: day/night cycles. Distilled water and sterilized food for mice were available *ad libitum*. At the end of 1 week of acclimation, the mice were 6 weeks old on their 1st day of VOCs mixture exposure.

VOCs inhalation: Benzene, Toluene and Xylene (BTX) and FA were purchased from the Sigma Co. (St. Louis, MO, USA). Four VOCs mixture exposure groups and an unexposed control group were used in this study. The concentrations of four VOCs mixture exposure groups corresponded to respectively 10, 30, 50 and 100 times of indoor air quality standard in China. Animals were exposed for 2 weeks at 2 h/day (from 14:00-16:00) and 5 days/week in an inhalation exposure chamber. An interscan 4160 digital electrochemical analyzer (4160-19.99 m, Interscan, USA) was used to measure the concentrations of gaseous formaldehyde. Gas Chromatograph (GC) (GC-2010, Shimadzu, Japan) was used to determine BTX levels. Ten mice were exposed in each of the five experiments (n = 50 mice).

Exposure conditions and analysis of chamber VOCs concentrations: The cylindrical experimental chamber (100 L) was made from glass and consisted of two compartments: the animal exposed chamber and the VOCs injection chamber, respectively. The two compartments were connected via a circular perforated baffle palate with ventilator fan to diffuse VOCs into the animal exposed chamber. A hole (5 cm diameter) was located in the VOCs injection chamber for VOCs injection. The other two holes (5 cm diameter) were located in the animal exposed chamber to determine the concentrations of FA and BTX. FA and BTX gas were generated in 100 mL injector using BTX High Performance Liquid Chromatographic (HPLC) purity and FA analytical reagent by 50°C oven and were

injected into the chambers in turn to achieve the desired gas concentrations. In order to keep VOCs concentrations in exposure chamber relatively stable, VOCs concentrations in exposure chamber were determined every 20 min and VOCs mixture were supplemented in time. The air in the exposure chamber was conditioned to 22±2°C and 50±5% humidity. Environmental chemical components such as VOCs and formaldehyde were determined in breeding room and no significant chemical components were detected in breeding room (detection limit 0.05 μg m^{-3}). The mean VOCs concentrations were achieved over the 2 week study period (Table 1).

Collection of samples: One day following the final VOCs mixture inhalation for 2 weeks, body weight was measured and the mice were sacrificed by cervical dislocation. Heparinized blood from the right ventricle was collected and the animals were perfused with PBS through the left ventricle to remove the blood. Tissue samples (lung and liver) (n = 5) of approximately 150 mg were collected from each mice. Bronchia Alveolus Lavage Fluid (BALF) was collected by cannulating the trachea and lavaging the lung four times with 1 mL of sterile saline as described previously (Fujimaki *et al*., 1997). The recovered fluids about 3.5 mL were pooled. BALF and blood were centrifuged and the resulting supernatant was collected.

LDH of BALF: Lactate Deshydrogenase (LDH) of BALF in mice was determined using a cytotoxicity detection LDH kit (Nanjing Jiancheng Biological Product, China) according to the manufacturer instructions. In this colorimetric assay, h-nicotinamide adenine dinucleotide was reduced to NADH through the conversion of lactate to pyruvate by LDH and then NADH reduced tetrazolium dyes to formazan dyes in the presence of diaphorase. In brief, 25 μL of BALF supernatants were mixed with 75 μL of the LDH substrate mixture in a 96-well plate. After incubation for 1 h at room temperature, the reaction was stopped by adding 100 μL of 1 mol L^{-1} HCl and the absorbance was read at 570 nm. Values of LDH level were expressed as U/L.

Table 1: VOCs concentrations during exposure experiments

Groups	Formaldehyde (mg m^{-3})	Benzene (mg m^{-3})	Toluene (mg m^{-3})	Xylene (mg m^{-3})
Control	-	-	-	-
1	0.94±0.08	1.05±0.11	1.97±0.19	2.04±0.22
2	3.11±0.17	3.23±0.38	5.98±0.49	6.10±0.55
3	5.14±0.63	5.88±0.51	9.77±1.12	10.14±1.08
4	9.66±0.93	10.87±1.42	19.11±2.28	21.25±2.49

Concentrations of each exposure chamber were determinated every 20 min. Number of determinations: 6; values were mean±SD

Measurement of intracellular ROS level: ROS generation was measured using the method described by other researchers (Wang and Joseph, 1999).

Just before the exposure to HCHO or H_2O_2, cells (5×10^5 cells/sample) isolated from the liver and lung tissues were incubated with culture medium containing 20 μmol L^{-1} DCFH-DA for 30 min at 37°C and 5% CO_2. DCFH-DA was then removed and cells were washed with PBS and exposed to HCHO or H_2O_2 as described. After exposure, fluorescence of each well was read immediately using a Spectra Max Gemini XPS microplate reader (RF-5301PC, Shimadzu, Japan) with an excitation filter set at 485 nm and an emission filter set at 530 nm. Data was expressed as the fluorescence intensity.

Antioxidant enzyme activities: The livers were homogenized in 1 mL of ice-cold 50 mmol L^{-1} sodium phosphate (pH 7.0) that contained 1% Polyvinyl Polypyrrolidone (PVPP). The homogenate was centrifuged (30,000 g for 30 min) and the supernatant was used for assays of activities of SOD, CAT, GSH, GSH-Px and the Total Anti-Oxidation Capacity (T-AOC) with a commercial-available kit (Nanjing Jiancheng Biological Product, China).

SOD kit uses a xanthine-xanthine oxidase system to determine the inhibition of Nitroblue Tetrazolium (NBT) Reduction due to superoxide anion generation. Briefly, 500 μL of supernatant was mixed with 50 μmol L^{-1} xanthine and 2.5 μmol L^{-1} xanthine oxidase in 50 μmol L^{-1} potassium phosphate buffer and incubated at 37°C for 40 min. Then, NBT is added. Total nitrite (nitrite+nitrate) produced by the oxidation of oxyamine was measured by detecting the absorbance at 550 nm. A unit of SOD was defined as the enzyme amount causing 50% inhibition of the NBT reduction rate. The SOD activity was expressed as U mg^{-1} prot.

CAT activity was assayed with hydrogen peroxide as substrate using a method based on the direct measurement of H_2O_2 decomposition. The final volume of each enzyme assay was 3 mL substrate and 20 μL supernatant of lens homogenates. Assay was performed at 25°C and 240 nm. The amount of enzyme that decomposed 1 μmol of hydrogen peroxide per minute per milligram tissue protein was defined as 1 U of activity. The results were expressed as U mg^{-1} prot.

GSH activity was measured according to the procedure. Briefly, the supernatant was precipitated with 50% trichloro acetic acid and then centrifuged at 1000 g for 5 min. The reaction mixture containing 50 μL of supernatant, 200 μL of 0.2 mol L^{-1} Tris-EDTA buffer (pH 8.9) and 10 μL of 0.01 mol L^{-1} 5, 5, -dithiobis (2-nitro-benzoic acid) was kept at room temperature for

5 min and then measured at 412 nm. The GSH concentration was calculated using a GSH standard curve and expressed as μmol g^{-1} prot.

The activity of Gutathione Peroxidase (GSH-Px) Passayed by determination of the reduced GSH in the liver homogenate. In brief, 200 μL of the supernatant was mixed with 2.68 mL of 0.05 mol L^{-1} phosphate buffer (pH 7.0) containing 5 mmol L^{-1} of EDTA followed by the addition of 0.1 mL of 8.4 mmol L^{-1} Nicotinamide Adinucleotide Phosphate (NADPH), 0.01 mL of glutathione reductase, 0.01 mL of 1.125 mol L^{-1} NaN_2 and 0.1 mL of 0.15 mol L^{-1} glutathione. The enzymatic reaction was initiated by the addition of 0.1 mL of 2.2 mmol L^{-1} H_2O_2. The changes in absorbance at 340 nm were continuously recorded between 2 and 4 min after initiation of the reaction (converting NADPH to NADP). A GSH-Px unit was defined as the enzyme activity required to convert 1 mmol of NADPH to NADP per mg tissue protein. The GSH-Px activity was expressed as U mg^{-1} prot.

The spectrometric method was applied to evaluate T-AOC. In the reaction mixture ferric ion was reduced by antioxidant reducing agents and blue complex Fe^{2+}-TPTZ (2, 4, 6-tri (2-pyridyl)-s-triazine) is produced. The optical density was measured at 520 nm. The 1 U was defined as the amount that increased the absorbance by 0.01 at 37°C. Data were expressed as U mg^{-1} prot.

Lipid and protein peroxidation: MDA was assayed in the form of Thiobarbituric Acid Reacting Substances (TBARS) with a commercial-available kit (Nanjing Jiancheng Biological Product, China). A total of 100 μL of the supernatant was mixed with 1 mL of 20% trichloroacetic acid and 1.0 mL of 0.1% TBARS reagent and incubated at 95°C for 80 min. After cooling on ice, the mixture was centrifuged at 1,000 g for 20 min. The absorbance of the supernatant was measured at 532 nm. The amount of TBARS was determined using tetraethoxypropane as a standard. The content of TBARS, as an index of MDA was expressed as nmol mg^{-1} prot.

Protein carbonyls in the liver were determined using a spectrometric DNPH assay according to Fagan with minor modifications (Fagan *et al*., 1999). Briefly, liver tissues were homogenized by sonication in lysis buffer containing PBS (pH 7.2), 1% Triton X100, 1 mmol L^{-1} EDTA and 1×protease inhibitor cocktail and the insoluble cellular debris were removed by centrifugation. Aliquots of protein samples were precipitated with ten volumes of HCl-acetone (3:100) and then washed with 5 mL of 10% of TCA solution. Pellets were resuspended in 500 μL buffer solution and then reacted with 500 μL of 10 mmol L^{-1} DNPH (2 mol L^{-1} HCl) by vortexing for 15 min. Protein blanks were prepared with 500 μL of 2 mol L^{-1} of HCl solution. After mixing, 500 μL of 30% TCA was added to each tube, vortexed and placed on ice for 10 min. To remove unreacted DNPH, the centrifuged pellets were washed with 5 mL of 20% TCA and 5 mL of an ethanol: ethylacetate mixture (1:1) (v/v). The final precipitate was dissolved in 1 mL of 6 mol L^{-1} guanidine HCl and the absorbance 380 nm was determined for the sample treated with DNPH and HCl which was subtracted as background. The carbonyl content was calculated from the absorbance measurement at 380 nm and an absorption coefficient of 22, 000 mol cm^{-1}.

DNA damage: The 8-OHdg levels serum of was measured using the commercially available 8-OHdg ELISA kit (BIOXYTECH® OXIS International Inc., Foster City, CA, USA) following manufacturer's recommendations. Briefly, serum samples were passed through Amicon Ultra Centrifugal Filters (Fisher Scientific) to remove any large-molecular-weight substances. Samples and standards (50 μL) were then added to microtiter plates precoated with 8-OHdg. This was followed by the addition of 8-OHdg monoclonal antibodies (50 μL). Plates were then sealed and incubated at 4°C overnight. In this primary reaction, the 8-OHdg in the serum competes with the 8-OHdg already bound to the plate for the monoclonal antibody. Higher levels of 8-OHdg in the sample will lead to lower levels of antibody binding to the plate.

To remove the antibodies bound to 8-OHdg in the serum, the plates were washed with 250 μL diluted washing buffer and a second enzyme-labeled antibody (100 μL) was added to each well. This second antibody will bind to the 8-OHdg monoclonal antibody already attached to the plate. After repeating the plate wash, 100 μL of chromogen was added to each well and incubated in the dark for 15 min. The development of color is proportional to the amount of antibody in the plate which in turn is inversely related to the amount of 8-OHdg in the serum sample. Lower color means higher amounts of 8-OHdg. Results are expressed as ng mL^{-1}.

NOS and NO assay: The activities of Total Nitric Oxide Synthase (T-NOS) in the liver were spectrophotometrically measured with commercial-available kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) based on the oxidation of oxyhaemoglobin to methaemoglobin by nitric oxide. The inducible NOS (iNOS, calcium- independent) activity was measured by adding ethylene glycol-bis- (2-aminoethyl)-N, N, N, N Tetraacetic Acid (EGTA) to chelate free Ca^{2+} in the reaction mixture. The constitutive NOS (cNOS, calcium-dependent) activity was calculated by subtracting iNOS from T-NOS. The NOS activities were expressed as U mg^{-1} prot.

NO concentration assay in the liver was performed according to kit protocols (Nanjing Jiancheng Bioengineering Institute Jiangsu, China). The OD value was determined by a spectrophotometer (U-3010, Hitachi, Japan). Results of NO were read with OD value at 550 nm. The result was calculated using the following eqution:

$$NO\ (\mu mol\ L^{-1}) = \frac{(Asampale\text{-}Ablank)}{(Astandard\text{-}Ablank)} \times 20\ (\mu mol\ L^{-1})$$

The final level of NO in liver homogenates was calculated and expressed as μmol g^{-1} liver protein.

Statistical analysis: Statistical analysis was performed using Origin 6.0 program (OriginLab, Hampton, Massachusetts, USA). To test for significant differences between five groups, researchers used the Analysis of Variance (ANOVA). Duncan's multiple range test used for multiple comparison of means after ANOVA when statistically significant difference was found.

RESULTS AND DISCUSSION

Body weights of VOCs mixture exposed mice compared with control: Body weight of the male Kunming mice was measured before the 1st day of VOCs mixture exposure and after one day following the final VOCs mixture inhalation.

The body weight of mice in all groups showed increases. With VOCs mixture dosages increasing, the increases in weight were gradually decreased. Body weight increases were significantly decreased in VOCs mixture exposure mice when compared with control ($p<0.05$ or $p<0.01$) (Fig. 1). The same as body weight increase, mice drinking water and food consumption changes of mice showed a similar trend. Thus, VOCs mixture exposure influenced a reduction in body weight gain.

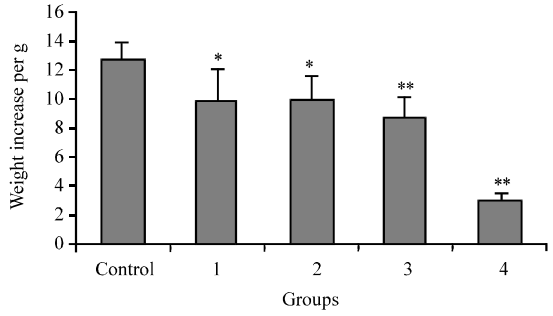

Fig. 1: Body weight change of adult male Kunming mice treated with VOCs exposure for 10 days. n = 10 in each group; Values were mean±SD. *p<0.05 **p<0.01 compared with control

LDH of BALF and ROS production in lung: LDH of BALF in mice was significantly affected (Table 2) which showed that VOCs exposure affected significantly cell membrane integrity in the alveolar area ($p<0.01$). With the increasing of VOCs concentrations, LDH activity became higher. These results indicated cell damage in the alveolar area. Lung intracellular ROS levels were measured by fluorescence spectrophotometer analysis using DCFH-DA staining to determine in VOCs mixture exposed mice. Intracellular ROS levels in the lung cells were also found to increase in mice exposure to VOCs and ROS in lung in treated groups were significantly higher than control ($p<0.01$) (Table 2). There was a positive correlation between LDH release in BALF and ROS production in lung ($r = 0.636$, $p = 0.00064$) (Fig. 2).

ROS production, lipid and protein peroxidation in liver, DNA damage in serum: Intracellular ROS levels in the liver cells in treated groups were significantly higher than control ($p<0.05$ or $p<0.01$) (Fig. 3a). The increases of MDA and carbonyl content were also observed in the liver in treated groups and the MDA and carbonyl contents were significantly higher in group 2, 3 and 4 ($p<0.05$ or $p<0.01$) (Fig. 3b and c). The 8-OHdg in the serum in group 3 and 4 were significantly higher than control (Fig. 3d). In addition with VOCs mixture concentrations increasing,

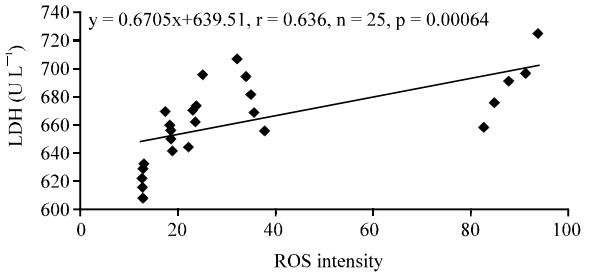

Fig. 2: Correlation between ROS in lung and LDH concentration in BALF in mice exposure to VOCs

Table 2: Effect of LDH in BALF and ROS production in lung in mice exposure to VOCs mixture

	Groups				
Index	Control	1	2	3	4
ROS	12.83±0.15	18.51±0.660**	23.15±1.050**	34.91±3.060**	88.25±5.880**
LDH (U L^{-1})	621.33±7.91	654.64±13.43*	670.67±26.08*	681.83±25.87*	692.52±34.21*

Values were means±SD. n = 5 in each group; *p<0.05 **p<0.01 compared with control

Table 3: Effect of antioxidative enzymes of liver in mice exposure to VOCs mixture

Groups	GSH (μmol g^{-1} prot.)	GSH-Px (U mg^{-1} prot.)	T-AOC (U mg^{-1} prot.)	CAT (U mg^{-1} prot.)	SOD (U mg^{-1} prot.)
Control	5.04±0.46	337.56±13.36	1.16±0.10	100.35±6.690	47.81±2.37
1	3.20±0.25**	278.36±17.14**	0.84±0.09**	115.59±17.17	40.85±4.76*
2	2.42±0.34**	258.11±25.75**	0.70±0.09**	115.91±18.25	43.80±1.91*
3	3.19±0.24**	237.35±13.26**	0.82±0.19**	131.57±13.68**	45.89±2.36
4	3.46±0.41**	138.41±20.79**	0.86±0.13**	86.30±11.23*	44.50±0.96*

Values were means±SD. n = 5 in each group; *p<0.05; **p<0.01 compared with control

Table 4: Effect of RNS of liver in mice exposure to VOCs mixture

Groups	TNOS (U mg^{-1} prot.)	iNOS (U mg^{-1} prot.)	cNOS (U mg^{-1} prot.)	NO (μmol g^{-1} prot.)
Control	0.87±0.05	0.41±0.07	0.45±0.09	0.57±0.10
1	0.99±0.19	0.56±0.07**	0.44±0.10	0.87±0.18*
2	1.18±0.19*	0.60±0.10**	0.58±0.11*	0.82±0.19*
3	1.27±0.23*	0.68±0.09**	0.60±0.15*	0.79±0.15*
4	1.28±0.20**	0.60±0.08**	0.67±0.11**	0.65±0.08

Values were means±SD. n = 5 in each group; *p<0.05**; p<0.01 when compared to control

ROS in liver was the most sensitive biomarker and there was a significant dose-effect relationship between ROS and VOCs mixture concentration.

Activities of antioxidative enzymes: In order to clarify whether the antioxidant defense changes were observed in the liver after exposure to VOCs mixture measured antioxidative enzymes in mice liver. The activities of GSH, SOD, T-AOC and GSH-Px were significantly lower than control, other than SOD in Group 2 There was a positive correlation between GSH, GSH-Px activity and VOCs mixture concentrations (Table 3). However, the activities of CAT in the livers were significantly higher in Group 3 ($p<0.01$) and lower in Group 4 ($p<0.05$) than control (Table 3) thus CAT in the mice liver was first increased and then decreased. On the other hand, GSH, T-AOC and GSH-Px were more sensitive to VOCs mixture than CAT and SOD.

NOS activity and NO level: The effects of exposure to short-term VOCs mixture on RNS responses in the liver of mice were investigated. The significant increases of NOS and iNOS activities and NO generation in the liver were observed in the treated groups ($p<0.05$ or $p<0.01$) (Table 4). NO in the whole liver homogenates would reflect NO produced for neurotransmission and NO produced by inflammatory responses that were initiated by VOCs mixture. VOCs mixture enhanced the expression of RNS and nitric oxide synthase of liver in mice.

Reactive Oxygen Species (ROS) including singlet oxygen, hydrogen peroxide, superoxide anion and hydroxyl radical can be produced by endogenous sources as well as through cellular aerobic metabolism and inflammation or through exposure to a variety of chemical and physical agents. Moreover, the overproduction of ROS would break down the balance of the oxidative/

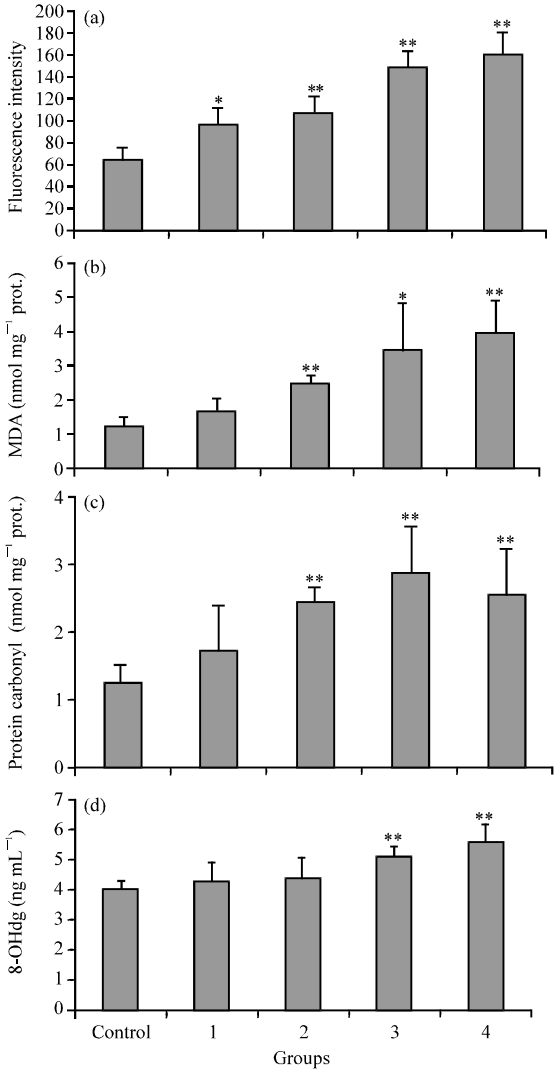

Fig. 3: Effect of ROS and products of oxidative damage in mice exposure to VOCs mixture, a) ROS generating of liver, b) MDA production of liver, c) Protein carbonal production of liver, d) 8-OHdg production of serum. n = 5 in each group. Values were means±SD. *p<0.05 **p<0.01 when compared to control

anti-oxidative system which is closely related to the reduction of the antioxidative enzymes like SOD, CAT and GSH-Px as well as nonenzymatic antioxidants like GSH in

the tissues resulting in the lipid and protein peroxidation and DNA damage. In the study, ROS of liver and lung was significantly increased (Table 2; Fig. 3a), the activities of SOD, GSH and GSH-Px were significantly inhibited (Table 3) and the total anti-oxidation capacity was decreased with the increasing of VOCs mixture doses. The reduction of antioxidative enzyme activities might be due to VOCs-induced overproduction of ROS to inhibit mRNA expression of SOD and GSH-Px. However, there were significant increasing CAT activities in Group 3 ($p<0.01$). Subsequently, the CAT enzymes were decreased significantly ($p<0.05$) at 10 days post exposure. The change trends in CAT activities were similar to some studies of single VOCs or two VOCs mixture (Rana and Kumar, 1994, 1995; Kum *et al*., 2007a; Stajkovic *et al*., 2009) which showed that toluene, xylene and methyl alcohol caused CAT activity increase in liver tissue under certain conditions. The initial CAT activities increase might plausibly be attributable to the fact that peroxidase is generated by product specific oxidase which are acted upon by CAT mainly in the liver (Rana and Kumar, 1995). Xylene (300 ppm), FA (6 ppm) or technical xylene + FA (150 + 3 ppm) for 6 weeks at 8 h day^{-1} did not change the activities of CAT and GSH in liver in adult and 4 weeks old rat (Kum *et al*., 2007a). FA exposure (an aqueous solution of formalin diluted to 1% FA by wt.) did not modify the activities of GPX, GSH, GST and CAT in lung (Lino-dos-Santos-Franco *et al*., 2011). FA (3 ppm) exposure for 24 h did not alter SOD activity in liver, too (Matsuoka *et al*., 2010). However, VOCs mixture (FA<1 ppm; xylene<2 ppm) exposure for 2 weeks at 2 h day^{-1} (static exposure) affected antioxidative enzymes in the study.

There are large amounts of Polyunsaturated Fatty Acids (PUFA) in the liver which play an important role in the tissues. But PUFA are easy to be invaded by ROS and cause impairments of cellular functions (Mates, 2000). ROS may induce lipids, proteins oxidative and DNA damage. MDA and carbonyl are markers of lipids and protein oxidative stress (Cederbegr *et al*., 2001; Cakatay *et al*., 2003). In general, lipid peroxidation is commonly regarded as a harmful process that leads to structural modifications of complex lipid protein assemblies such as biomembranes and lipoproteins and is usually associated with cellular malfunction (Kunn and Borchert, 2002). The data showed that the obvious production of ROS and lipid, protein peroxidation (MDA and carbonyl content increased) occurred in the liver of the mice treated with VOCs (Fig. 3) which indicated that these livers in mice exposure to VOCs mixture underwent severe oxidative stress. Similarly, formaldehyde was reported to cause oxidative stress in the mouse liver (Matsuoka *et al*., 2010) and rat liver, kidney, lung (Kum *et al*., 2007a, b; Hidir *et al*., 2008; Mukaddes *et al*., 2006; Sul *et al*., 2007). The 8-OHdg of tissues was significantly increased under VOCs pollution (Ma *et al*., 2010; Yoon *et al*., 2010) and as a sensitive biomarker (Pilger and Rudiger, 2006) it has been used to evaluated VOCs pollution indoors (Lu *et al*., 2007). The results also showed that 8-OHdg was significantly increased in Group 3, 4 ($p<0.05$) compared to control in serum. FA exposure (5 ppm) for 2 weeks at 6 h/day and 5 days/week could not induce lipid peroxidation and protein oxidation in lung in mice (Sul *et al*., 2007). FA (6, 10, 15 ppm) for 4 weeks at 6 h/day and 5 day/week could not induce DNA damage in serum (Speit *et al*., 2009). Xylene and FA (150 + 3 ppm) for 6 weeks at 8 h day^{-1} did not change MDA level in liver, too (Kum *et al*., 2007a). However, VOCs mixture (FA<5 ppm) induced DNA damage, lipid peroxidation (FA<3 ppm) and protein oxidation in the study.

Interactions between chemicals and cell membranes play a key role in pollutant-induced toxicity. Especially organic solvents are well known to damage cell membranes. The correlation between their hydrophobicity and induced toxicity has been reported (McKarns *et al*., 1997; Dreiem *et al*., 2003). Cytotoxicity was determined by the LDH assay to evaluate cell membrane integrity as a marker for toxicity *in vitro* (Pierre *et al*., 2011; Parisellia *et al*., 2009). Under the experimental conditions, LDH assay results showed that VOCs mixture exposure was able to induce LDH of BALF, indicating airway cell damage in the alveolar area. Induction of oxidative stress and necrosis is major mechanism of cell damage evoked by toxicants. Researchers therefore investigated ROS production in lung in mice in the experiment. Then, researchers found that there was a positive correlation between LDH release and ROS production. These results indicated that cell viability might be involved in ROS production. The toxic feature has been already described for H_2O_2 on human lung fibroblasts (Teramoto *et al*., 1999). Whether cell viability is involved in ROS production under VOCs pollution or not need to be further investigated *in vivo* experiments.

It has been demonstrated that NO plays dual roles in either neurotoxicity or neuroprotection under the oxidative stress (Holscher, 1997; Lin, 1999; Prast and Philippu, 2001). The depletion of the tissue GSH results in the increase of Nitric Oxide Synthase (NOS) (Heales *et al*., 1996). Exocytoic Ca^{2+} can stimulate cNOS activation (Holscher, 1997), suggesting that cNOS may respond quickly to tissue injury. Organic solvents (toluene and benzene) and their metabolites induced the formation of RNS (Myhre and Fonnum, 2001; Chen *et al*., 2005) and enhanced the expression of inducible Nitric Oxide

Synthase (iNOS) which can lead to the formation of excessive NO (Laskin *et al*., 2000). In this study, researchers investigated the effects of VOCs exposure on the activity of constitutive Nitric Oand Ioxide Synthase (iNOS) in the liver which are the rate-limiting enzymes for NO synthesis to indirectly reflect the changes of NO in the liver in mice. Researchers observed that accompanying with the decrease of GSH level in the liver in mice, the activities of cNOS and iNOS and NO level were significantly elevated by VOCs. The elevated cNOS activities might exhibit a protective effect on the exposure of VOCs. The increased iNOS activities may produce more NO and lead to the inflammatory response which is consistent with the increased generation of NO in the liver in mice. And NO can quickly react with ROS resulting in the formation of the peroxynitrite anion ($ONOO^-$) (Reynaert *et al*., 2005) which can cause the liver damage.

In China, the occupational exposure limit concentrations for formaldehyde, benzene, toluene and xylene proposed by the China State Bureau for Technical Supervision are following 3, 40, 100 and 100 mg/m^3. VOCs mixture (FA:3 mg/m^3; BTX<occupational exposure limit concentration) exposure for 2 weeks at 2 h day^{-1} (static exposure) led to oxidative damage. While researchers could not determine what combined toxicity is between FA and BTX in the present study. Researchers should consider the tolerance between rodents and humans against VOCs stress as well. Further studies could be required to determine these questions.

CONCLUSION

The results of this study show that inhalation of short-term VOCs mixture influenced the oxidative stress of mice so as to product excessive ROS. The overproduction of ROS could break down the balance of the oxidative/antioxidative system and cause impairments of cellular functions and also induce lipids, proteins oxidative and DNA damage as well as enhance the expression of RNS and nitric oxide synthase. ROS, GSH, GSH-Px, T-AOC, iNOS of liver could be used as biological sensitive makers of oxidative damage under short term VOCs mixture condition. These findings also provide the occupational exposure limit concentrations for VOCs with valuable reference.

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