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Changes in the Expression and Protein Level of Matrix Metalloproteinases after Exposure to Waterpipe Tobacco Smoke

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Abstract

Waterpipe smoking has become a worldwide epidemic with health consequences that only now are beginning to be understood fully. Because waterpipe use involves inhaling a large volume of toxicant-laden smoke that can cause inflammation, some health consequences may include inflammation-mediated lung injury. Excess matrix metalloproteinase expression is a key step in the etiology of toxicant exposure-driven inflammation and injury. In this study, changes in the level and mRNA of major matrix metalloproteinases (MMP-1, -9 and -12) in the lungs of mice following exposure to waterpipe smoke were investigated. Balb/c mice were exposed to waterpipe smoke for one hour daily, over a period of two or eight weeks. Control mice were exposed to fresh air only. ELISA and Real-Time PCR techniques were used to determine the protein and mRNA levels of MMP1, 9 and 12 respectively in the lungs. Our findings showed that MMP1, 9 and 12 levels in the lung significantly increased after both two ($P < 0.05$) and eight weeks ($P < 0.01$) exposures. Similarly, RT-PCR findings showed that mRNA of those proteinases significantly increased following two ($P < 0.01$) and eight weeks ($P < 0.001$) exposures. In conclusion, waterpipe smoking is associated strongly with lung injury as measured by elevation in the expression of MMPs in the lung tissue.

Introduction

Cigarette tobacco smoke contains thousands of compounds, many of which are known to cause lung cancer and other serious chronic cardiovascular and pulmonary diseases

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(Stampfli and Anderson 2009). Not surprisingly, tobacco smoking is one of the most common cause of mortality (Samet 2013). Waterpipe smoking is a tobacco use method in which smoke generated from a flavored, charcoal-heated tobacco preparation passes through a water bubbler before it is inhaled by the user. The prevalence of this method of smoking has greatly increased in the past two decades in most regions of the world, especially among youth (Khabour et al. 2012b, Jawad et al. 2013). One factor contributing to this trend may be the lack of knowledge about the potential health consequences of waterpipe smoking.

Contrary to common perceptions that the water bubbler renders the smoke safe for consumption, a recent review by Shihadeh et al. (2015) has found that waterpipe smoke contains large quantities of many of the compounds associated with numerous diseases in cigarette smokers, ranging from less than one to hundreds of cigarette equivalents in typical use session (Table 1). Notably, the review found that only 82 chemical compounds (including 27 known or suspected carcinogens) have been quantified in waterpipe smoke to date, and approximately 300 compounds identified. These numbers represent a small fraction of the 9600 compounds quantified and/or identified in cigarette smoke to date (Rodgman and Perfetti, 2013). Thus comparatively little is known about the chemical composition of waterpipe smoke, and the degree to which it may resemble cigarette smoke. Even less is known about its potential health effects (El-Zaatari et al. 2015). This study focuses on smoke exposure-induced inflammation in the lung, a key step in the etiology of lung disease in cigarette smokers (Morris et al. 2008).

Inflammatory response is mediated by the release of chemokines, cytokines, interferones and enzymes such as metalloproteinases (MMPs) (Segura-Valdez et al. 2000, Grumelli et al. 2004, Morris et al. 2008). Elevated MMPs expression in the lungs of cigarette smokers leads to excessive degradation of the extracellular matrix (ECM), which in turn produces fragments that act as chemo-attractants and which recruit neutrophils and macrophages (Churg et al. 2003). As a consequence of this closed cycle of degradation of ECM, recruitment of more inflammatory cells and extensive obstruction and lung parenchymal cell degradation occur (Butler and Overall 2013).

While changes in MMP expression appear to be a sensitive biomarker of harm resulting from cigarette smoke exposure (Selman et al. 2003, Bracke et al. 2005, Morris et al. 2008, Moon et al. 2014), such changes have not been investigated previously for the case of waterpipe tobacco smoke. In this study, changes in the level and mRNA expression of major matrix metalloproteinases (1, 9 and 12) in the lungs of mice following two or eight weeks of exposure to waterpipe smoke were investigated.

Materials and Methods

Animals

Naïve Balb/c mice were used in this study. Animals were placed randomly into the following groups (n=12 per group, weight: 21.91 ± 1.56 gram): two weeks control, eight weeks control, two weeks waterpipe and eight weeks waterpipe. Waterpipe animals were exposed to waterpipe smoke for one hour daily for 2 weeks or 8 weeks. Control animals were exposed to fresh air drawn from a waterpipe that was not used for smoking before. Mice

were acquired from the Animal Care Facility at Jordan University of Science and Technology (JUST), and were maintained in the same facility. Mice were maintained in standard cages (n=3-5 per cage) and constant temperature ($24 \pm 1^{\circ}\text{C}$) with a 12 h light/ 12 h dark cycle, and were provided with ad libitum access to food and water. Animals' general body health was monitored weekly during the experiment. All procedures were approved by the Animal Care and Use Committee of JUST.

Whole Body Smoke Exposure

A whole-body waterpipe smoke exposure system was used as previously described (Khabour et al. 2012a). Briefly, this system consists of an automated positive pressure smoking machine coupled to an animal exposure chamber. The smoking machine draws smoke from a lit waterpipe in accordance with a standard 171-puff, 1 hour duration puffing regimen using the Beirut method (Katurji et al. 2010) and discharges it entirely into a 24 L exposure chamber housing the animals. The chamber is ventilated continuously by a manually controlled fresh air supply and is instrumented with a CO monitor. Fresh air flow into the chamber was controlled to maintain a constant CO concentration throughout each exposure session (Khabour et al. 2012a). Ten grams of Two Apples flavor, Nakhla brand tobacco preparation were loaded in the waterpipe prior to each exposure session. Shaban brand quick-light charcoal briquettes were used as the heat source and were replaced during exposure when necessary. The waterpipe bowl was drained and filled with 700 ml of tap water each session.

Determination of MMPs protein levels

Animals were sacrificed with Thiopental (40 mg/kg, I.P.) at the end of the treatment. Lungs were removed directly from each animal, washed with ice-cold normal saline, sliced on ice-cold filter papers, weighted in 1.5 mL cold tubes and immediately frozen in liquid nitrogen. Frozen samples were stored at -80°C degree until the time of examination.

Lung tissues were homogenized in ice-cold phosphate buffered saline supplemented with SIGMAFAST™ protease inhibitor cocktail (Sigma, USA) using sterile plastic pestles that accommodate 1.5 mL tubes. Homogenate was then freeze-thawed two times to facilitate destruction of cellular membranes, and centrifuged at $5000 \times g$ for 5 minutes at 4°C . Aliquots of the supernatant was prepared and stored at -20°C . Total protein concentration of each sample was measured using modified Lowry protein assay protocol as previously described (Khabour et al. 2010). MMP-1, -9 and -12 proteases in lung tissue homogenate were quantified using Enzyme-Linked Immunosorbent Assay kits (CUSABIO, Wuhan, China) specific to each type of protease, according to the manufacturer's instructions. Absorbance was measured at 450 nm using an Epoch Biotek microplate reader (BioTek, Winooski, VT, USA). Concentrations of different MMPs in the samples were determined from the standard curve and the software supplied with the plate reader. Samples were assayed in duplicates in a blind manner.

Gene expression Assay test

Level of expression of MMP-1, -9 and -12 mRNA in mouse lung tissues was determined using real time PCR (RT-PCR) as follows. RNA was extracted from frozen lung tissues

using total RNA purification kit as described by manufacturer (Norgen's RNase-free DNase I Kit, Norgen Biotek Corp, Ontario, Canada). In brief, ice-cold lysis buffer was added to frozen tissue (600 uL lysis buffer/ 10 mg of tissue). Tissue was grinded on ice using cooled DEPC-treated plastic sticks that completely fit the shape of 1.5 mL tube. Tissue lysate was transferred to an RNase-free tube, then, it was centrifuged for two minutes at 14000 ×g at 4°C. Resulted supernatants were transferred to RNase-free tubes, mixed with 70% ethanol and RNA was isolated using RNA binding columns provided by the kit. Concentration of purified RNA was measured using NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA). Thermo Scientific RevertAid First Strand cDNA Synthesis Kit was used to convert the purified RNA to its complementary cDNA as described by the kit instructions. The resulting reverse transcription reaction products were analyzed using the same NanoDrop described above. Real-time PCR was carried out using the following mouse TaqMan gene expression assays (Applied Biosystems, Wilmington, DE, USA): MMP-1: 4351372, MMP-9: 4331182, and MMP-12: 4331182. The RT-PCR conditions were as recommended by the manufacturer. Equal amounts of cDNA were used in each reaction, and each reaction was run in triplicate for each sample. Amplification and analysis were performed using BIO-RAD CFX384 Touch™ Real-Time PCR Detection System (Hercules, Ca, USA). Changes in the expression of MMPs were performed using Ct method. Samples were assayed in triplicates in a blind manner.

Statistical Analysis

Data were analyzed using GraphPad Prism software, version 6 (La Jolle, Ca, USA). One-Way ANOVA and Tukey's test were used to make multiple comparisons between different treatment groups. Kruskal-Wallis followed by Dunn's test was performed to analyze data collected from the Real-Time PCR technique. P value < 0.05 was considered significant. Since no statistical differences were detected in MMPs mRNA and protein levels between 2 weeks and 8 weeks control groups, the two groups were merged together as one group (control group) to simplify the analysis.

Results

No obvious changes in general body health of the animals were observed during the experimental period. The average carbon monoxide exposure for the two weeks group was 1066 ± 70 PPM, whereas for eight weeks waterpipe group it was 1096 ± 57 PPM.

Protein expression

The 2 weeks exposure of animal group demonstrated significant elevation in the levels of MMP-1 and MMP-12 in lung tissue (Figure 1A and 1C, $P < 0.05$). In addition, an increase in the level of MMP-9 was detected after eight weeks exposure but this increase was not statistically significant ($P > 0.05$). On the other hand, exposure of mice to waterpipe smoke for 1 hour per day for 8 weeks (chronic exposure) significantly induced increase in all examined MMPs (Figure 1, $P < 0.05$).

mRNA expression

The results showed significant increases in the levels of MMP-1,-9 and -12 mRNA after both two and eight weeks of exposure (Figure 2, $P < 0.05$). The magnitude of increase was greater in the lungs of the eight weeks – waterpipe exposed group compared to the two weeks exposed group (Figure 2, $P < 0.05$).

Discussion

The association between waterpipe smoking and lung injury was investigated by examining changes in the levels of MMPs proteases in lungs of mice after exposure to waterpipe smoke. The results showed that the expression of MMP-1, MMP-9 and MMP-12 proteins and mRNA was elevated significantly after two and eight weeks of exposure to waterpipe smoke.

These findings are similar to those from previous studies of the effects of cigarette smoke on MMP expression (Selman et al. 2003, Bracke et al. 2005, Morris et al. 2008, Moon et al. 2014). For example, 3 days exposure of mice to cigarette smoke using whole-body exposure induced marked elevation in MMP-9 (Morris et al. 2008) and MMP-12 (Leclerc et al. 2006) proteins in the lung. Using guinea pigs, Selman et al., (2003) demonstrated that MMP-1 and MMP-9 activities were elevated in BALF of animals after exposure to cigarette smoke for one, two and four months.

Consistent with the results of the protein expression assays, the realtime PCR data showed strong elevation in the expression of MMP-1, -9 and -12 mRNA after exposure to waterpipe smoke. These results also are supported by literature from cigarette where exposure to tobacco cigarette smoke has been shown to elevate MMP mRNA levels in the lungs of mice and rats (Bracke et al. 2005, Marumo et al. 2014). Additionally, human studies showed elevations of MMPs mRNA in cells derived from the lungs (Atkinson et al. 2011). Thus, similar to cigarette smoking, waterpipe smoking induces expression of MMPs in the exposed lungs.

Previous work that examined health effects of waterpipe smoke on lung showed that exposure of mice to one hour of waterpipe smoke for 7 days using a whole body exposure system induced elevation in BALF neutrophils, macrophages, and lymphocytes as well as TNF- α and IL-6. In addition, changes in the lung oxidative stress markers such as glutathione peroxidase and catalase were also observed (Khabour et al. 2012a). Similar findings were also reported by Nemmar et al., (2015) using nose only exposure in mice for 30 min per day for 5 days. Elevation of MMPs expression after exposure to waterpipe smoke for 2 weeks observed in the current study confirms the inflammatory effects of this type of smoking after short-term exposure for two weeks. In addition, the high magnitude of change in the expression of MMPs by 8 weeks exposure suggests that severity of lung injury increases with duration of use. These results suggest that elevation of metalloproteinases might play an important role in lung inflammation induced by waterpipe smoking via degradation of the extracellular matrix, leading to a greater inflammatory response (Churg et al. 2003). Taken together, these data suggest strongly that regular waterpipe use can lead to the same inflammation-mediated lung diseases as are associated with cigarette smoking,

namely chronic obstructive pulmonary disease and lung cancer (Patel et al. 2008, Al Zaabi et al. 2011, Waked et al. 2011, Tageldin et al. 2012, Hawari et al. 2013, She et al. 2014).

Limitations of this study include the possibility that inflammatory responses to waterpipe smoke exposure in humans may be different than in mice. In addition, response in humans could differ according to sex, age, and smoking behavior of waterpipe smoker. Therefore, more studies are required that examine such changes in cells derived from human waterpipe smokers. Furthermore, only CO was monitored during exposure to waterpipe smoke while total particulate matter was not measured. Monitoring of total particulate matter as a parameter of exposure to smoke in future studies will enable establishing stronger correlations between waterpipe tobacco use and lung inflammation biomarkers.

In conclusion, an association between waterpipe smoking and changes in expression and protein levels of MMPs was found, suggesting that waterpipe tobacco smoking may induce inflammatory response in the lungs of smokers.

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Figure 1. Levels of MMPs in Balb/C mice lung tissue after two and eight weeks using whole body waterpipe smoke exposure

Histograms show fold change in levels of MMPs protein in Balb/C mice lungs of after treatment. Control animals were exposed to fresh air only, waterpipe groups were exposed to waterpipe smoke (1 hr/day) for 2 weeks or 8 weeks. Level of expression of MMP1, 9, and 12 was assessed by ELISA. Waterpipe smoke exposure induced significant changes in the levels of MMPs proteins. *, $P < 0.05$; **, $P < 0.01$; compared to control group.

Figure 2. Real-Time PCR assessment for changes in expression of MMP 1, 9, and 12 mRNAs in lungs of Balb/c mice

Histograms show fold change in levels of MMPs mRNA in lungs of Balb/C mice after exposures. Controls were exposed to fresh air only, waterpipe groups were exposed to waterpipe smoke (1 hr/day) for 2 weeks or 8 weeks. Level of expression of MMP1, 2, and 12 mRNAs was assessed by Real-Time PCR. Waterpipe smoke exposure induced significant changes in the levels of mRNA of MMPs. *, $P < 0.05$; **, $P < 0.01$; compared to control group.

Table 1

Nicotine, carbon monoxide, nitric oxide, and yields of measured known carcinogens (IARC Class 1) found in waterpipe tobacco smoke when measured using the Beirut Method. Data for cigarette smoke shown for comparison. Data extracted from Shihadeh et al. (2015).

Toxicant yield in mainstream smoke	Single cigarette	Waterpipe use session
Tar/Nicotine/CO/NO		
nicotine, mg	0.1-3	>0.01-9.29
CO, mg	14-23	5.7-367
nitric oxide, mg	0.100-6.00	0.325-0.440
Volatile organic compounds		
benzene, µg	20-70	271
Volatile aldehydes		
formaldehyde, µg	20-100	36-630
Tobacco-specific nitrosamines		
NNK, ng	80-770	LOD-46.4
NNN, ng	120-3700	34.3
Primary aromatic amines		
2-naphthylamine, ng	1-334	2.84
Polycyclic aromatic hydrocarbons		
Benzo[a]pyrene, ng	20-40	ND-307
Heavy metals		
Chromium, ng	4-70	250-1,340
Arsenic, ng	40-120	165
Beryllium, ng	0.5	65