Nose-only water-pipe smoking effects on airway resistance, inflammation, and oxidative stress in mice

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Nemmar A, Raza H, Yuvaraju P, Beegam S, John A, Yasin J, Hameed RS, Adeghe E, Ali BH. Nose-only water-pipe smoking effects on airway resistance, inflammation, and oxidative stress in mice. J Appl Physiol 115: 1316–1323, 2013. First published July 18, 2013; doi:10.1152/japplphysiol.00194.2013.—Water-pipe smoking (WPS) is a common practice in the Middle East and is now gaining popularity in Europe and the United States. However, there is a limited number of studies on the respiratory effects of WPS. More specifically, the underlying pulmonary pathophysiological mechanisms related to WPS exposure are not understood. Presently, we assessed the respiratory effects of nose-only exposure to mainstream WPS generated by commercially available honey flavored “moasel" tobacco. The duration of the session was 30 min/day and 5 days/wk for 1 mo. Control mice were exposed to air only. Here, we measured in BALB/c mice the airway resistance using forced-oscillation technique. Lung inflammation was assessed histopathologically and by biochemical analysis of bronchoalveolar lavage (BAL) fluid, and oxidative stress was evaluated biochemically by measuring lipid peroxidation, reduced glutathione and several antioxidant enzymes. Pulmonary inflammation assessment showed an increase in neutrophil and lymphocyte numbers. Likewise, airway resistance was significantly increased in the WPS group compared with controls. Tumor necrosis factor α and interleukin 6 concentrations were significantly increased in BAL fluid. Lipid peroxidation in lung tissue was significantly increased whereas the level and activity of antioxidants, including reduced glutathione, glutathione S transferase, and superoxide dismutase were all significantly decreased following WPS exposure, indicating the occurrence of oxidative stress. Moreover, carboxyhemoglobin levels were significantly increased in the WPS group. We conclude that 1-mo nose-only exposure to WPS significantly increased airway resistance, inflammation, and oxidative stress. Our results provide a mechanistic explanation for the limited clinical studies that reported the detrimental respiratory effects of WPS.

chronic obstructive pulmonary disease (COPD) is a preventable disease with a prevalence of more than 10% worldwide among adults 40 yr of age and older, and it affects over 5% of adult population in Western countries (11), imposing heavy physical, financial, and social burdens. It is the fifth leading cause of mortality worldwide and is predicted to be the third leading cause by 2020 (22). COPD includes chronic bronchitis and emphysema and is characterized by progressive airflow limitation and abnormal inflammatory responses in the lung (39). The primary cause of COPD is tobacco smoke (through tobacco use or second-hand smoke) (38). Total deaths from COPD are projected to increase by more than 30% in the next 10 years without interventions to cut risks, particularly exposure to tobacco smoke (44).

Water pipe smoking (WPS; also called Hubble bubble, shisha, hookah, or narghile) is a traditional method of tobacco smoking commonly practiced especially in the Arabian Peninsula, Turkey, India, and China and is now increasing in all over the world including in Europe and North America (8, 16, 18, 19). Estimates of the equivalence between cigarette and water-pipe smoking varies between 2 and 10 cigarettes for occasional and daily WPS, respectively, and 100 cigarettes for a 200-puff WPS session (23, 43).

Although the adverse health effects related to cigarette smoking (CS) have been extensively described, there are only a few studies that have investigated the respiratory effects of WPS (16).

A recent study involving two separate meta-analyses on six eligible cross-sectional studies reported a significant decrease in the forced expiratory volume in 1 s (FEV1) in the WPS group compared with the nonsmokers (33). However, no difference was found in FEV1, forced vital capacity (FVC), or FEV1/FVC between the WPS and CS groups. It was concluded that the significant decrease in FEV1 among water-pipe smokers not only is clinically relevant in terms of its minimal important difference but also supports a possible role for WPS in the development of COPD (33). Similarly, Hakim et al. (12) reported that one session of WPS resulted in significant increases in carboxyhemoglobin (COHb) concentrations, systolic and diastolic blood pressure, and heart and respiratory rates. A decrease was observed in peak expiratory flow rate (PEFR) and forced expiratory flow between 25 and 75% of forced vital capacity (FEF25–75%)%. These authors suggested that cardiopulmonary changes observed in healthy volunteers were similar or even greater than those reported in cigarette smoking (12).

Clinical studies have reported some difficulties in studying the isolated effects of WPS because most of the smokers are also current or past cigarette smokers. Therefore, experimental studies investigating the pathophysiological mechanisms underlying the respiratory adverse effect are critical and much needed. Experimental studies on the respiratory effects of WPS are very scarce. As far as we are aware, only one study has recently reported that exposure to WPS using a whole body...
exposure system 1 h daily for 7 days caused an increase in tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and glutathione peroxidase (GPx) activity in bronchoalveolar lavage fluid (15).

Therefore, the aim of this study is to investigate the possible effects of exposure to WPS for 1 mo, using a relevant exposure system, namely nose-only exposure, on a comprehensive set of respiratory indexes including 1) lung inflammation assessed by bronchoalveolar lavage (BAL) fluid analysis and histology; 2) concentrations of proinflammatory cytokines including TNFα, IL-6, and granulocyte macrophage-colony stimulating factor (GM-CSF) in BAL fluid; 3) airway resistance measured invasively using forced-oscillations technique; and 4) pulmonary lipid peroxidation (LPO) and reduced glutathione (GSH) concentration, and the activities of the antioxidant enzymes glutathione S-transferase (GST) and superoxide dismutase (SOD).

MATERIALS AND METHODS

Animals and Treatments

This project was reviewed and approved (A7-12, April 8th, 2012) by the Institutional Review Board of the United Arab Emirates University, College of Medicine and Health Sciences, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

WPS Exposure

BALB/c mice (Taconic Farms, Germantown, NY) were housed in a conventional animal house and maintained on a 12:12-h light-dark cycle. The animals were placed in cages and supplied with pelleted food and water ad libitum. Following 1 wk of acclimatization, animals were randomly divided into air (control) and WPS-exposed groups. Mice were anesthetized with an intraperitoneal injection of pentobarbital (100 mg/kg, i.p.) and were intubated with a 18-gauge needle into the trachea. Mice were connected to a computer-controlled small animal ventilator and quasi-sinusoidally ventilated with a tidal volume of 10 ml/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cmH2O to achieve a mean lung volume close to that during spontaneous breathing. After measurement of a baseline, each mouse was challenged by 10 ml of 0.9% saline delivered through the ventilator for 5 s, with increasing concentrations (0, 0.625, 1.25, 2.5, 5, and 10 mg/ml). Rf was measured using a "snapshot" protocol each 20 s for 2 min. The mean of these five values was used for each methacholine concentration, unless the coefficient of determination of a measurement was smaller than 0.95. For each mouse, Rf was plotted against methacholine concentration (from 0 to 10 mg/ml) (24, 30).

Collection and Analysis of BAL Fluid

The collection and analysis of BAL has been performed according to a previously described method (13, 25, 26, 30). In brief, in separate animals (n = 8), after WPS or air exposure, the mice were killed with an overdose of pentobarbital sodium. The trachea was cannulated and lungs were lavaged three times with 0.7 ml (a total volume of 2.1 ml) of sterile NaCl 0.9%. The recovered fluid aliquots were pooled. No difference in the volume of collected fluid was observed between the different groups. BAL fluid was centrifuged (1,000 g × 10 min, 4°C). Cells were counted and the differentials were microscopically performed on cytocentrifuge preparations fixed in methanol and stained with Diff-Quick (Dade, Brussels, Belgium). The supernatant was stored at −80°C until further analysis.

Histology

On the same animals used for BAL, after the lavage, the right lung was fixed with 10% formaldehyde (42). Seven-micrometer sections were prepared from right lung paraffin blocks and stained with hematoxylin and eosin. A histologist examined slices from different lung lobes and evaluated without prior knowledge of the animals’ treatment. The degree of interstitial infiltration by inflammatory cells, in response to WPS exposure, was evaluated by counting the number of inflammatory cells in the lung at high-power field (×100) as previously described (27).

Measurement of LPO, GSH Concentration, and Antioxidant Enzyme Activities in Lung Tissue

In separate mice (n = 8 in each group), following the exposure to WPS or air, animals were killed by an overdose of pentobarbital sodium. Lungs from air-exposed control and WPS-exposed mice were collected and rinsed with ice-cold PBS (pH 7.4) before homogenization in 0.1 M phosphate buffer, pH 7.4, containing 0.15 M KCl, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride at 4°C. Homogenates were centrifuged for 10 min at 3,000 g at 4°C to remove cellular debris, and supernatants were used for further analysis. Protein content was measured by Bradford’s method as described before (34).

Measurement of LPO. NADPH-dependent membrane LPO was measured as thiobarbituric acid-reactive substance using malondialdehyde as standard (Sigma-Aldrich Fine Chemicals, St. Louis, MO) (35).

Measurement of GSH concentrations and GST activity. These were carried out in control and WPS-exposed animals by standard procedures as described in previous publications (35).

Measurement of SOD activity. This was measured as the conversion of nitro blue tetrazolium (NBT) to NBT-diformazan according to the vendor’s protocol (R & D Systems, Minneapolis, MN). The extent of reduction in the appearance of NBT-formazan was used as a measure of SOD activity present in the experimental samples.

Measurement of TNFα, IL-6, and GM-CSF in BAL Fluid

The concentrations of IL-6, TNFα, and GM-CSF in BAL fluid were determined using ELISA Kits (Duo Set, R & D Systems).

Measurement of COHb

In separate animals, following WPS or air exposure mice were anesthetized intraperitoneally with pentobarbital sodium (45 mg/kg), and then blood was drawn from the inferior vena cava in heparin.
Immediately after that, COHb levels were measured using an Illex cooximeter (IL-682; Instrument Laboratory; Lexington, MA).

Statistics

All statistical analyses were performed with GraphPad Prism Software version 5. To determine whether parameters were normally distributed, the KS normality test was applied. Normally distributed data were analyzed using the unpaired t-test for differences between groups. Non-normally distributed data (Neutrophils, lymphocytes, and GM-CSF in BAL fluid) were analyzed using Mann-Whitney test for differences between groups. All the data in figures are reported as means ± SE. P values ≤ 0.05 are considered significant.

RESULTS

Lung Histology

Sections of lung from air-exposed mice showed a normal appearance under light microscopy. Compared with the air-exposed group, lung sections obtained from mice exposed for 1 mo to WPS showed the presence of interstitial infiltration of inflammatory cells, including neutrophils and lymphocytes. The number of neutrophils per high-power field (×100) in the lung of WPS-exposed mice was significantly increased compared with the control group (P < 0.05; Fig. 1C). The number of lymphocytes in the WPS-exposed group was increased compared with the air-exposed group (Fig. 1D). However, this increase did not reach statistical significance (P = 0.06).

Cell Composition and Number in BAL Fluid

Compared with the control group, 1 mo nose-only exposure to WPS caused a significant increase in neutrophil numbers (Fig. 2, B–E). Similarly, there was a significant influx of lymphocytes in BAL fluid (Fig. 2, C–E). The number of lymphocytes was significantly increased in the WPS-exposed group compared with the air-exposed group. However, the number of macrophages was not affected by WPS compared with the control group exposed to air (Fig. 2A). The total numbers of cells in BAL fluid in air- and WPS-exposed mice were 0.36 ± 0.01 × 10⁵/ml and 0.43 ± 0.04 × 10⁵/ml, respectively.

TNFα, IL-6, and GM-CSF Concentrations in BAL Fluid

TNFα concentration in BAL fluid was significantly increased (P < 0.05) following nose-only WPS exposure com-
pared with the air-exposed mice (Fig. 3A). Similarly, IL-6 concentration was also increased \((P < 0.01)\) after nose-only WPS exposure (Fig. 3B) compared with air-exposed mice. The concentrations of GM-CSF observed in BAL fluid following WPS exposure did not vary significantly compared with the air-exposed group (Fig. 3C).

**Airway Hyperreactivity to Methacholine**

Figure 4 shows airway resistance, measured by the forced-oscillations technique after increasing concentrations of methacholine (0–10 mg/ml), following nose-only exposure to air or WPS. Compared with the air-exposed group, WPS exposure induced a dose-dependent increase in airway resistance. From the resistance-methacholine dose-response curve, an index of airway responsiveness was calculated as the slope of the linear regression using 0–10 mg/ml concentrations. The latter showed a significant increase of airway resistance in WPS-exposed mice compared with air-exposed ones \((P < 0.01, \text{Fig. 4B})\).

**LPO in Lung Tissue**

Figure 5 illustrates the effect of 1 mo nose-only WPS or air exposure on LPO in lung tissue. One month of nose-only WPS exposure induced a significant increase \((P = 0.01)\) in lipid peroxidation compared with the air-exposed group (Fig. 5).

**GSH Concentration and GST and SOD Activities in Lung Tissue**

Figure 6 illustrates the effects of 1 mo of nose-only WPS exposure on the level of antioxidants in the lung including reduced GSH concentration, and GST and SOD activities. At the end of the 1-mo exposure period, we found a significant decrease of GSH concentration \((P < 0.0001)\) in lung tissue compared with the control group (Fig. 6A). The activities of both SOD and GST were decreased \((P = 0.05 \text{ and } P < 0.01, \text{ respectively})\) in WPS-exposed mice vs. those exposed to air (Fig. 6, B and C, respectively).

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**Fig. 2. Number of macrophages (A), polymorphonuclear neutrophils (PMN, B), and lymphocytes (C) in bronchoalveolar lavage (BAL) fluid at the end of the 1-mo exposure period to air (control) or WPS. Data are means ± SE \((n = 8)\). Cytocentrifuge preparation of cells obtained by lavage (D and E). The cells were recovered from lungs of mice exposed to air (D) or WPS (E), 1 mo after the exposure. The micrographs demonstrate the presence of macrophages (thin arrow) and influx of neutrophils (thick arrows) and lymphocytes (thick arrowhead).
Measurement of COHb Levels in Blood

A significant increase in COHb was found in mice exposed to WPS compared with those exposed to air (P < 0.0001) (Fig. 7).

DISCUSSION

The present study confirmed that 1-mo nose-only exposure to WPS induces pulmonary inflammation as evidenced by the influx of neutrophils and lymphocytes assessed by histology and BAL, and the increase of both TNFα and IL-6 in BAL fluid. The airway resistance was also augmented. The lipid peroxidation levels were increased and GSH concentration decreased in lung tissue. The activities of the antioxidant enzymes GST and SOD were inhibited by WPS. Moreover, the level of COHb has significantly increased following WPS exposure.

A number of exposure systems are currently used to study the effect of tobacco smoke exposure in mice (36, 37, 45). The smoking machines that have been used with animal models include systems that use nose-only or whole body exposures. The downside of using whole body exposure is that the animals may ingest nicotine or tar substances when cleaning their fur. The nose-only exposure system avoids this problem and most likely best resembles the human situation (36, 37). Yet, only limited studies have used nose-only exposure systems to study the pulmonary and systemic effect of tobacco smoke (1, 3, 27, 28, 36).

We have recently demonstrated that short-term (4 days) nose-only exposure to cigarette smoke causes lung inflammation and increase of airway resistance mediated at least partly through the oxidative stress (27). Using a whole body exposure...
Khabour et al. (15) investigated the effect of short-term exposure (1 h daily for 7 days) to WPS and compared it to CS. They found that both CS and WPS exposure resulted in elevation of total white blood cell count, proinflammatory markers (TNF-α and IL-6) in BAL fluid, and oxidative stress markers, including GPx activity in lungs (15). Presently, we aimed at assessing the effects of nose-only WPS exposure (30 min daily, 5 days/wk, for 1 mo) on various respiratory end points, including lung inflammation (histology and BAL fluid analysis), proinflammatory cytokines (IL-6, TNF-α, and GM-CSF), oxidative stress (LPO, GST, GSH, and SOD), and their possible impact on a relevant physiological end point, namely airway resistance in vivo.

Our data show that 1 mo exposure to WPS causes pulmonary inflammation evidenced by interstitial infiltration of inflammatory cells, including neutrophils and lymphocytes, and an increase of neutrophil and lymphocyte numbers in BAL fluid. Moreover, while the increase of GM-CSF did not reach statistical significance, we found an increase of both IL-6 and TNFα in BAL fluid. An increase of neutrophil and lymphocyte numbers along with IL-6 and TNFα was observed in BAL fluid of mice exposed 1 h daily for 7 days to WPS in a whole body exposure system (15). With respect to CS, similar observations have been reported in mice exposed 1 h daily for 7 days to WPS in a whole body exposure system (15). With respect to CS, similar observations have been reported in mice exposed 3 mo to CS in a nose-only exposure system (36). Likewise, whole body CS exposure for 4 wk was reported to cause an influx of inflammatory cells in the lung, including neutrophils and lymphocytes (7, 14). Non-smoking emphysematous patients exhibit increased circulating TNF-α and IL-6 concentrations compared with those of healthy never-smoker males, and these patients show a reduction in both cytokine concentrations after resection of inflammatory emphysematous tissue by lung volume reduction surgery (20). This finding suggests that TNFα and IL-6 are key cytokines in the perpetuation of cigarette smoke-induced inflammation even after smoking cessation. Others have previously shown that TNF-α drives the majority (~70%) of cigarette smoke-related emphysema in the mouse (9).

To evaluate pulmonary function in mice, noninvasive, unrestrained whole body plethysmography allows easy and repeatable measurements in mice without anesthesia, but it has been criticized by many authors as being an inadequate method to measure mechanical lung function (4, 17, 32). Despite its invasiveness, evaluation of lung and airway mechanics with the forced-oscillation technique provides precise information, and physiological variables can be measured accurately (10, 41). We have recently demonstrated that short-term exposure to particulate air pollution (25, 29) or CS (27) causes an increase of airway resistance. Our data demonstrate that 1 mo exposure to WPS caused a significant and a dose-dependent increase of airway resistance. This novel finding corroborates the human studies that reported impairment of pulmonary function and respiratory symptoms in WP smokers (6). Also, clinical studies reported a significant decrease in the FEV₁ (33), respiratory rate, PEFR, and FEF₂₅–₇₅% (12) in the WPS group compared with the nonsmokers.

Tobacco smoke contains many oxidants and free radicals and organic compounds, such as superoxide and nitric oxide (5). In addition, inhalation of tobacco smoke into the lung also activates endogenous mechanisms, such as accumulation of neutrophils and macrophages, which further increase the oxidant injury (5). Indeed, oxidative stress and airway inflammation together form a vicious cycle that is responsible for the disease progression in patients with COPD (2). Oxidative stress results from an imbalance between radical-generating and radical-scavenging systems leading to cell membrane impairment or DNA damage (40, 46). To further evaluate the mechanism underlying the increase of airway resistance following WPS exposure, we measured the degree of oxidative stress by determining LPO levels in lungs. Our data show that WPS exposure causes a significant increase in LPO in lung

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Fig. 6. Reduced glutathione (GSH, A) levels, and superoxide dismutase (B) and glutathione S transferase (GST, C) activities in lung tissues at the end of the 1-mo exposure period to air (control) or WPS. Data are means ± SE (n = 8).

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Fig. 7. Carboxyhemoglobin (COHb)% in venous blood at the end of the 1-mo exposure period to air (control) or WPS. Data are means ± SE (n = 6).
tissue. Our results are in line with experimental (27) and clinical (21) studies that reported that CS exposure increase LPO levels. Unexpectedly, Hakim et al. (12) found a decrease in 8-isoprostane levels after WPS in healthy volunteers. The authors explained their findings by the high levels of carbon monoxide, a potent anti-inflammatory agent, observed in WP smokers (12).

Moreover, we have presently measured the concentration of the free radical scavenger GSH and the activities of two antioxidant enzymes (GST and SOD). Our data show a significant decrease in GSH and inhibition of the activities of these antioxidant enzymes after WPS exposure. Two major mechanisms may have played a role in the decrease of these antioxidant enzymes activities (5, 31). The first possible mechanism involves consumption of antioxidant during the breakdown of free radicals and the high level of H2O2 or the inhibition of the enzyme by these radicals, while the second involves the direct inhibition of GSH synthesis and activities of SOD and GST by WPS. An increase of GPx and SOD has been reported following 7-day exposure to WPS in mice (15). We have also recently demonstrated that short-term (4 days) exposure to CS causes an increase of antioxidant enzymes including GST, SOD, and GSSG (27). However, in the present study, the decrease of GSH and inhibition of antioxidant enzymes observed in 1–mo post WPS exposure suggests an increase in oxidative stress and possible depletion of antioxidant enzymes caused by a longer exposure period of time to WPS.

In the present study, the exposure session to WPS lasted 30 min. The latter is similar to the exposure session reported in the human study (12). We measured the levels of COHb, which is a useful marker for tobacco smoke absorption. Our results showed that COHb% increased significantly in mice exposed to WPS (37.3 ± 3.3%) compared with those exposed to air (1.9 ± 0.1%). The levels of COHb observed in our study are higher than those reported in human subjects (12, 47). COHb concentrations of 9.49 ± 5.52% were reported in humans by Hakim et al. (12) following 30 min of WPS compared with before WPS 1.4 ± 0.5%. In the latter study (12), it was reported that the post-WPS COHb concentration was 10–15% in 10 subjects, 15–20% in one subject, and >20% in three subjects (≤26%). Zahran et al. (47) showed that levels of COHb of sheesha smokers following 10–40 min of WPS (10.0 ± 2.5%) were distinctly different from those of both cigarette smokers (6.4 ± 2.7%) and nonsmokers (1.6 ± 0.7%).

We conclude that 1 mo nose-only exposure to WPS causes airway inflammation and increases airway resistance through release of proinflammatory cytokines and oxidative stress. Additional studies are required to further investigate the pathophysiological mechanisms underlying the effects of WPS and to compare the short-term and long-term effects of WPS, and to study the effects of additives that are supplemented to tobacco added in shisha. Our results provide a plausible mechanism explanation for the limited clinical studies which reported that WPS is harmful.

REFERENCES


