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In vitro Cytotoxicity and Mutagenicity of Mainstream Waterpipe smoke and its Functional Consequences on Alveolar Type II Derived Cells

Mayyasa Rammah*, Farah Dandachi†, Rola Salman‡, Alan Shihadeh**, and Marwan El-Sabban††

*Department of Anatomy, Cell Biology and Physiological Sciences, Faculty of Medicine, American University of Beirut, Beirut 1107 2020. mr37@aub.edu.lb †Department of Anatomy, Cell Biology and Physiological Sciences, Faculty of Medicine, American University of Beirut, Beirut 1107 2020. farahdandashi@yahoo.com ‡Department of Mechanical Engineering, American University of Beirut, Beirut 1107 2020. rs107@aub.edu.lb **Department of Mechanical Engineering, American University of Beirut, Beirut 1107 2020. as20@aub.edu.lb

Abstract

Introduction—While waterpipe tobacco smoking has become a global phenomenon, its potential health consequences are poorly understood. In this manuscript, we report the in-vitro mutagenicity of waterpipe smoke condensate (WSC), the alteration in cellular parameters of lung alveolar cells in response to WSC exposure and discuss the implication of cellular responses in the pathophysiology of chronic obstructive pulmonary disease (COPD).

Methods—The mainstream WSC was generated using a standard laboratory machine protocol. We assessed its mutagenicity using Ames test. In addition, we studied the effect of WSC on the proliferation and cell cycle of alveolar type II cells and vascular endothelial cells. We also assessed the effect of WSC on the expression of genes involved in cell cycle arrest and inflammation.

Results—Within the range of tested doses, WSC did not elicit sufficient response to be considered mutagenic in any of the strains tested (TA98, TA100, TA102, and TA97a) but were found to be toxic for strains TA97a and TA102 at the highest tested doses. However, WSC induced cell cycle arrest and cellular senescence mediated by the p53-p21 pathway. Also our study indicated that WSC induced an increase in the transcriptional expression of matrix metalloproteinases, MMP-2 and MMP-9 and an immune response regulator, Toll Like Receptor-4.

Conclusion—The data reported here represent the first in vitro demonstration of the effect of waterpipe smoke on cellular parameters providing evidence of the potential involvement of WPS in the pathogenesis of COPD through impairing cellular growth and inducing inflammation.

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††corresponding author: Department of Anatomy, Cell Biology and Physiological Sciences, Faculty of Medicine, American University of Beirut, Bliss Street, P.O.Box: 11-0236, Riad El-Solh 1107 2020, Beirut-Lebanon, me00@aub.edu.lb, Tel: +961-1-350000, FAX: +961-1-3500004913.

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Declaration of Interests

None declared.

Keywords

waterpipe smoke; mutagenicity; cytotoxicity; inflammation; alveolar lung cells; COPD

1. Introduction

Waterpipe (also known as narghile, argile, shisha, hookah, goza, hubble bubble) smoking (WPS), a form of tobacco smoking, is widespread in the Middle East and is rapidly spreading globally (Azab et al., 2010; Combrink et al., 2010; Dar-Odeh et al., 2010; Dugas et al., 2010; Johnston et al., 2011; Primack et al., in review; Taha et al., 2010). Additionally, WPS is becoming prevalent among adolescents (Maziak, 2011). One reason for its spread may be the fact that this form of tobacco use is often overlooked in tobacco control policy, which, in turn, may stem from the limited evidence available about its health effects.

While the health effects of cigarette smoking have been studied for many years, there have been comparatively few reports on the health effects of WPS. Evidence from systematic reviews states that WPS likely increases the risk of bronchogenic carcinoma, lung, oral, and bladder cancers as well as causes low birth weight (Akl et al., 2010). The assumption that WPS is associated with cancer is not surprising, given that, recent studies on the chemistry of waterpipe smoke demonstrate that it contains carcinogens. Sepetdjian et al. (2008) identified 16 polycyclic aromatic hydrocarbons (PAH) in the mainstream smoke of waterpipe including Benzo[a]pyrene. Furthermore, Al-Rashidi et al. (2008) detected significant amounts of formaldehyde in the mainstream smoke of waterpipe. Schubert et al. (2011) also found that waterpipe smoke contains carcinogenic tobacco specific nitrosamines. Recent studies on human subjects have addressed the genotoxic effects of waterpipe smoking (El-Setouhy et al., 2008; Khabour et al., 2011; Yadav and Thakur, 2000). In addition, evidence that waterpipe smokers are systemically exposed to carcinogens was recently provided by Benowitz's group who showed that carcinogen metabolites showed up in the urine after a single waterpipe use. Thus not only do we know that the waterpipe smoke contains carcinogens, but also that waterpipe smokers are systemically exposed to them (Jacob et al., 2011).

In addition to the genotoxic effect of WPS, some studies evaluated the effect of WPS as a risk factor for chronic obstructive pulmonary disease (COPD). COPD is the most common lung disease and is a leading cause of death. COPD, caused by noxious particles or gas, most commonly from tobacco smoking, triggers several pathogenic processes such as inflammation, alterations of cell growth, cellular apoptosis, abnormal cell repair, extracellular matrix destruction, and oxidative stress. These processes are mediated by a growing number of molecules, many of which affect more than one pathogenic process (Yoshida and Tuder, 2007). A recent review by Raad et al. (2011) suggests a possible role of WPS in the pathogenesis of COPD, thus illustrating that WPS may be as harmful as cigarette smoking in the development of COPD. Moreover, a recent epidemiological study in Lebanon determined an association between COPD prevalence and waterpipe smoking (Waked et al., 2011).

Since all evidence for the genotoxic and health effects of WPS was generated from studies done on human subject, there is a need for more research to identify the causal relationship between WPS and clinical outcomes. The first purpose of this study is to provide basic information regarding the mutagenicity and cytotoxicity of mainstream waterpipe smoke condensate (WSC) generated using "ma'ssel", a highly popular form of tobacco smoking. Additionally, since smoking is a risk or a causative agent in chronic obstructive pulmonary disease and cardiovascular disease, we studied its effect on A549 cells - alveolar type II cells

commonly used as a model for lung epithelial cells (Smith, 1977), and ECV, an endothelial cell line. The second purpose of this paper is to evaluate the alteration in the cellular profile of A549 in response to WSC exposure and discuss the implication of certain cellular responses in the pathophysiology of COPD. Our study represents the first in vitro demonstration of the effect of waterpipe smoke on cellular parameters and suggests a possible mechanism of cellular responses to waterpipe smoke insult.

2. Materials and Methods

2.1 Smoking machine protocol and WSC preparation

A standard smoking protocol (Beirut Method) was used as described by Shihadeh et al. (2005) and Katurji et al. (2010), which consists of a total of 171 puffs of 0.53 L volume, a puff duration of 2.6s, and an inter puff interval of 17s. The Waterpipe was prepared by filling the head with 10g of “*Nakhla*” brand tobacco mixture known as “two apples”, covering it with aluminum foil and perforating the foil to allow air passage. A charcoal, *Three Kings* brand quick-light briquette was ignited and placed on the top of the head at the beginning of the smoking session. Another half charcoal briquette was added at puff number 105. Water in the water bowl was changed at the beginning of every smoking session.

For the Salmonella reverse mutagenicity assay, smoke condensate was collected using a concentric tube electrostatic precipitator (ESP, In-Tox Products) operating at 6600 V. The ESP was used to collect condensate for the salmonella reverse mutagenicity assay because it allowed concentration condensate concentration of 100 mg/ml, which was unachievable by the standard method of collection on glass fiber pads. The ESP method showed comparable amounts of TPM and PAH as collected on filter pads, consistent with previously published data from our group (Sepetdjian et al., 2008). In addition, condensate collected using the ESP and the glass filter pads at the same concentration was tested on A549 cells and showed equivalent effect on viability and proliferation.

The ESP collection tube was capped and weighed at the beginning and the end of each run to measure the amount of condensate collected. A backup filter (Pall Type A/E glass fiber) was placed downstream of the ESP to trap particulate matter, if any, that was not collected by the ESP. Additionally, the setup allows the collection of a fraction of the gaseous phase (5.19 L of a total of 90.4 L drawn), in a Teflon bag to calculate the weight of Carbon monoxide CO produced in each session. Heads and Charcoal were weighed at the beginning and the end of each session to measure the weight of charcoal and tobacco that has burned. Based on the weight of TPM collected in the ESP, DMSO (Dimethyl Sulfoxide, Sigma) was added to yield a final concentration of 100mg/ml in case of waterpipe. The ESP tube was capped, shaken well and then the content poured into a 50ml conical (Corning), sealed tube wrapped on the outside with aluminum foil to prevent light from degrading the sample. A total of 5 smoking sessions were conducted for this assay. 10 ml WSC from each session was pooled with the rest, aliquoted and stored at -80°C until the day of the assay.

Cigarettes, Marlboro Red- purchased at retail outlets adjacent to the American University of Beirut, were smoked using the same smoking machine, with the same setup of ESP. The smoking regimen used was the MDPH (Massachusetts Department of Public Health) protocol. Smoking was stopped 1mm before reaching the filter. Cigarette smoke condensate was prepared as described for the WSC, to yield a final concentration of 10mg/ml. A total of 5 cigarettes were smoked consecutively with the TPM of all five collected together in the ESP.

For testing the effect of WSC on human cells, the condensate was collected using Pall Type A/E glass fiber filters as described by Shihadeh and Saleh (2005). Filters were stored in air-

tight containers and at -20°C . Cell culture incomplete media (without FBS) was added to each filter to yield a concentration of 40mg/ml, the filter was then pressed in a syringe to ensure recovery of media added. All recovered media was then mixed together, sterilized using 0.22 μm filters (Costar) and stored at -20°C for not more than one month.

2.2 Ames Mutagenicity Assay

The potential mutagenic activity of WSC was investigated using the Salmonella Reverse Mutagenicity Assay. A salmonella/microsome reverse mutagenicity assay kit was purchased from Moltox, Inc. The plate incorporation method was employed using Salmonella typhimurium tester strains TA97a, TA98, TA100, and TA102 in the presence and absence of an external metabolic activation system S9. Liver homogenate (S9) was prepared from adult male Sprague-Dawley rat induced with Aroclor 1254 according to the method of Ames et al. (1975). The concentration of S9 in the S9 mix was 5% (V/V). The assay was conducted as described by Maron and Ames (1983), with the pre-incubation method described by Yahagi et al. (1975). The WSC was diluted with DMSO to 10 doses: 10000, 7500, 5000, 2500, 1000, 500, 250, 100, 50, and 25 μg TPM/plate. Three independent experiments were performed each with triplicate plates of each concentration.

To confirm sensitivity of the test organisms, concurrent positive and negative controls were tested in the assay. Negative controls included solvent control (DMSO) and the wash obtained by rinsing an empty ESP aluminum tube with DMSO. Positive controls included specific controls for each strain. Table 2 and 3 describes the list of chemicals tested in each strain of bacteria with or without metabolic activation. Additionally, compounds that are found in cigarette smoke condensate (CSC) that are known to cause mutagenicity as well as Cigarette smoke condensate collected were included in the assay (Table 4).

2.3 Cell Culture, Cytotoxicity and Proliferation Assays

A549 cells were grown in DMEM high glucose (4.5g/l) culture media, while ECV-304 cells were grown in RPMI 1640 (Lonza). Both culture media were supplemented with Penicillin G 100 U/ml and Streptomycin 100 μg /ml (Gibco), and 10% Fetal Bovine Serum (Sigma). Additionally, A549 cell culture media was supplemented with Sodium Pyruvate (Lonza).

For the cytotoxicity and proliferation assays, cells were seeded at a density of 10^4 cells/cm². Treatment started 24 hours post-seeding. For the cytotoxicity assay, treatment was performed for 24 hours by mixing extract and complete media to the desired final concentration (0.5, 1, 3, 4, 6 and 8 mg/ml). For the proliferation assay, the cells were treated with a final concentration of 4 mg/ml. This treatment was either repeated daily for 3 consecutive days (Repeated Exposure), or was done only once and was replaced with fresh complete media 24 hours later (Single Exposure). The cells were counted every 24 hours using the Trypan blue dye to differentiate between dead and live cells.

2.4 Cytotoxicity Assay by Real-time cell impedance analysis

To validate our previous cytotoxicity and proliferation results, Real-Time Cell Analyzer (RTCA) xCELLigence System (Roche Applied Science, Mannheim, Germany) was used to dynamically monitor cell proliferation rates. The system monitors cellular events at set intervals via measuring electrical impedance across microelectrodes on the bottom of tissue culture E-plates. The impedance measurement provides information about cell number, viability, morphology and adhesion. The RTCA Software supplied by the manufacturer analyzes these measurements and calculates the doubling time of the cells based on Cell Index (CI). A549 cells were seeded at a density of 7000 cells/well on an E-Plate. Treatment started 24 hours post-seeding by mixing extract and complete media to the desired final concentration (0.5, 1, 3, 4, 6 and 8 mg/ml). Dynamic CI values were monitored in 45-min

intervals for 24 hours after treatments. The results are presented as doubling time. Standard deviations of duplicates of wells with different treatments were analyzed with the RTCA Software.

2.5 Nuclear Staining and Cell Cycle Analysis

Cells were seeded in 12 well plates containing cover slips at a density of 10^4 cells/cm². Cells were either treated daily for 3 consecutive days (Repeated Exposure), or were treated only once and then replaced with fresh complete media 24 hours later (Single Exposure). Cells were then washed with 1× PBS (Phosphate Buffered Saline, pH=7.4) and fixed with ice-cold absolute methanol solution. The cover slips were then incubated with Hoechst 33342 (10 mg/ml) (Molecular Probes, Eugene, OR, USA) at a dilution of 1/10,000 for 10 minutes, then washed twice with PBS for 10 minutes each. The cover slips were then mounted on glass slides using Prolong Antifade®. Images were taken using a laser scanning confocal microscope (LSM-710, Zeiss, Germany).

Analysis of the cell cycle was carried out by flow cytometry using propidium iodide stain (Molecular Probes, Eugene, OR, USA). The cells were plated in 6-well plates at a density of 10^4 cells/cm². The cells were either treated once or repeatedly for 3 consecutive days. DNA content was conducted as described by Bazarbachi et al. (1999) and then analyzed by flow cytometry (Beckton Dickinson, FacSort) while Data acquisition was performed using Cell Quest. Data analysis was performed using Flow Jo, applying the Watson Pragmatic Model for fit analysis.

2.6 Annexin V/PI staining by flow cytometric analysis

Detection of early apoptotic cells was performed with the annexin V-FITC (Roche Diagnostic, IN, USA) in combination with propidium iodide by flow cytometry. Briefly, cells were plated in 6-well plates at a density of 10^4 cells/cm². The cells were either treated once or repeatedly for 3 consecutive days. Treated and untreated cells were trypsinized, spun down at 100g, re-suspended in PBS, washed twice, and then incubated with FITC-conjugated annexin V and PI for 15 min at 20°C in a Ca²⁺-enriched binding buffer in the dark for 15 minutes. They were immediately analyzed on the flow cytometer (Beckton Dickinson, FacSort) in their staining solution. For each sample, data from 10,000 cells were recorded on logarithmic scales. Data analysis was performed using Flow Jo software on cells characterized by their forward/side scatter (FSC/SSC) parameters. Samples stained with Annexin V-FITC and PI, are represented by dot plots of PI versus Annexin V intensity. Such dot plot is divided into four regions of cells where the lower left region includes cells that stain negatively for both Annexin V and PI and are considered undamaged; the lower right region includes cells stained with Annexin V but are still PI negative and are considered early apoptotic; the upper right includes cells stained with Annexin V and PI and are classified as late apoptotic or necrotic; and the upper left region includes cells that are both Annexin V negative but PI positive and are dead cells.

2.7 Western Blot Analysis

Expression of proteins regulating cell cycle was analyzed by western blot. Cells grown in different conditions were lysed using 2x sample buffer (126 mM of TRIS/HCL, 20% glycerol, 4% SDS, 0.02% Bromophenol blue). Protein quantification was performed using BioRad DC quantification kit according to the manufacturer's instruction. 50 µg of total lysate were separated on 12% SDS page gels. Proteins were transferred to an activated PVDF membrane. Primary antibodies against Actin (Sigma, A-2066), p21 (Santa Cruz, biotechnology sc-397), p53 (B.D Pharmingen 14091A), phosphor-p53 (Ser15) (cell signaling, 92845) and Phospho-Histone H3 (Ser10) (H3P) antibody (cell signaling, 9701),

were used with adequate HRP conjugated secondary antibodies. Films, exposed in the linear range, were then analyzed using Image J®. All bands were normalized against actin.

2.8 Immunofluorescence

A549 cells were grown, treated and fixed as mentioned in section 2.5 for immunolabeling. Non-specific antigen sites were blocked with 3% normal goat serum (NGS) (Santa Cruz Biochemicals, Santa Cruz, CA) in PBS for 1 hour. Slides were incubating with rabbit anti-trimethyl-Histone H3 (lys9) primary antibody (Millipore, 07-442) of 1 mg/ml concentration diluted 1:500 in PBS containing 1% NGS overnight at 4°C. Slides were rinsed and incubated for 1 hour with a fluorescent goat anti-rabbit secondary antibody conjugated to Cy3 diluted 1:250 (concentration). Slides were then washed in PBS and nuclei were then counterstained with Hoechst 33342. The stained cells were washed three times for 5min with PBS, mounted on glass slides using Prolong Antifade® (Invitrogen) and stored at 4°C overnight. Slides were analyzed using the Zeiss LSM 710 confocal microscope.

2.9 Measurement of NO concentration

The amount of NO produced in the cultured medium by treated and untreated cells for 72 hours was assessed by measuring the concentration of nitrite, a stable degradation product of NO using the Griess Reagent Kit (Molecular Probes, Eugene, OR, USA, G-7921) as described previously by Mendoza et al. (2008). The obtained concentration was divided by the number of counted cells corresponding to each condition and plotted relative to the control.

2.10 RNA extraction, reverse transcription and quantitative PCR

Total RNA was isolated from cells exposed to WSC at a concentration of 4 mg/ml. After 24, 48 and 72 hours incubation, cells were washed in sterile cold PBS, RNA were isolated by Nucleospin® RNA II (Macherey Nagel) according to manufacturer's instructions. RNA concentration was determined by NanoDrop® (ND-1000, Thermo Fisher Scientific). Reverse-transcription and real-time quantitative PCR were performed using Revert Aid™ first strand cDNA synthesis kit (Fermentas) and IQ™ SYBR® Green supermix according to manufacturer's instructions. Forward and reverse primers for TLR-4, MMP-2, MMP-9 and GAPDH were designed as follows:

MMP-2 Forward: 5'-ttgacggtaaggacgggactc-3'; Reverse: 5'-acttgagctactcccatcg-3'

MMP-9 Forward: 5'-tgacagcgacaagaagtgg-3'; Reverse: 5'-gccattcagctcgtctcttat-3'

TLR-4 Forward: 5'-ccgcttctggtcttatcat-3'; Reverse: 5'-tctgctgcaactcatttcat-3'

GAPDH Forward: 5'-tggtgctcagtgtagcccgag-3'; Reverse: 5'-ggacctgacctgccgtctag-3'

Cycle parameters were 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for TLR-4 and 55°C for MMP-2 and MMP-9 for 15 s, and extension at 72 °C for 30 s. Specificities of the amplification products were controlled by melting curve analysis. No amplification of non-specific products was observed for any of the primer sets used. In order to obtain a normalized target value, the house-keeping gene GAPDH was used. The gene specific threshold cycle (Ct) for each sample (TLR4, MMP-2 and MMP-9) was corrected by subtracting the Ct for the housekeeping gene GAPDH. Results were expressed mean $\pm$ SEM of the relative gene expression calculated for each experiment in folds ($2^{-\Delta\Delta Ct}$) compared to untreated cells used as calibrator using BioRad CFX software.

3. Results

3.1 Consistency of the smoking sessions

Table 1 summarizes the average tobacco consumed, as well as the condensate and carbon monoxide (CO) yields for each of the condensate collection methods (ESP and glass fiber pads). All results were consistent with previously published data using the same smoking machine (Shihadeh and Saleh, 2005).

3.2 Evaluation of potential mutagenic activity of WSC using Salmonella Reverse Mutagenicity Assay

The Salmonella Reverse Mutagenicity Assay is an in vitro test used to detect mutations in bacterial tester strains induced by chemical agents. This assay is based on the occurrence of reverse mutations (as mutagenic events) that cause histidine-requiring mutants to revert to their non-histidine requiring state. The spontaneous reversion of the strains fell within the range provided by the supplier (table 2). The number of revertant colonies formed in the positive controls supplied by the kit (ICR-191, Daunomycin, Sodium Azide, Mitomycin C and 2-aminoanthracene) fell within the expected ranges as well (table 3). As for the cigarette positive control, results were all positive, and significantly higher than the spontaneous reversion (table 4). The mutagenicity of cigarette in TA98 and TA100 was found to be consistent with previous published studies (TA98: 357.3 revertants/250µg TPM, TA100: 320.3 revertants/250µg TPM from Kentucky reference cigarette K1R4F) (Doolittle et al., 1990).

Across the range of tested doses, WSC did not induce an increase in the number of revertant colonies in TA97a, TA98 and TA102 strains, neither with nor without the metabolic activation system. However, WSC was toxic to TA97a and T102 at the highest tested doses. Also WSC did not exhibit a dose-related mutagenic response in TA97a, TA98 and TA102 strains either with or without S9 metabolic activation. It did however induce a dose-dependent increase in the number of revertants at the concentrations ranging from (2500–10000µg TPM/plate) in TA100 with metabolic activation which failed nonetheless to reach a two-fold increase. Due to the lack of a dose-responsive trend and the observation of increase in the number of revertants colonies in only one of the tested strains that didn't reach a two-fold increase at the tested doses, we inferred that the mainstream waterpipe smoke can only be described as a weak mutagen. Figures 1A, B, C and D summarize the results of the assay.

3.3 WSC induced growth inhibition of alveolar and endothelial cells

In addition to testing the mutagenicity of WSC, we also tested the cytotoxicity of WSC on the A549 and ECV cells. Upon exposure to various concentrations of waterpipe smoke condensate (WSC) for 24 hours, the A549 cell density and viability indicated that WSC reduced cell proliferation in a dose-dependent manner. We found that the A549 proliferative capacity was maintained in the presence of up to 1 mg/ml of WSC; however a dose of 4 mg/ml rapidly inhibited cell growth after 24 hours of exposure but the cells sustained their viability. When applied at a dose of 8 mg/ml, the WSC induced a cytotoxic effect (figure 2A). We repeated such study using a Real-Time Cell Analyzer (RTCA) based assay which makes it possible to monitor dynamic changes in the properties of A549 cells during exposure to WSC. Our results showed that WSC increased the doubling time in a dose-dependent manner (figure 2B). Thus consistent with our previous findings, WSC at doses lower than 1 mg/ml did not change the doubling time while a higher dose was required to increase the doubling time. Therefore, a 4 mg/ml concentration of WSC was used in this study.

Next we tested the effect of 4 mg/ml of WSC on A549 and ECV cell proliferation for 24, 48 and 72 hours as described in 2.3. WSC at the dose of 4 mg/ml interfered with the growth of A549 and ECV cells. When treated repeatedly, the increase in cell number was significantly lower than the control. The decrease in number of cells was not due to an increase in cell death as evident by the ability of the cells to exclude the cell membrane impermeable trypan blue dye. Moreover, when treated only once, the cells regained their proliferative capacity after treatment cessation (figure 2C). Next, we investigated the reversibility of the effect seen after repeated exposure. The cells were treated for 4 consecutive days with 4 mg/ml of WSC, and then at day 5 post-seeding, the treatment was stopped and replaced by fresh media. Cells were counted for 2 subsequent days. The effect of the WSC was reversible in terms of cells doubling.

3.4 WSC induced cell cycle arrest but not apoptosis

Based on the finding that WSC treatment inhibited cell proliferation, we investigated whether this effect was due to an actual inhibition of cell proliferation or due to an increase in apoptosis. Therefore A549 and ECV cells were stained with Hoechst and observed by fluorescence microscopy for detection of apoptotic bodies. In addition, combined annexin V and PI staining were performed to complement the Hoechst staining results. WSC-treated cells did not have higher number of apoptotic bodies relative to the control (figure 3A). Similarly, Annexin V/PI double staining as shown in figure 3B indicated a very low level of cell death upon WSC treatment because more than 80% of the cells were not stained (annexin V⁻/PI⁻). Relative to the basal level of apoptosis (the percentage of the untreated cells that are positive for apoptosis), upon single exposure or repeated exposure A549 cells have a very low if any percentage of apoptotic cells (figure 3B).

To further confirm these results and to study the effect of WSC on cell cycle distribution of the two cell lines, we conducted a cell cycle analysis. Consistent with our Hoechst findings and annexin V/PI staining, WSC did not induce a significant increase in the percentage of apoptotic/necrotic cells (PreG0/G1 cells) (data not shown). However, WSC resulted in the accumulation of the cells in G0/G1 and a decrease in the percentage of cells in the S phase, suggesting therefore a cell cycle arrest at G0/G1 in the two cell lines used (figure 4A and B). This effect was sustained as long as the treatment continued and was abolished upon removal of the treatment and replacement with fresh complete media as evident by the increase in the percentage of cells in the S phase (figure 4B).

In addition, our observation was verified through assessing the phosphorylation of histone H3. Phosphorylation of histone H3 on Ser-10 is a powerful marker to identify mitotic cells (Juan et al., 1998; Hendzel et al., 1997). In response to treatment, A549 cells expressed low levels of phosphorylated histone H3 relative to the control, and upon removal of the treatment the phosphorylated histone H3 levels were reestablished in the cells (figure 4C).

3.5 WSC induced cell cycle arrest through the p53-p21 pathway

To elucidate the mechanism by which WSC induced cell cycle arrest, we studied the effect of WSC on the activity and accumulation of p53 and the cyclin dependant kinase inhibitor p21waf1/ink4. p53 is a tumor suppressor which inhibits cell cycle progression from the G1 to the S phase. Hence, it is commonly implicated in the cell cycle arrest at the G0/G1 phase of the cell cycle. Our western blot results showed that WSC induced an increase in p53 expression (figure 5A). In addition to triggering the accumulation of p53, WSC induces the phosphorylation of p53, a post-translational modification that regulates the transcriptional activating functions of such protein (figure 5B). Moreover, upon treatment with 4 mg/ml of WSC, a marked increase in p21 expression level was observed (figure 5C). P21 is a key target for transcriptional activation by p53. It acts as an inhibitor of the cell cycle through

binding and inhibition of Cyclin Dependant Kinases (CDKs) as well as through binding to PCNA (Proliferating Cell Nuclear Antigen) thus inhibiting its translocation to the nucleus and initiation of DNA synthesis (Abbas and Dutta, 2009). Our results suggested a role of p53-p21 pathway in mediating cell cycle arrest in response to WSC.

3.6 WSC induced cellular senescence

Since WPS induced cell cycle arrest with an accumulation of p21, we hypothesized that WPS induces A549 senescence. One of the mechanisms implicated in establishing cellular senescence is the formation of chromatin structures called senescence-associated heterochromatic foci (SAHF). SAHF are compacted foci of DNA densely stained by DAPI and enriched for histone modifications including lysine9-trimethylated histone H3 (H3K9me3). SAHF plays a role in transcriptional silencing of proliferation promoting genes (Dimauro and David, 2009).

To study this, we aimed to detect the accumulation of the senescence-associated heterochromatic marker H3K9me3. As documented by immune-fluorescence microscopy images (figure 6), the treated and untreated cells showed pronounced differences in levels of H3K9me3. The data showed that A549 cells exposed to WSC form H3K9me3-rich heterochromatin domains and unlike arrested cells, the untreated cells did not form such domains. In addition, the treated cells acquired an additional feature of senescence that is represented by the flat and enlarged cellular morphology. These results supported our previous conclusion of the western blots and cell proliferation results, suggesting that the presence of elevated heterochromatin markers such as H3K9me3 is consistent with cellular arrest.

3.7 WSC induces NO production

Nitric oxide (NO), as reactive signaling molecule is involved in several diverse physiological as well as pathophysiological processes. Several studies showed that NO production is associated with pro-inflammatory and damaging effects. Previous studies indicated that NO production is specifically up-regulated in airway epithelial and inflammatory cells in response to cytokines in vitro thus describing it as a mediator of the inflammatory response (Nelson et al., 1997). Moreover, Louhelainen et al. (2008) concluded that NO may play a role in the pathogenesis and progression of COPD. It is believed that the formation of free radicals, superoxide or nitric oxide result in lung oxidant toxicity through subsequent chain reactions resulting in uncontrolled destructive oxidation (Kluchová and Tká ová, 2006). As an inflammatory mediator, we studied the effect of WSC on the production of NO from A549 cells. Our results showed that cells treated with WSC produce NO fourfold more than untreated cells (figure 7A). This indicates that WPS induced an increase in NO production.

3.8 Increased Matrix Metalloproteinase Expression in response to WSC

One of the important aspects of senescent cells is the increased production of pro-inflammatory mediators and enhanced matrix protease activity thus acting as a source of chronic inflammation and stimulating active tissue destruction (Ren et al., 2009). Matrix metalloproteinases (MMPs) are a large family of proteolytic enzymes that degrade the components of the extracellular matrix. In the MMP family, MMP-9 is a major elastolytic MMP, responsible for tissue remodeling and repair through the degradation of basement membrane type IV collagen and other matrix proteins (Ohbayashi, 2002). For this reason, we studied the effect of WSC on the expression of the MMP gelatinases, MMP-2 and MMP-9. Such gelatinase family of MMPs has pro-inflammatory effect and is known to play a role in the remodeling of lung tissue in response to chronic local and systemic inflammation (Corbel et al., 2000). The real-time PCR results of our study indicates that

WSC induced and increased the mRNA expression of both MMP-2 and MMP-9 which is maximal after 72 hours of exposure to WSC (figure 7B).

3.9 WSC induced increased TLR-4 expression

Another inflammation associated gene that is up-regulated in senescent cells is the Toll-like receptor 4 (TLR-4) (Ren et al., 2009). The TLR-4 plays important role in the lung through sustaining its integrity by modulating oxidant generation and directing immune responses against local inflammation through the release of cytokines and chemokines (Basu and Fenton, 2004). In the present study, we detected the influence of WSC on the expression level of TLR-4. The results indicated an increase in the TLR-4 mRNA upon exposure to WSC and such increase was maximal after 72 hours of exposure (figure 7C).

4. Discussion and Conclusion

The absence of mutagenic activity of the waterpipe smoke condensate at the doses 0–500 µg TPM/plate is comparable to results in studies on cigarettes that heat rather than burn tobacco. Doolittle et al. reported the absence of mutagenic or cytotoxic effect in the Ames Salmonella reverse mutagenicity assay with and without metabolic activation at doses ranging from 0–500µg TPM from cigarette that heats tobacco (Doolittle et al., 1990). Studies conducted later on a cigarette that heats tobacco were consistent with these findings (Bombick et al., 1997). The similarity between the cigarette that heats tobacco and water pipe is the relatively low temperature of tobacco when compared to regular cigarette smoking. The highest temperature that the tobacco mixture can reach, just under the burning coal, is 450°C and goes down to 50°C at the outlet of the head (Shihadeh, 2003). When White et al. (2001), studied the effect of pyrolysis temperature of tobacco on the mutagenicity of the cigarette smoke condensate in TA98 and TA100, it was found that CSC obtained is only mutagenic when the temperature of the tobacco exceeds 400 to 475°C, which further confirms our findings. It is nevertheless important to note that although tobacco temperature in the water pipe reaches 450°C at max, the charcoal burns to reach much higher temperatures. Monzer et al. (2008) found that charcoal contributes to more than 95% of Benzo[a] Pyrene production in the water pipe mainstream smoke. However, the contribution of charcoal in the TPM formation is much lower in a way that the toxicants produced by charcoal are diluted by TPM produced by the tobacco. The observed lower mutagenic activity of waterpipe relative to CSC may also be due to the fact that per unit mass of condensate, WSC likely contains significantly lower quantities of biologically active constituents. Although a single waterpipe smoking session yields far greater quantities of carcinogenic PAH and aldehydes than a single cigarette, these constituents are delivered in circa 1500 mg of particulate matter compared to circa 30 mg with the cigarette, potentially resulting in a considerably more dilute toxicant mixture. The mutagenicity results of the WSC using the salmonella reverse mutagenicity assay are not conclusive given the limitation of this assay. These limitations include the use of prokaryotic living organisms rather than eukaryotic ones, dose limitations, and the use of a solvent that is not naturally occurring in lungs. In fact, when studying the predictability of salmonella assay for rodent carcinogenicity in 1987, Zeiger found that a positive result in the salmonella reverse mutagenicity assay can predict rodent carcinogenicity while a negative result in salmonella can't infer negative rodent carcinogenicity (Pagano and Zeiger 1987). A better assay to assess the mutagenic potential of WSC must be adapted in future studies.

On the other hand, the results of our study demonstrated that highest non-cytotoxic concentration of WSC interfered with human cell proliferation. Moreover, one of the two pathways that lead to the inhibition of cell cycle progression and thus cellular senescence is the p53-p21 pathway. Our results showed that A549 cells established cell cycle arrest by the activation of the p53 pathway. Furthermore, Forrester et al. (1996) established a correlation

between NO and p53, where NO causes the stabilization and accumulation of p53 thus leading to increased p53 activity. Activation of the p53 in turn promotes cell cycle arrest and is typical feature of cellular senescence (Beauséjour et al., 2003). These results suggested a role for WSC-induced NO production in the senescence of A549.

One characteristic of cellular senescence is the increased production of matrix metalloproteinase. There are abundant data indicating that MMP-9 and MMP-2 may play an important role in the systemic inflammatory process in COPD. Supporting evidence for the involvement of MMP-2 and MMP-9 in COPD was provided by the study of Segura-Valdez et al. (2000) who through analyzing lungs of COPD patients showed a markedly increased expression of both MMP-2 and MMP-9. Therefore, our results suggested that WSC might be a risk factor for COPD through elevating MMP-2 and MMP-9 expression in alveolar epithelial cells.

Moreover, one of the pathways that trigger inflammation in cells is the TLR-4 pathway. Therefore, we hypothesized that TLR-4 signaling would be one of the pathways that triggers the inflammatory process in A549 response to WSC exposure. Also we proposed that the increased expression of TLR-4 in response to WSC might be a contributing factor in the pathology of COPD. A recent study by Pace et al. (2011) addressed the role of TLRs in COPD with acute respiratory failure and indicated an increased expression of TLR-4 in the airways of COPD patients. Other studies suggested that in COPD, TLR-4 signaling is important in the inflammatory process and in abnormalities contributing to loss of lung structural integrity (Thorley and Tetley, 2007).

In conclusion, concurrent with other studies (Waked et al., 2011; Raad et al., 2011), our data suggested that waterpipe smoke might be a contributing factor in the pathogenesis of COPD through impairing cellular growth and inducing inflammation (figure 8). This is due to the observations that WPS modified several physiological processes at the highest non toxic concentrations that contribute to the disease. One process that is involved in the development of COPD is the alteration of cellular growth thus leading to abnormal cell repair. After being injured by inhaled toxins, such as WPS, the alveolar epithelium must initiate repair responses to cover defects resulted from the injury. One requirement for the repair response to occur is the proliferation of the alveolar epithelial cells. The failure of the epithelium to repair itself results in structural and functional disruptions that facilitate the progression of pulmonary diseases. In addition, our data showed that WSC-induced cellular senescence produced higher levels of matrix metalloproteinases. Increased levels of matrix metalloproteinases in-vivo is likely to lead to alveolar destruction, airspace enlargement, loss of surface area for gas exchange and thus loss of the elasticity that is required for normal lung function.

These results complement recent laboratory studies of toxicant yield and delivery which demonstrate the potential harm of this burgeoning tobacco use method, and provide further impetus for a comprehensive assessment of health effects arising from regular waterpipe use.

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Highlights

The potential health consequences of WPS are poorly understood.

We present the first in vitro demonstration of the effect of WPS on cellular parameters.

WSC induced cell cycle arrest and cellular senescence mediated by the p53-p21 pathway.

WSC induced an increase in the transcriptional expression of MMP-2 and MMP-9 and TLR-4.

WPS might be a contributing factor in the pathogenesis of COPD.

Figure 1.

Results of the Salmonella reverse mutagenicity assay with and without metabolic activation.

(A) Mutagenicity of WSC at different concentration for strain TA97a with and without S9.

(B) Mutagenicity of WSC at different concentration for strain TA98 with and without S9.

(C) Mutagenicity of WSC at different concentration for strain TA100 with and without S9.

(D) Mutagenicity of WSC at different concentration for strain TA9102 with and without S9.

The results are shown as the mean of the revertant colonies of three independent experiments with triplicate plates of each concentration $\pm$ S.E.M.

Figure 2.

WSC induced growth inhibition. (A) Dose-response of WSC on A549 cells viability 24 hours post-treatment using (1) Trypan blue dye and (2) xCELLigence system. (B) Effect of WSC on cells proliferation. Growth curve of A549 and ECV cells treated (single and repeated exposure) and untreated. The graphs represent average of duplicates of a single representative experiment $\pm$ S.D of three independent experiments.

Figure 3.

WSC did not induce apoptosis of A549 and ECV cells. (A) Hoechst staining of cells 72 hours post-treatment with WSC. The first panel represents untreated cells, panel 2 represent cells singly treated, and panel three represent repeatedly treated cells (40x). (B) Annexin V/PI staining of A549 cells 24, 48, and 72 hours post-treatment with WSC.

Figure 4.

WSC induced cell cycle arrest of A549 and ECV cells. (A) Example of histograms and dot plot for cell cycle of untreated and treated A549 cells. (B) Effect of WSC on cell cycle distribution of A549 and ECV cells. (1) the percentage of cells in G_0/G_1 and S phase of control cells and cells treated once (single exposure) for 24, 48 and 72 hours, (2) the percentage of cells in G_0/G_1 and S phase of control cells and cells treated repeatedly (repeated exposure) for 24, 48 and 72 hours as a percentage of the control. The results are shown as percentage of the negative control value and represent means $\pm$ S.E.M of three independent experiments. * Represents statistical significance ($p < 0.05$) using student's t-test. (C) Phosphorylation of Histone 3 (Ser 10) western blot analysis after 24, 48 and 72 h of

treatment. Equal loading was determined through reprobing with antibodies to β -actin. Western blotting data are representative of three experiments.

Figure 5.

WSC induced cell cycle arrest through the p53-p21 pathway. p53 (A), phospho-p53 (B), and p21 (C) expression in A549 cells untreated and treated (S.E and R.E) with 4 mg/ml of WSC for 24, 48 and 72 hours using western blot. The histogram shows the expression of p53, phospho-p53 and p21 normalized against actin. Results are percentage of negative control and represent means of 4 independent experiments $\pm$ S.D. * represents statistical significance ($p < 0.05$) using student's t-test. (CTRL: control, S.E: single exposure, R.E: repeated exposure).

Figure 6. WSC induced senescence (A) Immunofluorescence labeling for the H3K9me3 of A549 cells untreated and treated (S.E and R.E) with 4 mg/ml of WSC for 72 hours. Hoechst and H3K9m3 are both nuclear, where blue is Hoechst and red is H3K9m3. The lower panels are the DIC light microscopy images of the same field.

Figure 7.

WSC induced inflammation. (A) NO production in A549 cells untreated and treated with 4 mg/ml of WSC for 72 hours (R.E). The histogram represents the NO concentration per number of cells. Results are percentage of negative control and represent means of 2 independent experiments $\pm$ S.D. (B) MMP-2 and MMP-9 gene expression in A549 cells untreated and treated with 4 mg/ml of WSC for 24, 48 and 72 hours. The histogram represents the expression of MMP-2 and MMP-9 normalized against GAPDH. Results are percentage of negative control and represent means of 2 independent experiments $\pm$ S.D. (C) TLR-4 gene expression in A549 untreated and treated with 4 mg/ml of WSC for 24, 48 and 72 hours. The histogram represents the expression of TLR-4 normalized against GAPDH.

Results are percentage of negative control and represent means of 2 independent experiments $\pm$ S.D. (CTRL: control, S.E: single exposure, R.E: repeated exposure).

Figure 8.

Proposed model for the pathway through which WSC induce inflammation in alveolar epithelial cells. After exposure to WSC, the cells increase their NO production which in turn activates the p53-p21 pathways. The p53-p21 pathway induces cell cycle arrest and thus contributes to the cellular senescence. Senescent cells increase production of matrix proteases and TLR-4 thus causing inflammation.

Table 1

The amount of tobacco consumed, TPM and carbon monoxide produced

	ESP in mg (S.E.M)	Cambridge Filter Pads in mg (S.E.M)
Tobacco Consumed	4908 (54)	5425 (103)
TPM	1485 (21)	1439 (21)
Carbon Monoxide	140 (5)	170 (6)

The average tobacco consumed, as well as the condensate (TPM) and carbon monoxide (CO) yields for each of the condensate collection methods (ESP and glass fiber pads). TPM was collected using the ESP for the salmonella mutagenicity assay and the Cambridge filter pads for studying its effect on tested cells.

Table 2

The spontaneous reversion range and the positive control of 4 tested strains

Strain	Spontaneous Reversion Range	Positive Control without S9	Positive Control with S9
TA97a	75–200	ICR-acridine	2-aminoanthracene
TA98	25–60	Daunomycin	2-aminoanthracene
TA100	90–220	Sodium Azide	2-aminoanthracene
TA102	200–350	Mitomycin	2-aminoanthracene

The spontaneous reversion range provided by the supplier and the positive control inducing mutagenic response in each of the 4 tested strains with and without metabolic activation.

Table 3

Revertant colonies upon treatment with the chemicals

Strain	Control Substance	S9 activation	Number of Revertant Colonies/Plate	S.E.M.
TA97a	DMSO ICR-acridine	– –	116 1356	21
TA97a	DMSO 2-aminoanthracene	+ +	115 792	24 70
TA98	DMSO Daunomycin	– –	24 1215	3
TA98	DMSO 2-aminoanthracene	+ +	24 1984	2 90
TA100	DMSO Sodium Azide	– –	114 528	3
TA100	DMSO 2-aminoanthracene	+ +	113 792	4 70
TA102	DMSO Mitomycin	– –	241 1180	6
TA102	DMSO 2-aminoanthracene	+ +	230 1734	3 110

The number of revertant colonies in each strain upon treatment with the chemicals that are known to be mutagenic for each strain with and without metabolic activation fell within the expected ranges.

Table 4

Revertant colonies in response to cigarette smoke and its components

	TA97a (75–200)	TA98 (25–60)	TA100 (90–220)	TA102 (200–350)
CSC	-	336 (16)	360 (4)	-
NNK	-	50 (0.5)	230 (28)	-
IQ	-	>3000	2008 (409)	-
Benzo[a]pyrene	-	270	586 (5)	-
Formaldehyde	-	-	-	426 (30)
ESP collection tube wash	24	129	106	256

The number of revertant colonies in the 4 strains in response to cigarette smoke and its components that are known to be mutagenic. DMSO: Dimethylsulfoxide, IQ: 2-amino-3-methylimidazol [4, 5-f] quinoline, NNK: 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone.