Evaluation of waterpipe smoke toxicity in C57BL/6 mice model

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ABSTRACT

Waterpipe smoking is a popular pastime worldwide with statistics pointing to an alarming increase in consumption. In the current paper, the evaluation of sub-chronic waterpipe smoke exposure was undertaken using C57BL/6 female mice using a dynamic exposure setting to emulate smoke exposure. Mice were daily subjected to either one (single exposure, SE) or two sessions (double exposure, DE) of waterpipe-generated smoke (two-apple flavor) for a period of two months. Although lungs histopathological examination pointed to a minor inflammation in smoke-exposed mice compared to control air-exposed (CON) group, the lung weights of the waterpipe-exposed mice were significantly higher (+72% in SE and +39% in DE) (p < 0.01) when compared to CON group. Moreover, changes in the protein expression of several proteins such as iNOS and JNK were noted in the lungs of smoke-exposed mice. However, no changes in p38 and EGFR protein levels were noted between the three groups of mice. Our results mainly show a significant increase in urea serum levels (+28%) in SE mice along with renal pathological damage in both SE and DE mice compared to CON. Additionally, severe significant DNA damages (p < 0.05) were reported in the lungs, kidneys, bone marrow and liver of waterpipe-exposed animals, using MTS and COMET assays. These findings highlight the significant risks posed by sub-chronic waterpipe smoke exposure in the selected animal model and the pressing need for future better management of waterpipe indoor consumption.

1. Introduction

Smoking is a major worldwide health problem with an estimation of 5–6 million deaths reported each year [1]. The prevalence of waterpipe tobacco smoking has increased dramatically in recent years and has become a popular pastime in numerous countries worldwide including the Middle East [2]. Consumers are lured and addicted to this smoking device by the variety of flavored tobacco [3,4] available on the market. There are misconceptions among waterpipe consumers that bubbling the generated smoke through water before lung inhalation can remove all if not most of the harmful components [5,6]. These misconceptions together with cheap prices of the waterpipe smoking apparatus and the sweetened flavored tobacco itself led to a consumption epidemic [6]. The popularity of waterpipe together with lax regulatory requirements on consumption are contributing to severe public health challenges worldwide [7].

The health impacts associated with tobacco smoking have been reported in the literature. The harms caused by tobacco are mainly associated with cancers and mainly lung cancer, respiratory disease such as chronic obstructive pulmonary disease, cardiovascular disease [8] (mainly coronary heart disease), reduction in fertility in both men and women, Alzheimer’s and vascular dementia [9]. Besides, smoking has been reported to be a major risk factor for stroke, blindness, deafness, back pain, osteoporosis, and peripheral vascular disease. However, there are still wide gaps in the knowledge associated with waterpipe smoke toxicity. A meta-analysis based on thirteen case-control studies showed a positive correlation with lung and esophageal cancer [10]. Numerous publications highlighted the waterpipe’s effects in humans and animal models focusing on lactation and memory [11], airway inflammation [13,14], cardiovascular biomarkers [12], vascular receptors [15], renal biomarkers [16] and chromosomal damages [17,18]. However, studies with standardized exposure measurements are still needed to evaluate the effect of waterpipe smoking on the development of chronic diseases.

In this study, the toxic damage triggered by sub-chronic double apple (Nakhla brand) waterpipe smoke exposure was assessed using C57BL/6 female mice. Among all the flavored waterpipe flavors that we tested and reported previously [19], double apple flavored (Nakhla Brand) was the most toxic by comparison to other tested flavors such as lemon and mint (both Mazaya and El Fakher brands), grape and mint (Mazaya brand) as well as watermelon and mint (Mazaya brand). The double apple generated smoke in the waterpipe apparatus has previously shown the most significant (80%) drop of viability after 50 min of smoke exposure [19]. The originality of the approach, used in the present study, lies in the novelty of the multilevel custom made exposure setting that was designed to assess smoking and the exposure rates. In the mixing chamber setting, waterpipe smoke was mixed with fresh air [20,21] prior to channeling smoke to the custom made exposure chambers housing the animals. Furthermore, the study explored the toxic effects (two-month daily expo-
2. Materials and Methods

The protocols used in the present study were approved by the Animal Care and Use Committee (ACUC) at the Lebanese American University (NRC 2011) [22]. All animal investigations adhered to the Guide for the Care and Use of Laboratory Animals published by the US National Research Council committee [23].

2.1. Reagents and chemicals used

Double apple flavored tobacco (Nakhla brand, Egypt) was purchased from reputable local suppliers. NaCl (S/3120/60) was purchased from Fisher Scientific, Tris (BP152-1) from Fisher BioReagents, Triton X-100 (TB0198) from Bio Basic, and phenylmethylsulfonyl fluoride (329-98-6) from Acros Organics. Trypsin (59427C) and EDTA (T9285) were purchased from Sigma-Aldrich and formaline (F/1501/PB17) from Fisher Chemical. Quantification of viable cells and DNA strand breaks was performed using the MTS assay from Promega (G1111) and the COMET assay from Trevigen (4253-096-K). The major components for Mason's trichrome stain were hematoxylin (C.I. 75290; Merck), ponceau xylidine (C.I. 16150; Fluka), acid fuchsin (C.I. F8129; Sigma-Aldrich), phosphotungstic acid (C.I. 79690; Sigma-Aldrich) and fast green (C.I. 42053; Merck). The major components for Periodic Acid Schiff were basic fuchsin (C.I. 42510; Merck) and sodium hydrogen sulfite (Himedia). The major component for picrosirius red was sirius red (C.I. 35780; BDH).

2.2. Waterpipe apparatus

The waterpipe apparatus used was selected based on the common waterpipe setting used in cafes and hookah bars worldwide [24] (Figure S1). The waterpipe apparatus was thoroughly cleaned after each smoking session. Fresh water was added to the vase prior to initiating the smoking session. The hose was also cleaned thoroughly by passing water through it to remove any nicotine residues and tar buildup.

2.2.1. Waterpipe tobacco and charcoal used

The tobacco selected for this study consisted of Nakhla brand double apple flavored tobacco (0.5% nicotine) commonly available in the market. The choice of Nakhla double apple flavor was the result of previous in vitro research on waterpipe where we reported that this flavor was the most toxic among a range of selected and tested commercially available flavored tobacco products [19]. Prior to exposure, the head of the waterpipe was loaded with 22 g of flavored tobacco, covered with perforated aluminium foil in order to allow air circulation prior to adding the lit charcoal. The charcoal used consisted of Spinneys supreme brand (produced in Indonesia) that has been formu-

lated from coconut shells with zero Sulfur. The charcoal was lit using a 220 V resistance prior to be added to the waterpipe head.

2.3. C57BL/6 mice upkep and exposure protocols

Ninety adult female C57BL/6 mice (Lebanese American University animal breeding program), aged 8–10 weeks, were used in this study. Animals were kept in groups of five per cage under 12 h light/12 h dark cycle conditions and at a constant temperature (22 ± 2 °C) at LAU animal facility. Mice were allocated into three groups (control, single smoke exposure/day and double smoke exposure/day) of 30 animals each. The smoke-exposed animals were randomly allocated in the various levels of the exposure chambers to ensure exposure uniformity to generated waterpipe smoke. These animals were subjected to a 60 min-session of waterpipe smoke (controlled by a computer controlled pump) either once a day, in the early morning, (SE) or twice a day, in the early morning and late afternoon, (DE) (Fig. 1) for a 2 month period as per published methodology [25,26]. Every time SE and DE mice were subjected to the waterpipe smoke, the control group of mice (CON) was used and exposed to fresh ambient air at a flow rate of 3 L/min (controlled by Cole palmer flowmeter) in the same exposure tower and under the same conditions. All animals were deprived of food and water during exposure and were returned to their respective cages afterwards.

2.4. Animal exposure setting

The exposure tower was constructed from Plexiglas and designed using computer software. The individual chambers (30 × 20 ×10 cm) were stacked on top of each other in a multilevel tower consisting of nine levels. The total volume of the exposure tower was 67,710 cm³. The bottom and top of each chamber was perforated to allow smoke flow from the lower to upper chambers. The top of the tower allowed purging the smoke to the external environment. The control mice were placed in level 1 below the mixing chamber where only fresh air was pumped while waterpipe smoke was pumped into level 3 and diffused upward in the tower as a result of mixing with the air generated by the pump [22]. The various gas concentrations (VOC, NO2, CO, CO2) and particulate matter PM2.5 and PM10 generated by the waterpipe fumes have been quantified.

The computer operated pump consisted of a modified Cole-Parmer Compact Syringe Pump. The operation was electronically controlled by an on board circuit hooked to a PC to control the various pump puffing regimes to achieve the desired volume output at set times (Fig. 1).

The puffing regime was simulated by the pump software according to reported waterpipe puffing parameters in the literature described as the Beirut method [27,28]. This protocol outlined an inter-puff time of 17 s together with puffing parameters as described by Shihadeh and Saleh (2005) [21]. The dynamic methodology further elucidated smoke exposure by mixing the water-
pipe smoke with fresh airflow as per previous publication by Khalil et al. (2019) [19,22].

2.5. Animal investigations

During the two months of waterpipe smoking exposure (five animals per exposure chamber in the exposure tower), the weight of CON, SE, and DE mice was recorded weekly. Upon the end of exposure, mice were weighed and humanely killed. Heart, lungs, liver and kidneys were harvested and weighed. Harvested tissues were either processed for genotoxic damage or fixed in 10% neutral buffered formalin for histopathological experiments or preserved in liquid nitrogen for Western blot analysis.

2.6. Blood serum analysis

Blood was collected from 12 mice in each group through the inferior vena cava using a sterile syringe. The maximal volume of blood collected from one mouse is around 400 μl, while the volume of serum needed to perform all serum measurements should be at least between 600 and 800 μl. Therefore, in order to ensure a proper final serum volume for analysis, the collected blood from three different animals in each group (~1.2 ml in total) was mixed with 5% EDTA in Eppendorf tubes to prevent coagulation and centrifuged at 900 G for 15 min at 4 °C. The obtained serum (600–800 μl) was then separated from the blood fractioning and stored at −80 °C for further analysis [29] using a Cobas C311 analyzer. Four independent measurements were then performed. Sodium (mM), Potassium (mM), Chloride (mM), Albumin (g/dl), Alanine aminotransferase (ALT; U/L), Aspartate aminotransferase (AST; U/L), Blilirubin (mg/dl), Cholesterol (mg/dl), Creatinine (mg/dl), Globulin (g/dl), Glucose (mg/dl), Phosphates (mg/dl), Total Protein (g/dl), Triglyceride (mg/dl), Uric Acid (mg/dl) and Urea (mg/dl) were measured.

2.7. Animal organs cell harvesting, cytotoxic and genotoxic assessment

Cells from harvested tissues were isolated by mincing the tissue followed by digestion in a Trypsin solution overnight at 4 °C in an orbital shaker as previously described [29]. The next day, cells were isolated by pipetting the trypsin solution up and down a few times, followed by centrifugation of supernatant and resuspension of cells in fresh media for viability determination. Viability determination was ensured by Trypan blue exclusion. The viable cells were then assessed for mitochondrial and DNA damage using the MTS and Comet assay methodology previously described [30,31].

2.7.1. MTS cytotoxicity assay

The MTS assay is a colorimetric based assay that measures mitochondrial dehydrogenase activity in cells [19]. In living cells, mitochondria will convert the Formazan product in the MTS from yellow to red. The color is read spectrophotometrically at a wavelength of 492 nm [19,32–34].

2.7.2. Comet DNA damage assay

The DNA damage assay (single-cell gel electrophoresis) measures both single and double strand DNA breaks in cells. Briefly, cells were embedded in agarose on a slide and subjected to a series of chemical manipulations before electrophoresis [35]. Post electrophoresis, the living cells with undamaged DNA will be seen under a microscope with a cloud around them. The DNA damaged cells will display a comet representing the migrating DNA. The comets were measured using CASP software to report a number of parameters that can be translated to DNA damage [19,29,31,33,36].

2.8. Histopathology

Harvested specimens (heart, lungs, liver and kidneys) from 6 different mice in each group were fixed in 10% neutral buffered formalin, dehydrated in 95% ethanol and then embedded in paraffin as described by Zeeni and her colleagues [37]. Sections from paraffin embedded specimens were stained with hematoxylin and Eosin [32]. The heart and kidney sections were stained using Periodic Acid Schiff and Masson’s trichrome methods, while Picrosirius red staining was performed for the liver sections. For each mouse, two different sections were obtained from each organ and screened under a light microscope (Zeiss) by an experienced board certified pathologist. Disease parameters/loci (e.g., steatosis, inflammation) were reported as present or not. Heart samples were analyzed for interstitial fibrosis (presence or absence of collagen fibers between cardiac myocytes on the trichrome stain) and inflammation (presence or absence of lymphocyte aggregates within the interstitial space). Liver samples were analyzed for fibrosis (sinosoidal and portal), steatosis (presence or absence of intracellular clear vacuoles of lipids) and necro-inflammation (present or not). Kidney samples were analyzed for glomerular sclerosis, glomerular retraction, interstitial fibrosis, tubular dilatation, and chronic interstitial inflammation, all reported as present or not. Lung samples were analyzed for hemorrhage and peribronchial inflammation using ImageJ program by applying a threshold to the acquired pictures and creating selections of the damaged areas. Four to five images were analyzed for each mouse in each of the three groups.

2.9. Preparation of protein extracts from the lungs

Right and left lungs isolated from CON, SE and DE mice were homogenized as described by Harra and his colleagues in iced-cold buffer containing (in mmol/L): NaCl 150, Tris-HCl (pH 7.5) 50, EDTA 2, PMSF 1 and supplemented with 1% Triton [38]. Protein lysates were on ice for 20 min and then centrifuged at 16,560 g at 4 °C for 15 min. Supernatants were subjected for protein measurement by Nanodrop and then used for Western blotting [39,40]. Immunoreactive bands were revealed and quantified with ChemiDoc Imaging Systems (Bio-Rad). INOS was detected with a rabbit anti-iNOS antibody from Abcam (ab3523, 1:200). p38 was detected with a rabbit anti-p38 antibody from Abcam (ab32142, 1:1000). EGFR was detected with a rabbit anti-EGFR antibody from Abcam (ab40815, 1:1000). JNK was detected with a rabbit anti-JNK antibody from Abcam (ab179461, 1:1000). Actin was detected with anti-actin antibody from Santa Cruz, CA (SC-8432) [39].

2.10. Statistical analysis

All quantitative data was reported as means ± SEM. Statistical analysis was performed with the IBM SPSS statistics [22]. Mann-Whitney Test was used to compare each parameter. Values of p < 0.05 were considered significantly different.

3. Results

3.1. Waterpipe smoke analysis

The generated smoke (Double Apple flavor) channeled to the exposure tower was diluted with filtered air (Fig. 1) prior to analysis for the following gases (CO, CO2, PM2.5, PM10 and Volatile Organic Compounds (VOCs)) as reported in Table 1. Similar readings in the mixing chamber and upper levels of the exposure tower indicated good mixing and distribution of waterpipe smoke emission in the apparatus. The following compounds (Cadenadioenoic acid; Linoleic acid; Nicotine [22]; Palmitic acid and Vanillin) have also been detected using GC/MS [19].

<table>
<thead>
<tr>
<th>VOC</th>
<th>waterpipe/air mixture (mg/m³)</th>
</tr>
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<tbody>
<tr>
<td>PM2.5</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>PM10</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>CO</td>
<td>77 ± 12</td>
</tr>
<tr>
<td>CO2</td>
<td>2317 ± 521</td>
</tr>
<tr>
<td>VOCs</td>
<td>3079 ± 621</td>
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</table>
3.2. Waterpipe smoke exposure impact on body and organs weight in C57BL/6 mice

Female C57BL/6 mice were exposed to 60 min-session of waterpipe smoke either once a day (SE) or twice a day (DE) over a two-month period as described in Materials and Methods. The effect of waterpipe smoke on animals was assessed for anatomical changes. The weight of the different organs was reported (Table S1).

The lung weight of the exposed mice was significantly higher (+72% in SE and +39% in DE) than that of the CON group whereas the weight of body, heart and kidneys and liver did not display any significant difference between exposed and controls. (Table S1). The results were similar when the organ weights were normalized to body weight (Fig. 2).

3.3. Waterpipe smoke exposure impacts on C57BL/6 mice blood parameters

Serum collected from each mice group was analyzed for potential changes resulting from two month-SE and DE waterpipe smoke exposure using clinical chemistry (Table 2). The tabulated results from four independent measurements showed no significant effects on the serum levels of the following measured parameters for SE and DE cohorts when compared to controls (Sodium, Potassium, Chloride, ALT, AST, Bilirubin, Creatinine, Globulin, Glucose, Phosphates, Total protein, Triglycerides and Uric acid).

Nevertheless, waterpipe smoke exposure induced a significant increase in urea levels (mg/dL) in SE mice compared to CON group (Controls: 42.7 ± 3.4; n = 4 vs SE: 54.7 ± 2.1, n = 4; p < 0.05). Furthermore, the serum levels of albumin, cholesterol and uric acid were significantly decreased in DE mice compared to SE group.

3.4. Waterpipe smoke exposure pathological impacts in C57BL/6 mice organs

The reported significant increase in lung weight in SE and DE mice compared to control warranted further investigation of the potential pathological damages triggered by smoke exposure. Therefore, lungs as well as all harvested organs were stored in formalin and processed for pathological assessment. Data in Fig. 3 represent the results of six independent histological sections of the lungs and kidneys from SE, DE and control mice.

Cross examination of the lungs showed normal bronchial and alveolar structure in CON mice (Fig. 3A). However, in waterpipe exposed mice, different grades (mild to severe) of peribronchial chronic inflammatory infiltrate, with effacement of the underlying alveolar architecture were shown in 83% of SE (Figs. 3B) and 100% of DE lungs (Fig. 3C). Quantitative analysis of the inflammation revealed a damaged area estimated to be in average 18% in both SE and DE lungs (Figure S2). Furthermore, in the smoke exposed mice, we observed a significant dilatation of the proximal convoluted tubules in the kidney of all SE and DE mice compared to control (Fig. 3E, F and 3H). Besides, our results showed chronic renal interstitial inflammation (Fig. 3G) in 50% of SE and 100% of DE mice as well as a renal glomerular retraction (Fig. 2I) in all SE and DE mice. No significant pathological changes were noted in the heart and liver of the three mice groups (data not shown).

3.5. Waterpipe smoke exposure impact on protein expression in exposed and control mice lungs

The absence of significant inflammatory induction in the lungs as reported in Fig. 3 led to further investigations of various proteins expressions in the lungs using western blots technology. Fig. 4 shows a significant downregulation of iNOS level in SE mice compared to CON, which seems to be reversed in DE vs SE mice. Whereas no changes in p38 and EGFR expression were noted between the three groups, an increase in both JNK isoforms levels (p54 and p56) was observed in DE lungs compared to CON.

3.6. Waterpipe smoke exposure mutagenic induction in exposed and control mice organ derived cells

The mutagenic potential of waterpipe smoke components previously reported in the literature together with the findings reported in Figs. 2–4 warranted the investigation of smoke inhalation impact on cellular genotoxic damage. Isolated cells from various organs from exposed and control mice were subjected to comet assay to measure genotoxic potential (29,36). The comets were assessed using CASP software and data reported in Table 3.

The data shown in Table 3 represented the assessment of cells harvested from various organs from CON, SE and DE mice for DNA damage. The comet tail length data, a good indicator of DNA damage showed significant damage in SE (46 ± 1, 119 ± 18, 60 ± 17 in 5 mice) and DE cells (84 ± 11, 264 ± 92, 110 ± 18 in 5 mice) with the bone marrow, lungs and liver, respectively by comparison to controls (15 ± 2, 6 ± 2, 20 ± 8 in 5 mice) (p < 0.05). Furthermore, the data generated from the comet assay (Table 3) was utilized to calculate several genotoxic indices as reported in Fig. 5. The presented data pointed to significant DNA cellular damage in all the harvested organs (bone marrow, kidneys, lungs, and liver).

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Table 2

<table>
<thead>
<tr>
<th></th>
<th>CON (n = 4)</th>
<th>SE (n = 4)</th>
<th>DE (n = 4)</th>
</tr>
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<tbody>
<tr>
<td>Sodium (mM)</td>
<td>184 ± 10</td>
<td>176 ± 6</td>
<td>180 ± 5</td>
</tr>
<tr>
<td>Potassium (mM)</td>
<td>3.8 ± 0.3</td>
<td>4.0 ± 0.4</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>Chloride (mM)</td>
<td>106 ± 4</td>
<td>106 ± 1</td>
<td>104 ± 2</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>2.9 ± 0.1</td>
<td>3.3 ± 0.1</td>
<td>2.8 ± 0.1</td>
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<tr>
<td>ALT (U/L)</td>
<td>21.8 ± 2.2</td>
<td>23.3 ± 2.2</td>
<td>20.7 ± 3.1</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>64.9 ± 7.2</td>
<td>51.3 ± 19</td>
<td>55.0 ± 2.4</td>
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<tr>
<td>Bilirubin (mg/dL)</td>
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<td>0.03 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>47 ± 3</td>
<td>55 ± 3</td>
<td>43 ± 5</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.16 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.16 ± 0.01</td>
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<tr>
<td>Globulin (g/dL)</td>
<td>1.6 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>165 ± 12</td>
<td>185 ± 18</td>
<td>152 ± 13</td>
</tr>
<tr>
<td>Phosphates (mg/dL)</td>
<td>5.9 ± 0.6</td>
<td>5.6 ± 0.4</td>
<td>6.0 ± 0.7</td>
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<tr>
<td>Total Protein (g/dL)</td>
<td>4.6 ± 0.2</td>
<td>4.3 ± 0.2</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>62 ± 5</td>
<td>60 ± 4</td>
<td>67 ± 4</td>
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<tr>
<td>Uric Acid (mg/dL)</td>
<td>1.5 ± 0.5</td>
<td>2.3 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>42.7 ± 3.4</td>
<td>54.7 ± 2.1</td>
<td>50.5 ± 2.9</td>
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**Fig. 2. Organ weight ratio.** All data expressed as mean ± S.E.M. The number of mice for each group is shown in brackets above the bars. Abbreviation used: BW, body weight. Statistically significant differences between CON and SE or CON and DE are, respectively, indicated as **+, p < 0.01; ***, p < 0.001.
Fig. 3. Representative of organ histopathology (lung and kidney) results in CON (A and D), SE (B, E, F) and DE mice (C, G, H, I). Lung: A (normal bronchial and alveolar structures, Hematoxylin and Eosin, ×10); B (severe peribronchial chronic inflammatory infiltrate with effacement of the underlying alveolar architecture, Hematoxylin and Eosin, ×10); C (mild peribronchial chronic inflammatory infiltrate, Hematoxylin and Eosin, ×10). Kidney: D (normal, Hematoxylin and Eosin, ×20); E (dilation of the proximal convoluted tubules, Hematoxylin and Eosin, ×20); F (dilation of the proximal convoluted tubules, Periodic acid–Schiff, ×40); G (chronic interstitial inflammation, Hematoxylin and Eosin, ×20); H (dilation of the proximal convoluted tubules, Masson’s Trichrome, ×20); I (glomerular retraction, Periodic acid–Schiff, ×20).

Fig. 4. Expression of iNOS, p38, EGFR and JNK in CON, SE and DE mice. Equal amounts of proteins extracted from the lungs of CON, SE and DE mice were separated on SDS/PAGE and revealed with specific antibodies for iNOS, p38, EGFR and JNK. Actin was used as a loading control. (A) Shown are representative blots. (B) Quantification of all data obtained in several immunoblots from CON, SE and DE samples and expressed as mean ± S.E.M. The number of mice for each group is shown in brackets above the bars. Statistically significant differences between CON and SE, CON and DE or SE and DE are, respectively, indicated as **, p < 0.01; *, p < 0.05; ***, p < 0.001.

4. Discussion

Waterpipe smoke contains a cocktail of known carcinogens as well as numerous toxic chemicals generated from the process of tobacco heating by the charcoal and that have been well publicized in the literature [28,41,42]. In this study, we assessed the toxicological impacts of waterpipe generated smoke exposure in female C57BL/6 mice in a multi-leveled exposure tower (Fig. 1). A number of parameters were measured in control mice (CON) exposed to air-
flow only, Single Exposure (SE) exposed once a day or Double Exposure (DE) exposed twice a day to 1 h of waterpipe smoke session for a two-month period.

Our results showed that the body weight of mice after the exposure to waterpipe generated smoke was not different from that of the CON mice. The effect of waterpipe smoke on body weight is somewhat controversial in the literature. While some studies showed a significant decrease in mice body weight after exposure to waterpipe smoke [43], others indicated no change [44] while some long-term studies (six-month exposure) showed significant increase in body weight [45]. Moreover, no significant change was noted in the heart, kidneys, and liver weight between exposed and control mice while a significant increase in lung/BW index was observed in SE and DE mice. Data on the effect of waterpipe smoke on lung weights has been well documented in the literature [43]. Serum of mice exposed to waterpipe smoke showed a significant increase in urea levels (54.7 ± 2.1 mg/dL in SE vs 42.7 ± 3.4 in CON) compared to serum obtained from CON mice. This increase could be pointing to smoke inhalation affecting kidney functions mainly their detoxifying ability, an important function essential for removal of poisonous materials from the body [46]. Hence, this finding is in agreement with published reports on the impacts of waterpipe smoking on renal functions in mice [46] and rats [47]. Surprisingly, the lack of effect in some serum markers in DE exposed mice when compared to CON mice was noted. One would expect a higher toxicity in the DE group compared to the SE exposed mice when it comes to the increase in lung weights and pathology. The absence of a linear correlation between the dose and the effects reported in exposed mice could be explained at this stage by a saturating single exposure inducing the maximal toxicity that could be reached within two months. Hence, a longer period of exposure of mice to waterpipe smoke would allow a better characterization of the progression of the lung damages. The results on the increase in urea levels in SE mice were further corroborated by the renal histological section data. In the smoke exposed mice, we observed a significant dilation of the proximal convoluted tubules as well as a chronic renal interstitial inflammation and a renal glomerular retraction in waterpipe smoke exposed mice attesting a toxic effect of the waterpipe smoke on the kidneys as previously reported [48].

Despite the increase in Lungs/BW ratio in exposed mice compared to CON, our results on pulmonary histological sections were not conclusive as we mainly noted minimal to mild peribronchial chronic inflammatory
trate with effacement of the underlying alveolar architecture in most of the smoke exposed mice. The damaged area in most SE and DE mice was estimated to be in average around 18%, which is a modest rate of inflammation. This was not in agreement with published literature on cigarette smoke exposure aggravating lung inflammation in C57BL/6 mice [49]. However, some studies reported that cigarette-smoke induced pulmonary inflammation and emphysema are associated with mice strains and length of the smoke exposure [50]. In our study, the two-month exposure period to waterpipe smoke might not have been sufficient to induce a regular high grade inflammation in the lungs of SE and DE mice but has certainly led to early damage in DNA integrity and to changes in the expression of some proteins.

The impact of diluted chronic waterpipe generated smoke exposure on various protein expressions using Western Blots techniques pointed to a significant downregulation of iNOS in SE by comparison to CON. To our knowledge, this is the first study that reports a change in iNOS expression in whole lung extracts exposed to waterpipe smoke. iNOS was reported in the literature as a key player in regulating the inflammatory responses in mice model exposed to cigarette smoke [51,52]. The literature mainly referred to an upregulation of this protein by cigarette smoke exposure while some researchers reported cigarette derived-smoke decreased iNOS in lung epithelial cells [53].

The p38 are mitogen-activated protein kinases playing an important role in modulating cytokine expression [54]. They play a critical role in cell differentiation and apoptosis [55]. Our data showed no significant upregulation in this protein in our in vivo model although there are some reports of its upregulation in the mice model [56].

The data also pointed to no significant change in EGFR expression in SE and DE lungs. EGFR expression is common in many cell types and plays an important role in cell proliferation and cancer onset [57,58]. These findings are not in accordance with numerous publications correlating cigarette smoke to EGFR induction [59] and with some reports linking nicotine to EGFR induction [60,61]. This could perhaps be explained by the dilution of generated smoke (with a flow of 3 L/min of filtered air) in our apparatus in order to emulate smoke exposure. This resulted in lower concentrations of smoke components inhaled by the mice, which would fall below the threshold required to affect cell proliferation induction, and cancer onset reported in the literature.

The mice exposure to smoke resulted in a significant upregulation in JNK expression in DE lungs. Actually, JNK plays an important role in apoptosis and response to genotoxic stress [55]. JNK activation was also reported in mice exposed to tobacco smoke for 12 weeks [56]. This increase in the JNK expression was associated with a damaged DNA structure. Cells isolated from exposed and control mice lung-derived cells were subjected to the comet assay (single and double strand DNA damage) and the various parameters were calculated by the CASP analysis software. The data was used to calculate a number of parameters such as the Olive Tail Moment (OMT) and Tail Moment Index (TMI) presented [29,36]. The OMT and TMI clearly indicated significant DNA damage in lungs, bone marrow, kidney and liver cells with the highest levels of damage reported in the lung cells. This agrees nicely with published literature on cigarette smoke induced DNA damage in various cells using the comet assay [62–64]. However, data on the genotoxic impacts of waterpipe cigarette smoke in bone marrow, kidney and liver cells [65] are limited.

It is important to note that the study had several limitations. The use of the mouse model clearly indicated various damages triggered by waterpipe smoke inhalation during the study period. The correlation to humans is challenging as the mouse model is different from human exposure. There are numerous ethical issues associated with recruitment of humans for this invasive study. The data generated from the mouse concurs with the in vitro data on waterpipe smoke inhalation. Synergies could be observed mainly at the protein expression and DNA damages using human derived lung cells as previously reported [19]. Other limitations included the use of female mice in this study. Although many studies either did not investigate the sex-related functional consequences of waterpipe smoke on pulmonary toxicity [13,66] or characterized exclusively the effect of waterpipe smoking on the lungs in males [66], some reported that female smokers are more susceptible to increased risk of small airways disease compared to mala.

ers seem to experience an accelerated decline in their pulmonary function compared to male smokers with similar smoking exposure in mild to moderate COPD [68]. These pathologic changes may be attributed to female sex hormones and need further investigations. Moreover, male and female mice exhibited differential sensitivity for acute waterpipe smoke exposure in both C57BL/6j and BALB/c\j mouse strains, and females showed increased susceptibility to pulmonary toxicity compared to males in both the strains [69]. Hence, we sought to characterize the effect of waterpipe smoke toxicity on the lungs in female mice, knowing that the evaluation of the same toxic effects in males would be crucial. The selection of the most potent flavored tobacco (Double Apple Nakhla brand) based on previous in vitro dynamic experimentation [70].

5. Conclusions

The evaluation of sub-chronic health impacts resulting from waterpipe smoke exposure in mice was undertaken in this study. The research clearly indicated severe health impacts in exposed animals whether to a single or double session a day during the study period. Mice exposed to waterpipe smoke displayed a significant increase in urea serum level along with a significant dilation of the proximal convoluted tubules as well as a chronic renal interstitial inflammation and a renal glomerular retraction. Although lung histopathological analyses pointed to minor inflammation in smoke exposed female mice compared to CON group, increases in lung weights and protein expression, JNK, were noted in the lungs of smoke exposed mice by comparison to controls. Lastly, severe DNA damage was detected and measured in bone marrow, kidney, liver, and lungs of smoke exposed mice. Taken together, our results pointed to a systemic toxicity triggered by the waterpipe smoke, highlighting the significant health, risks associated with waterpipe smoke exposure.

The significant health impacts from waterpipe consumption will require better public health policies to curb consumption and reduce government healthcare spending on tobacco related illnesses and diseases.

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Credit author statement

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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Abbreviations

SE Single exposure


