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Acute exposure to waterpipe tobacco smoke induces changes in the oxidative and inflammatory markers in mouse lung

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Abstract

Context—Tobacco smoking represents a global public health threat, claiming approximately 5 million lives a year. Waterpipe tobacco use has become popular particularly among youth in the past decade, buttressed by the perception that the waterpipe “filters” the smoke, rendering it less harmful than cigarette smoke.

Objective—In this study, we examined the acute exposure of waterpipe smoking on lung inflammation and oxidative stress in mice, and compared that to cigarette smoking.

Materials and methods—Mice were divided into three groups; fresh air control, cigarette and waterpipe. Animals were exposed to fresh air, cigarette, or waterpipe smoke using whole body exposure system one hour daily for 7 days.

Results—Both cigarette and waterpipe smoke exposure resulted in elevation of total white blood cell count, as well as absolute count of neutrophils, macrophages, and lymphocytes ($P < 0.01$). Both exposures also elevated proinflammatory markers such as TNF- α and IL-6 in BALF ($P < 0.05$), and oxidative stress markers including GPx activity in lungs ($P < 0.05$). Moreover, waterpipe smoke increased catalase activity in the lung ($P < 0.05$). However, none of the treatments altered IL-10 levels.

Discussion and conclusion—Results of cigarette smoking confirmed previous finding. Waterpipe results indicate that, similar to cigarettes, exposure to waterpipe tobacco smoke is harmful to the lungs.

Keywords

Acute waterpipe smoke exposure; cigarette smoking; inflammation; lungs; oxidative stress

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Declaration of interest

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Introduction

Tobacco smoking is a global public health threat with more than 5 million deaths each year attributed to tobacco use, an annual toll that may double within the next two decades (World Health Organization, 2009). Tobacco is commonly smoked in different ways including cigarette, cigar, and waterpipe (a.k.a. hookah, narghile, or shisha). The popularity of waterpipe tobacco smoking is growing in the eastern Mediterranean and throughout the world including the USA and other western countries, especially among youth (Eissenberg et al., 2008; Maziak, 2008; Primack et al., 2008; Warren et al., 2009; Azab et al., 2010). This rise in popularity could be, in part, due to the common misperception that the waterpipe “filters” the smoke, rendering it less harmful and less likely to cause dependence than cigarette smoke (Smith-Simone et al., 2008; Primack et al., 2010). The popularity may also be due to the type of tobacco used in the waterpipe, called ma’assel, which is sweetened and available in many flavors.

Tobacco smoke contains chemical substances that are highly toxic and diverse in their effects on human health. It causes cancers of the lung, oral cavity, esophagus, stomach, and other organs (Jha, 2009). Moreover, smoking increases the risk for cardiovascular disease (Wells, 1994; Glantz & Parmley, 1995). The mechanisms by which tobacco smoking might cause such hazardous health effects include promotion of tissue inflammation and the production of high amount of free radicals in the bodies of smokers. These free radicals are remarkably reactive and randomly attack various cellular constituents as in the case of initiation of lipid, protein and DNA damage, (Frei et al., 1991; Reznick et al., 1992; Fahn et al., 1998; Khabour et al., 2011) resulting in tissue damage that can adversely affect different body organs including lung. For example, inflammatory cells (neutrophils, macrophages, and lymphocytes) were elevated in the airways, lungs parenchyma, and bronchoalveolar lavage fluid (BALF) of mice exposed to cigarette smoke (D’Hulst et al., 2005; Betsuyaku et al., 2008). With regard to oxidative stress, chronic exposure to cigarette smoke in mice was associated with increased reactive oxygen species (ROS) generation along with elevation in lipids, proteins and DNA oxidation (Cotgreave et al., 1987; Howard et al., 1998; van der Vaart et al., 2004; Menegali et al., 2009; Rueff-Barroso et al., 2010; Tuon et al., 2010). Moreover, activities of oxidative enzyme systems, namely superoxide dismutase (SOD) and catalase, and lipid peroxidation, were all increased in BALF and lungs of cigarette smoke-exposed mice as a response to elevated oxidative stress induced by smoke exposure (Valenca et al., 2008).

The short-term and long-term health effects of water-pipe smoke still need to be investigated. While many of the same toxicants have been found in cigarette smoke and waterpipe smoke (Shihadeh, 2003; Shihadeh et al., 2004; Al Rashidi et al., 2008; Sepetdjian et al., 2008), charcoal is used to heat and partially burn the tobacco in a waterpipe as opposed to burning it completely in the cigarette (Shihadeh, 2003). Moreover, the smoking behavior of waterpipe users (i.e. their puff topography) differs substantially from that of cigarette smokers, such that the amount of smoke inhaled during a single waterpipe use episode that takes about 45 min is 60 times greater than the amount of smoke inhaled when smoking a single cigarette that takes about 5 min (Cobb et al., 2011). Depending on the toxicant considered, the smoke from a single waterpipe tobacco smoking episode may contain 10–100 times the amount found in the smoke of a cigarette (Shihadeh, 2003; Sepetdjian et al., 2008). Therefore, even occasional waterpipe tobacco smoking may lead to harm to the body including tissue inflammation.

In this study, we adapted methods used to study the effects of cigarette smoking on lung function to an examination of the acute inflammatory effects of water-pipe tobacco smoke inhalation on major organs in mice. Outcomes included expression of inflammatory and

oxidative markers and changes in different white blood cells in the lung alveolar fluid. Results of this study are relevant to predicting the health effects of waterpipe tobacco smoking in the millions of youth who use them across the globe, and also to health promotion campaigns aimed at informing waterpipe users and others of the potential risks of this method of tobacco use.

Materials and methods

Animals

Six weeks old naïve BALB/c mice, equal numbers of each sex, were used in the study. The animals were maintained in the animal care unit at $24 \pm 1^\circ\text{C}$ with a 12:12 light/dark cycle (light on at 07.00 h). Mice were caged in groups of 4 in plastic cages with wire lids and food and water were available *ad libitum*. The study procedure was approved by the Animal Care and Use Committee at Jordan University of Science and Technology.

Waterpipe smoke exposure system

A waterpipe smoke whole body animal exposure system was developed for this study. The system (Figure 1) is comprised of two main components: a waterpipe smoking machine and an exposure chamber. The smoking machine utilizes a variable flow positive displacement diaphragm pump to draw from a waterpipe and discharge into the chamber. As is the case during normal use, the smoke from the waterpipe passes first through the water before it was drawn into the exposure chamber (Figure 1). Water was replaced before each exposure session. The pump is automatically controlled to provide 171 puffs of 2.6 s duration with an interpuff interval of 17 s, in accordance with the Beirut Method (Katurji et al., 2010). During each exposure session, puff volumes were monitored and recorded using a waterpipe puff topography instrument, (Shihadeh et al., 2004) and the diaphragm pump flow rate was manually adjusted to maintain the 530 mL puff volume specified by the Beirut Method. This regimen was chosen because it approximates, on average, human puff topography during waterpipe smoking (Shihadeh et al., 2004).

The exposure chamber ($38 \times 25 \times 25$ cm, L×W×H) was constructed from transparent polycarbonate, and had a removable ceiling fitted with flow ports for the smoke inlet, fresh air inlet, excess flow outlet, and smoke sampling line. A 3 cm fan was suspended from the lid to ensure that the chamber contents were well mixed during each exposure session. Carbon monoxide (CO) concentration was continuously monitored in the chamber using an electrochemical sensor (Bacharach Monoxor II) drawing a flow rate of 0.3 LPM through a 47 mm glass fiber filter (Pall Type A/E). The filter was weighed prior to and after each exposure session to determine total suspended particulate matter concentration in the chamber. Fresh air was continuously pumped into the chamber, resulting in an air change rate of approximately 1.5 changes per hour.

It should be noted that likely due to the highly turbulent nature of the flow inside the diaphragm pump, a significant fraction of the smoke particulate matter drawn from the waterpipe deposited in the pump. By comparing dilution-corrected particulate matter concentration sampled at the waterpipe mouthpiece and in the chamber, we estimated a 2/3 particle loss fraction (by mass) in the pump. As a result, waterpipe smoke in the chamber was relatively denuded of particulate matter.

Whole body smoke exposure

The animals were randomly assigned to three groups ($n = 8$ per group: 4 males and 4 females); control (C), cigarette smoke (CS, as positive control), and waterpipe smoke (WS). C animals were placed in the inhalation chamber and exposed to fresh air only. WS animals

were exposed to mainstream waterpipe smoke using the whole body exposure system described above for one 60 min smoking session/day for 7 days. CS animals were placed in the same inhalation chamber used for the waterpipe group and were exposed to sidestream cigarette smoke by hanging a smoldering cigarette in the upper middle half of the chamber. The cigarettes were regular (red) MarlboroTM (Philip Morris, USA) purchased in Irbid, Jordan. For each exposure session (1 h), approximately 5.6 cigarettes were consumed (Santiago et al., 2009).

We based the daily exposure period (60 min/day) on the published literature for cigarette smoke (Zhang et al., 2002; Churg et al., 2003). To provide a degree of commensurability between CS and WS inhalation exposure, the ventilation system in the chamber was adjusted so that CS and WS animals experienced similar levels of CO (mean $\pm$ SD 980 $\pm$ 94 ppm CS, 915 $\pm$ 94 ppm WS). The resulting observed TPM concentrations were higher in WS by approximately 25% [963 $\pm$ 380 mg/m³ (CS) and 1230 $\pm$ 540 mg/m³ (WS)], though the difference was not statistically significant ($P > 0.05$).

Preparation of alveolar fluid

After the last exposure, animals were terminated with a high dose of anesthetic (40 mg/kg of thiopental i.p.). Directly after termination, the lungs were washed using a cannula inserted into the trachea and the lungs were instilled using three 0.5 mL aliquots of sterile phosphate-buffered saline. Aliquots were combined for each animal. Part of the BALF was used for differential cell count and the remainder was used for analysis of inflammation bio-markers (see below). For lung tissue experiments, different sets of animals (treated identically as described above) were used. After termination, the lungs were dissected out from the animal and cut longitudinally into pieces and washed with cold and sterile phosphate-buffered saline. They were, then, immediately frozen into liquid nitrogen followed by storage at -80°C until used.

Differential count of white blood cells

BALF was centrifuged at 300 $\times$ g and fixed with 0.5 mL of methyl violet fixative for total cell counts. Differential staining of white blood cells was performed using Hema-Gurr-stained cytopspins kit as described previously (Morris et al., 2008). Count for each leukocyte type was determined by multiplying its percentage with the total number of cells for each sample. Counts were expressed as cells per milliliter BALF.

Molecular measurement in BALF and lung tissue

To determine the levels of the inflammatory markers IL-10, TNF- α and IL-6, aliquots of BALF were centrifuged at 300 $\times$ g to remove cells and then supernatants were assayed. All markers were analysed using ELISA technique as described by the manufacturer. For IL-10, IL-6 and TNF- α eBioscience kits (San Diego, CA) were used and the absorbance was read at 450 nm (ELx800 plate reader, Bio-teak instruments, Winooski, USA). The same inflammatory markers were examined also in lung homogenates as described above.

For total protein determination in BALF, aliquots were first centrifuged to remove cells and then protein was determined in the supernatant using BioRAD procedure (Hercules, CA) as previously described (Khabour et al., 2010).

Examination of oxidative stress markers in the lungs

Tissues were homogenized in buffer manually using small pestle in ice-cold lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl₂, 1% Nonyl phenyl polyethylene glycol ether, 10% glycerol, 0.5 mM sodium vanadate, 1 mM polymethane sulfonyl fluoride) (Alzoubi et al., 2012), and protease inhibitor cocktail (Sigma-Aldrich Corp., MI, USA). Homogenates were

centrifuged at 14,000×g and 4°C for 5 min to remove insoluble material. For assaying GPx activity, 10 µL of tissue homogenate was mixed with 990 µL of reaction cocktail containing 0.25 mM NADPH, 0.5 unit/mL glutathione reductase, 2.1 mM reduced glutathione and 30 mM tert-butyl hydroperoxide (Sigma–Aldrich). Directly after mixing the reagents, the change in the absorbance of the reaction was measured every 10 s for 1 min at 340 nm using spectrophotometry (UV-1800, Shimadzu, Japan). Catalase activity was measured by adding 20 µL of lung homogenate to 50 µL of hydrogen in 100 mM phospheroxide working solution (12 mM H₂O₂ phosphate buffer, pH: 7.0). After 1 min of incubation, 50 µL of the catalase quencher (provided by the kit, Cell Biolabs, San Diego, USA) was added to stop the reaction. Then 200 µL of the catalase chromagen was added to the mixture. After 40 min of shaking of the mixture, absorbance was measured at 540 nm. Activity of SOD was measured using SOD kit provided by Sigma–Aldrich (MI, USA) as described by the manufacturer. Plates were read at 450 nm. SOD activity was expressed as unit/mg protein.

Statistical analysis

The collected data were analysed using GraphPad Prism software (version 5.0, La Jolla, CA). Groups were compared using One-way ANOVA followed by Tukey's test. The level of significance of hypothesis testing was $P < 0.05$.

Results

To investigate the effects of waterpipe smoke inhalation in the lungs of mice, we assessed several parameters of inflammation and oxidative stress. BALF total cellularity was higher in WS ($6.12 \pm 0.39 \times 10^5$; $P < 0.001$, Figure 2A) and CS ($6.50 \pm 0.21 \times 10^5$) groups compared to C group ($3.06 \pm 0.25 \times 10^5$). In all groups, the predominant type of cells was macrophages with relatively higher numbers in WS and CS groups ($P < 0.01$, Figure 2C). Neutrophils were observed in greater numbers in both WS ($P < 0.01$) and CS ($P < 0.01$) groups compared to C group (Figure 2B). Lymphocytes were the least abundant type of cells in all groups but their numbers were still higher in WS and CS groups ($P < 0.05$, Figure 2D). In all type of cells, there was no difference across tobacco products ($P > 0.05$, Figure 2). We also estimated protein leak across the alveolar-capillary barrier through measuring total protein concentration in the BALF (Kenyon et al., 2002). We found a significant increase in total protein in BALF of mice exposed to either cigarette smoke or waterpipe smoke ($P < 0.001$, Figure 3). Additionally, there was no difference in total BALF proteins between CS and WS groups ($P > 0.05$, Figure 3).

To investigate if changes in white blood cells are accompanied by changes in inflammatory cytokines, TNF- α , IL-6 and IL-10 were assayed in both BALF and lungs homogenates. The proinflammatory cytokines TNF- α and IL-6 were significantly elevated in BALF in both WS and CS (Figure 4A and 3B, $P < 0.05$), whereas, the anti-inflammatory cytokine IL-10 was not affected ($P > 0.05$, Figure 4C). Similarly, in homogenate derived from lungs, TNF- α was elevated in both WS and CS groups ($P < 0.05$, Figure 5A) and IL-10 was not affected ($P > 0.05$, Figure 5C). However, unlike BALF results, in lungs homogenate, IL-6 was not altered by both treatments ($P > 0.05$, Figure 5B).

As a marker of tissue injury, we examined the activity of the antioxidative enzymes catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD) in the lungs using the same treatment protocol. The activity of both catalase ($P < 0.05$, Figure 6A), and GPx ($P < 0.01$, Figure 6B) were elevated in lungs of mice treated with waterpipe smoke (WS group). Additionally, catalase activity was elevated in the lungs of CS group ($P < 0.0$, Figure 6A). In all groups, the activity of SOD in the lungs was similar (Figure 6C). In the study, equal numbers of male and female mice were used and we observed no significant sex differences in the response to waterpipe or cigarette tobacco smoke (data not shown).

Discussion

In this study, we investigated the acute inflammatory effects of waterpipe smoking on the lung. Results indicate that, similar to cigarette smoke, exposure to water-pipe smoke induces lung inflammation and injury.

The acute effects of cigarette smoking have been extensively studied (van der Vaart et al., 2004). In human studies, acute exposure to cigarette smoke induces elevation in peripheral blood neutrophils and granulocytes (Abboud et al., 1986; Hockertz et al., 1994). In the lungs of cigarette smokers, acute cigarette smoking has been shown to increase lung endothelial permeability (Ward et al., 2000) and oxidative stress biomarkers such as 8-iso-prostane, hydrogen peroxide and superoxide (Morrison et al., 1999; Guatura et al., 2000; Montuschi et al., 2000). In animal studies, acute cigarette smoke exposure increases alveolar macrophages and neutrophils in lung tissues and BALF (Sato et al., 2008). On the other hand, epithelial permeability and mast cells were elevated in the airways, whereas eosinophils were decreased after cigarette smoke exposure (Hulbert et al., 1981; Burns et al., 1989; Vitalis et al., 1998). In addition, cigarette smoke exposure increases the levels of inflammatory cytokines TNF- α , IL-6 and macrophage chemoattractant protein 1 (Churg et al., 2002; Churg et al., 2003; Sato et al., 2008). Regarding oxidative stress, most studies showed a direct increase in oxidative markers after exposure to acute cigarette smoke exposure. For example, in the lung and BALF, acute cigarette smoke induces imbalance in reduced and oxidized glutathione and increased nitric oxide (Cotgreave et al., 1987; Li et al., 1996; van der Vaart et al., 2004). In addition, activities of oxidative enzyme systems, namely SOD and catalase, and lipid peroxidation, were all increased in BALF and lungs of cigarette smoke-exposed C57/BL mice as a response to elevated oxidative stress induced by smoke exposure (Valenca et al., 2008). Finally, oxidative DNA damage as marked by 8-hydroxy-2'-deoxyguanosine was high in the lungs, liver and heart of BALB/c mice exposed acutely to cigarette smoke (Howard et al., 1998). Results of acute exposure to cigarette smoking presented in our study confirm the above findings.

Regarding waterpipe, only one published study has evaluated WTS effects on the inflammatory response of BALB/c mice (Mirsadraee et al., 2010). Groups of 6 mice were first sensitized to ovalbumin as a model of asthma and then exposed to either cigarette or waterpipe smoke for either 6 or 24 h: significant increases were observed in nitric oxide levels and BALF eosinophils for all smoke-exposed animals (Mirsadraee et al., 2010). In the current study, we showed for the first time that acute exposure to waterpipe smoke leads to inflammation to the lungs similar to that induced by cigarette smoking. This effect was shown by (i) elevation in WBC in BALF of exposed mice, (ii) increases in protein leak across the alveolar-capillary barrier, (iii) elevation of inflammatory cytokines TNF- α and IL-6 in both BALF and lung tissues and (iv) up-regulation of the antioxidative enzymes catalase and GPx. Thus, waterpipe tobacco smoke exposure is associated with lung damage similar to that seen following cigarette smoke exposure. These results provide no evidence that waterpipe tobacco smoking presents less health risk than cigarette smoking.

Recent work indicates that waterpipe smoking is a potential health threat (e.g., Akl et al., 2010; Raad et al., 2011; Hakim et al., 2011). A potential mechanism for the health threat involves the fact that, relative to a single cigarette, the smoke from a single waterpipe tobacco smoking episode contains eight times the CO, three times the nitric oxides, 4–15 times the acrolein, 6–31 times the formaldehyde, and 3–245 times the PAHs (Shihadeh, 2003; Shihadeh & Saleh, 2005; Al Rashidi et al., 2008). Importantly, many of these toxicants are associated with inflammation (Podechard et al., 2008; Gentner & Weber 2011) and oxidative stress (Kim & Lee, 1997; Wells et al., 1997; Bravo et al., 2011; Wegesser et al., 2010; Hanzalova et al., 2010). The results of our study provide direct evidence that

waterpipe smoke inhalation is associated with lung inflammation and injury. More studies are required to investigate the effect of waterpipe smoking on other major body organs both acutely and chronically.

The primary aim of this investigation was to examine the acute toxic effect of waterpipe tobacco smoking of the lungs. We used cigarette smoke as a positive control. The results showed that the response of the lungs to water-pipe tobacco smoke was similar to that of exposure to cigarette tobacco smoke. Previous studies showed that the level of exposure to toxic substances in one waterpipe smoking session is higher than that of a single cigarette (see discussion above). In addition, water in the bowl of the waterpipe is not expected to interact with the smoke; however, it exerts a small filtering effect that likely does not significantly modulate toxicity profile of the water-pipe smoke (Schubert et al., 2011). In our experiment, the ventilation system in the chamber was tuned so that CS and WS mice were exposed to similar levels of CO (mean $\pm$ SD 980 $\pm$ 94 ppm CS, 915 $\pm$ 94 ppm WS). Thus while the primary aim of the CS exposure condition was to include a positive control, this experimental design emphasizes differences between WS and CS exposure resulting from differing quality rather than quantity of smoke delivered; in reality, a single waterpipe smoking session typically delivers significantly greater quantities of toxicants than a single cigarette. It is also worth noting that animals in the cigarette group were exposed to sidestream smoke and that this smoke may be substantially different than that inhaled by smokers. A more appropriate comparison would utilize mainstream smoke generated by a cigarette smoking machine. In addition, in this study, we investigated the acute effect of exposing animals to waterpipe tobacco smoke for a short period of time (one 60 min smoking session/day for 7 days). Chronic exposures for long periods might produce different toxic responses between the two examined types of smoking. This will be a matter of future research.

In this study, we used whole body exposure to investigate the health threat of waterpipe smoking. In the literature of cigarette smoking, animals were exposed to tobacco smoke either directly to the nose of a restrained animal (nose only exposure) or to the cage of an unrestrained animal (whole body exposure) (Cheng et al., 2010). While nose only exposure has the advantage of being certain that the animal inhales a particular amount of smoke, its limitations are considerable. For example, these limitations include the use of stressful restraint that can, in itself, alter a variety of relevant outcome measures. The animal's inability to thermoregulate is another important concern (Cheng et al., 2010). Thus, whole body exposure is preferred over nose only exposure because there is no stressful restraint, although this model may involve exposure to smoke through ingestion (i.e., when the animal grooms itself). In fact, whole body exposure is commonly used in studying the effects of cigarette smoke in rodents (Mousa et al., 1988; Suemaru et al., 1992; Anderson et al., 2004; Simons et al., 2005; Small et al., 2010). The results presented in this study demonstrate that whole body waterpipe smoke exposure model that we have developed is useful to measure toxicant exposure and health effects of waterpipe tobacco smoke inhalation in experimental animals. This model will be helpful in filling the knowledge gap regarding the health effects of waterpipe tobacco smoking and thus inform public and policy makers about the harmful health effects of this method of tobacco use.

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Figure 1.
Schematic of the whole body smoke exposure system.

Figure 2.

Elevation in WBC in BALF of waterpipe and cigarette smoke-exposed mice. Mice were exposed to either waterpipe or cigarette smoke (WBE, 1 h daily for 7 days). Both cigarette and waterpipe smoke exposure resulted in elevation of total white blood cells count (A), absolute count of neutrophils (B), Macrophages (C), and lymphocytes (D). No difference was detected between the waterpipe and the cigarettes exposure groups. *Significant difference from the control group ($P < 0.01$, $n = 8$).

Figure 3.

Increases in protein leak across the alveolar-capillary barrier after exposure of mice to waterpipe or cigarette smoke. Mice were exposed to either waterpipe or cigarette smoke (WBE, 1 h daily for 7 days). Both cigarette and waterpipe smoke exposure resulted in elevation of total BALF proteins. *Significant difference from the control group ($P < 0.01$, $n = 8$).

Figure 4.

Changes in BALF inflammatory markers induced by cigarette or waterpipe smoke exposure. Mice were exposed to either waterpipe or cigarette smoke (WBE, 1 h daily for 7 days). Both cigarette and waterpipe smoke exposure resulted in elevation of TNF- α levels (A) and IL-6 (B). However, none of the treatments affected IL-10 levels (C). *Significant difference from the control group ($P < 0.05$, $n = 8$).

Figure 5. Changes in lung inflammatory markers induced by cigarette or waterpipe smoke exposure. Mice were exposed to either waterpipe or cigarette smoke (WBE, 1 h daily for 7 days). Both cigarette and waterpipe smoke exposure resulted in elevation of TNF- α levels in lung homogenate (A). However, none of the treatments affected IL-6 (B) and IL-10 levels (C). *Significant difference from the control group ($P < 0.05$, $n = 8$).

Figure 6. Changes in lung oxidative stress markers induced by cigarette or waterpipe smoke exposure. Mice were exposed to either waterpipe or cigarette smoke (WBE, 1 h daily for 7 days). Waterpipe smoke exposure resulted in elevation of lung catalase activity (A) whereas, both treatments induced elevation in GPx activity (B). However, none of the treatments affected SOD activity (C). *Significant difference from the control group ($P < 0.05$, $n = 8$).