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To cite this article: Nour A. Al-Sawalha, Ala'a M. Migdadi, Karem H. Alzoubi, Omar F. Khabour & Nidal A. Qinna (2017) Effect of waterpipe tobacco smoking on airway inflammation in murine model of asthma, *Inhalation Toxicology*, 29:2, 46-52, DOI: [10.1080/08958378.2017.1280105](https://doi.org/10.1080/08958378.2017.1280105)

To link to this article: <http://dx.doi.org/10.1080/08958378.2017.1280105>



Published online: 28 Feb 2017.



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RESEARCH ARTICLE

Effect of waterpipe tobacco smoking on airway inflammation in murine model of asthma

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ABSTRACT

Objective: There has been an increase in the popularity of waterpipe tobacco smoking (WTS) worldwide, especially in the younger population, including asthma patients. In this study, we investigated the effects of waterpipe smoking on airway inflammation, cytokine levels and oxidative stress markers in an antigen-driven murine model of asthma.

Materials and methods: Balb/c mice were divided into four groups; (1) control (received fresh air, ovalbumin sensitization and saline challenge), (2) WTS (received WTS, ovalbumin sensitization and saline challenge), (3) Ova S/C (received fresh air, ovalbumin sensitization and ovalbumin challenge) and (4) simultaneous WTS and Ova S/C (received WTS, ovalbumin sensitization and ovalbumin challenge). Airway inflammatory cells were evaluated in the broncho-alveolar lavage fluid. Cytokines [interleukin (IL)-13, 10 and 18] and oxidative stress markers [superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx)] were evaluated in the lung homogenates.

Results: Chronic exposure to WTS significantly increased the number of airway inflammatory cells in mice, specifically: eosinophils, neutrophils, macrophages and lymphocytes. The level of IL-13 in the lungs was increased and the level of IL-10 was reduced ($p < 0.05$) by WTS.

Chronic WTS potentiated the increase in inflammatory cells induced by Ova S/C ($p < 0.05$). The level of IL-13 in the lungs was increased by simultaneous WTS and Ova S/C ($p < 0.05$) while, levels of IL-10, IL-18, SOD, catalase and GPx in the lungs were not affected.

Conclusions: Chronic WTS exposure induced airway inflammation in control mice and enhanced airway inflammation in murine model of asthma.

ARTICLE HISTORY

Received 16 September 2016

Revised 5 January 2017

Accepted 5 January 2017

KEYWORDS

Cytokines; eosinophils; inflammatory cells; murine model of asthma; ovalbumin; oxidative stress; waterpipe smoking

Introduction

Asthma is a chronic airway inflammatory disease that is characterized by airway inflammation, mucus overproduction and airway hyperresponsiveness. The number of asthma patients is expected to reach 400 million in year 2025 (Masoli et al., 2004). Several triggers, such as tobacco smoking, have been found to exacerbate asthma (Ritz et al., 2006). The popularity of tobacco smoking has increased throughout the world; there are around 1.25 billion smokers worldwide and 75% of them are in developing countries (Centers for Disease Control and Prevention, 2004). It is also estimated that around 25% of asthma patients in developed countries are smokers (Thomson et al., 2004). Cigarette smoking increases the severity of asthma symptoms (Althuis et al., 1999), mucus production (Cerveri & Brusasco, 2010) and therefore, the need for rescue medications (Gallefoss & Bakke, 2003). In addition, smokers have a greater reduction in the forced expiratory volume in 1 s (FEV1) (Al-Fayez et al., 1988; Gallefoss & Bakke, 2003; Lange et al., 1998) and a reduced health-related quality of life (Gallefoss & Bakke, 2003) in comparison to nonsmokers.

Waterpipe tobacco smoking (WTS), also known as hookah, shisha, narghile and argile, has become an international trend of tobacco smoking (Maziak et al., 2004), especially among the younger population (Azab et al., 2010; Rahman et al., 2014). Around 100 million people worldwide use waterpipe daily (Gatrad et al., 2007). The prevalence of WTS has increased among asthma patients; ~19% of asthmatic adolescents smoke waterpipe (Martinasek et al., 2014), 48% of asthma patient in Jordan use waterpipe and, 25.6% smoke both cigarettes and waterpipe (Al-Sheyab et al., 2014). Recent data suggest that the daily consumption of WTS leads to similar nicotine absorption levels (Neergaard et al., 2007) and three times higher carbon monoxide (CO) exposure as compared to cigarette smoking (Eissenberg & Shihadeh, 2009). In addition, WTS contains significant amount of nicotine, tar and heavy metals (like arsenic, chromium and lead) (Shihadeh, 2003) as well as volatile aldehydes that are involved in the development of pulmonary diseases (Al Rashidi et al., 2008). Previous reports focused on studying the effect of WTS on airway inflammation. It has been found that acute WTS exposure increased airway recruitment of inflammatory cells, as well as enhanced the

production of pro-inflammatory mediators in mice (Khabour et al., 2012). Additionally, acute exposure to WTS in an antigen-driven murine model of asthma increased airway eosinophils levels (Mirsadraee et al., 2010). The goal of this study was to examine the effects of chronic WTS exposure on airway inflammation, cytokine level and oxidative stress markers in an antigen-driven murine model of asthma.

Materials and methods

Animals

Balb/c male mice (6–8 weeks old) were purchased from the Animal Care Unit at Jordan University of Science and Technology (Irbid, Jordan). All the procedures were reviewed and approved by the Animal Care and Use Committee (ACUC) of Jordan University of Science and Technology. Mice were kept in a 12:12 light/dark cycle at standard room temperature with free access to water and food.

All mice were sensitized to ovalbumin, and received either (1) fresh air and saline challenge (control group), (2) 1 h of WTS daily for 4 weeks and saline challenge (WTS group), (3) fresh air and ovalbumin challenge (Ova S/C group) or (4) 1 h of WTS daily for 4 weeks and ovalbumin challenge (WTS and Ova S/C group).

Ovalbumin sensitization and challenge

Mice were sensitized with three intraperitoneal injections of 2 mg/kg/day of ovalbumin (Ova) (Sigma-Aldrich, St. Louis, MO) with 2 mg of Alum (Imject Alum; Thermo Scientific, Pierce, Rockford, IL) as described (Thanawala et al., 2013). Mice were challenged with nebulized 1% Ova for 50 min (Ova S/C) (Hall et al., 2016) or saline (control) as illustrated in Figure 1.

Waterpipe smoke exposure

Mice in the WTS group were exposed to mainstream waterpipe smoke for 1 h/day for 28 days using whole body exposure system as described previously (Khabour et al., 2012). Briefly, the whole-body exposure apparatus was connected to smoking machinery. The smoking machinery was set to perform in accordance with the Beirut Method (Katurji et al., 2010). The pump was programed to provide 171 puffs

of 2.6 s with 17-s interval between puffs. The puffs were monitored by puff topography instrument to provide the mean puff volume of 530 ml, similar to human puff topography that was measured during waterpipe smoking session (Shihadeh et al., 2004). In each exposure session, the level of CO in the exposure chamber was monitored using an electrochemical sensor (Monoxor II, Bacharach Inc., PA). The level of CO concentration was maintained at similar levels (870 ± 94 ppm, mean $\pm$ SD) during all of the exposure sessions.

Bronchoalveolar lavage fluid (BALF) collection

Mice were killed on day 28 by administering 0.1 ml of ketamine/xylazine (containing 87.5 mg/kg ketamine and 12.5 mg/kg xylazine). The bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea and perfusing both lungs with 1 ml normal saline. After centrifuging BALF at $200 \times g$ for 15 min, the cell pellet was suspended in $1 \times$ PBS (1 ml) (Forsythe et al., 2004). The total cells in the BALF were counted using a hemacytometer (Hausser Scientific, Horsham, PA) and the differential cell count was determined by cytocentrifugation using a Cytospin 4 (Thermo Electron Corporation, Waltham, MA) at 2000 rpm for 5 min, followed by Wright-Giemsa staining (Sigma-Aldrich, St. Louis, MO).

Measuring interleukins level and oxidative stress markers in the lungs

Tissues were homogenized using a tissue homogenizer (Tissue Master-125, Omni International, Kennesaw, GA) in lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO) as described previously (Alzoubi et al., 2012; Khabour et al., 2012). Homogenates were centrifuged at $14\,000 \times g$ at 4°C for 5 min to remove insoluble debris. After centrifugation, the levels of IL-13, IL-10 and IL-18 (R&D Systems Inc., Minneapolis, MN) were measured by an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. The absorbance was read at 450 nm using an EL $\times 800$ plate reader (Bio-teak instruments, Winooski, VT). The cytokine level was normalized to the total protein in each sample.

The levels of anti-oxidative enzymes superoxide dismutase (SOD) (Sigma-Aldrich Corp.), catalase (Cayman Chemical, Ann Arbor, MI) and glutathione peroxidase (GPx) (Sigma-Aldrich Corp.) were measured in lung

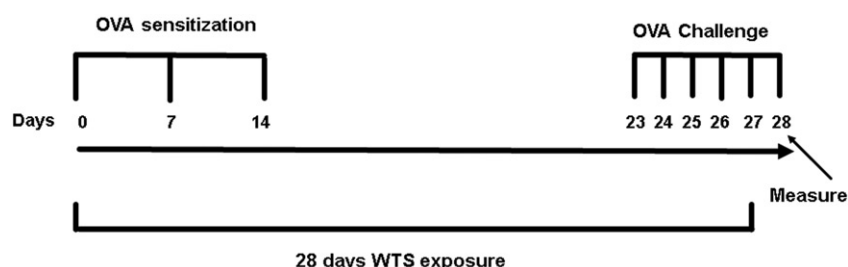


Figure 1. Treatment protocol. Mice received intraperitoneal (i.p.) ovalbumin (Ova) (2 mg/kg/day in 2 mg of alum) on days 0, 7 and 14; followed by nebulized ova (1%) for 50 min on days 23, 24, 25, 26 and 27. Waterpipe tobacco smoke (WTS) exposure in correspondent groups was for 28 days.

homogenates by ELISA as described previously (Khabour et al., 2012). The enzyme activity was normalized to the total protein in each sample.

Results

Effect of waterpipe smoke exposure on airway inflammation in antigen-driven murine model of asthma

To determine the effect of waterpipe smoking in an antigen-driven murine model of asthma, mice were exposed to WTS for 28 days and BALF was evaluated for inflammatory cells. The total cell count in the BALF of mice that were exposed to WTS ($3.16 \pm 0.06 \times 10^6$) was higher compared to the control group ($0.05 \pm 0.01 \times 10^6$, $p < 0.05$) (Figure 2A). The airway recruitment of inflammatory cells was also increased in Ova S/C mice ($6.01 \pm 0.05 \times 10^6$) compared to control group ($0.05 \pm 0.01 \times 10^6$, $p < 0.05$). WTS exposure for 28 days increased the number of total cells in Ova S/C mice

($8.471 \pm 0.10 \times 10^6$) as compared to all other groups ($p < 0.05$) (Figure 2A).

The Ova sensitization and challenge model of asthma is characterized by an eosinophilic type of airway inflammation. The number of eosinophils in the BALF of Ova S/C mice ($5.34 \pm 0.78 \times 10^5$) and WTS exposed mice ($3.30 \pm 0.22 \times 10^5$) were higher than the control group ($0.02 \pm 0.01 \times 10^5$, $p < 0.05$) (Figure 3B). WTS exposure increased the recruitment of eosinophils to the airways in Ova S/C mice ($17.8 \pm 0.74 \times 10^5$) compared to all other groups ($p < 0.05$) (Figure 2B).

Other types of inflammatory cells were also affected by WTS. WTS exposure elevated the levels of neutrophils, macrophages and lymphocytes in BALF ($4.94 \pm 0.32 \times 10^5$, $86.62 \pm 3.13 \times 10^5$ and $10.57 \pm 1.35 \times 10^5$, respectively, $p < 0.05$) compared to control ($0.03 \pm 0.01 \times 10^5$, $0.81 \pm 0.23 \times 10^5$ and $0.29 \pm 0.04 \times 10^5$, respectively, $p < 0.05$). In addition, WTS exposure and Ova S/C increased the level of airway recruitment of neutrophils and

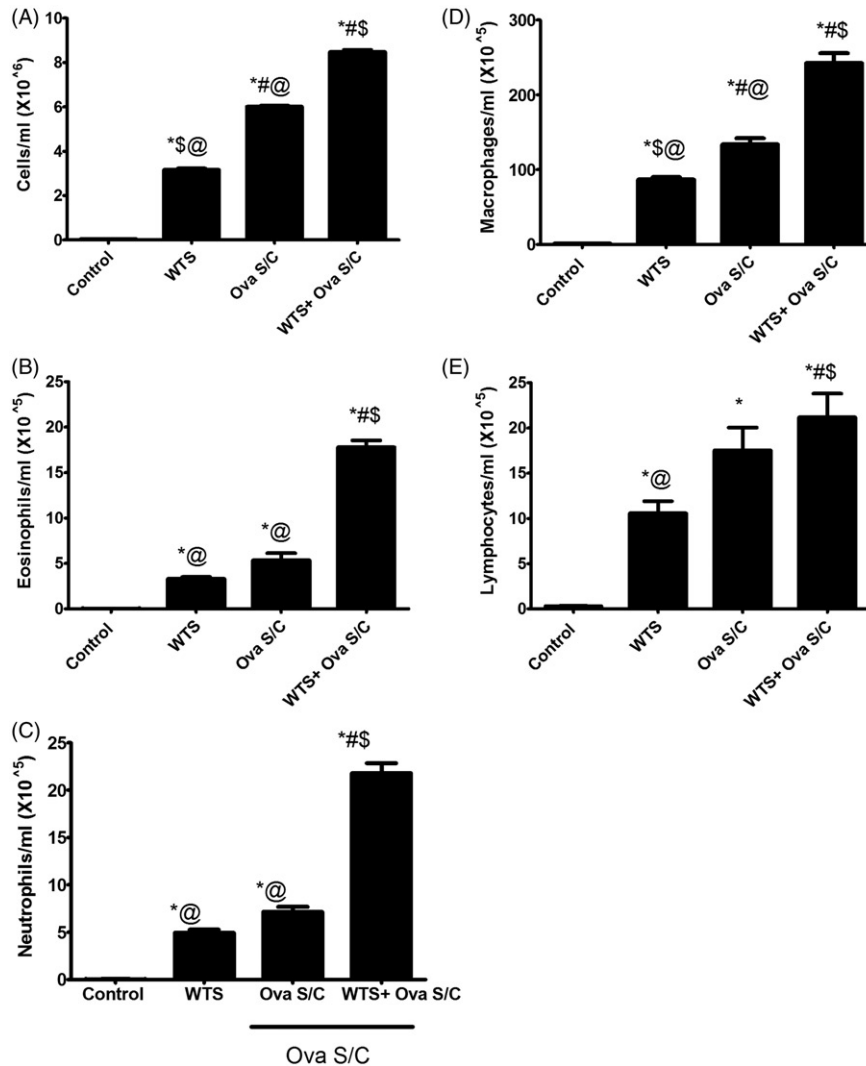


Figure 2. Effect of waterpipe smoke (WTS) exposure on inflammatory cells in bronchoalveolar lavage fluid (BALF). BALF was collected from mice that were exposed to fresh air and saline challenge (control group), 1 h of WTS daily for 4 weeks and saline challenge (WTS group), fresh air and ovalbumin challenge (Ova S/C group) or, WTS and ovalbumin challenge (WTS and Ova S/C group). BALF was collected 24 h after the last Ova challenge and last WTS exposure. BALF was analyzed for (A) total inflammatory cells, (B) eosinophils, (C) neutrophils, (D) macrophages and (E) lymphocytes. * indicates significant difference from control group, # indicates significant difference as compared to WTS exposure, \$ indicates significant difference as compared to Ova S/C, @ indicates significant difference as compared to Ova S/C and WTS exposure. Values represent the mean $\pm$ SEM of data from 6–8 mice in each group. $p < 0.05$ was considered significant.

macrophages ($21.80 \pm 1.05 \times 10^5$ and $242.3 \pm 13.16 \times 10^5$, respectively, $p < 0.05$) compared to the airways of Ova S/C mice ($7.14 \pm 0.55 \times 10^5$ and $133.7 \pm 8.10 \times 10^5$, respectively, $p < 0.05$). However, lymphocyte recruitment was not affected by WTS exposure in Ova S/C mice ($21.18 \pm 2.63 \times 10^5$ versus $17.54 \pm 2.52 \times 10^5$, $p > 0.05$) (Figure 2C–E).

Effect of waterpipe smoke exposure on cytokines levels in an antigen-driven murine model of asthma

WTS exposure increased the level of tissue IL-13 (4.56 ± 0.92 pg/mg) compared to control (2.01 ± 0.49 pg/mg, $p < 0.05$) and Ova S/C mice (1.08 ± 0.17 pg/mg, $p < 0.05$). Simultaneous WTS and Ova S/C increased the level of IL-13 in the lungs compared to Ova S/C mice (7.25 ± 0.95 pg/mg and 1.08 ± 0.17 pg/mg, respectively, $p < 0.05$) (Figure 3A). WTS exposure reduced the level of IL-10 in the lungs (0.33 ± 0.07 pg/mg) compared to control (1.98 ± 0.72 pg/mg, $p < 0.05$) (Figure 3B). However, the level of IL-18 in the lungs was not different among the different groups (34.78 ± 6.36 , 23.33 ± 3.67 , 28.51 ± 3.63 , 37.49 ± 18.49 pg/mg in control, WTS exposed, Ova S/C and simultaneous WTS and Ova S/C mice, respectively, $p > 0.05$) (Figure 3C).

Effect of waterpipe smoke exposure on the level of oxidative stress markers in antigen-driven murine model of asthma

The level of catalase in the lungs (403.00 ± 51.83 , 412.40 ± 73.17 , 413.20 ± 41.27 , 514.40 ± 80.59 μ M/mg in control, WTS exposed, Ova S/C and simultaneous WTS and Ova S/C mice, respectively) and SOD activity (5.67 ± 0.89 , 4.69 ± 0.82 , 6.55 ± 0.65 , 5.20 ± 0.63 units/mg in control, WTS exposed, Ova S/C and simultaneous WTS and Ova S/C mice, respectively) were not affected by WTS or Ova S/C ($p > 0.05$) (Figure 4A and B). WTS exposure reduced the SOD activity but the reduction was not significant ($p > 0.05$) (Figure 4B). The level of GPx in the lungs did not change in response to WTS exposure (2.25 ± 0.09 , 2.39 ± 0.05 , 2.36 ± 0.12 , 2.25 ± 0.08 units/mg protein in control, WTS exposed, Ova S/C and WTS and, simultaneous WTS and Ova S/C mice, respectively, $p > 0.05$) (Figure 4C).

Discussion

This study evaluated, for the first time, the effect of simultaneous exposure to WTS and an antigen in a murine model of allergic airway disease. Chronic exposure to WTS induced airway inflammation, which was characterized by increased airway influx of eosinophils, neutrophils, macrophages and lymphocytes. WTS exposure increased the level of interleukin (IL)-13 and reduced the level of IL-10 in the lungs. Sensitized mice that were exposed to both Ova and WTS had higher levels of airway inflammation with increased influx of eosinophils, neutrophils and macrophages. The level of IL-13 in the lungs was increased by the simultaneous exposure to WTS and antigen while levels of lung IL-10 and IL-18 were not affected. WTS exposure, either alone or with

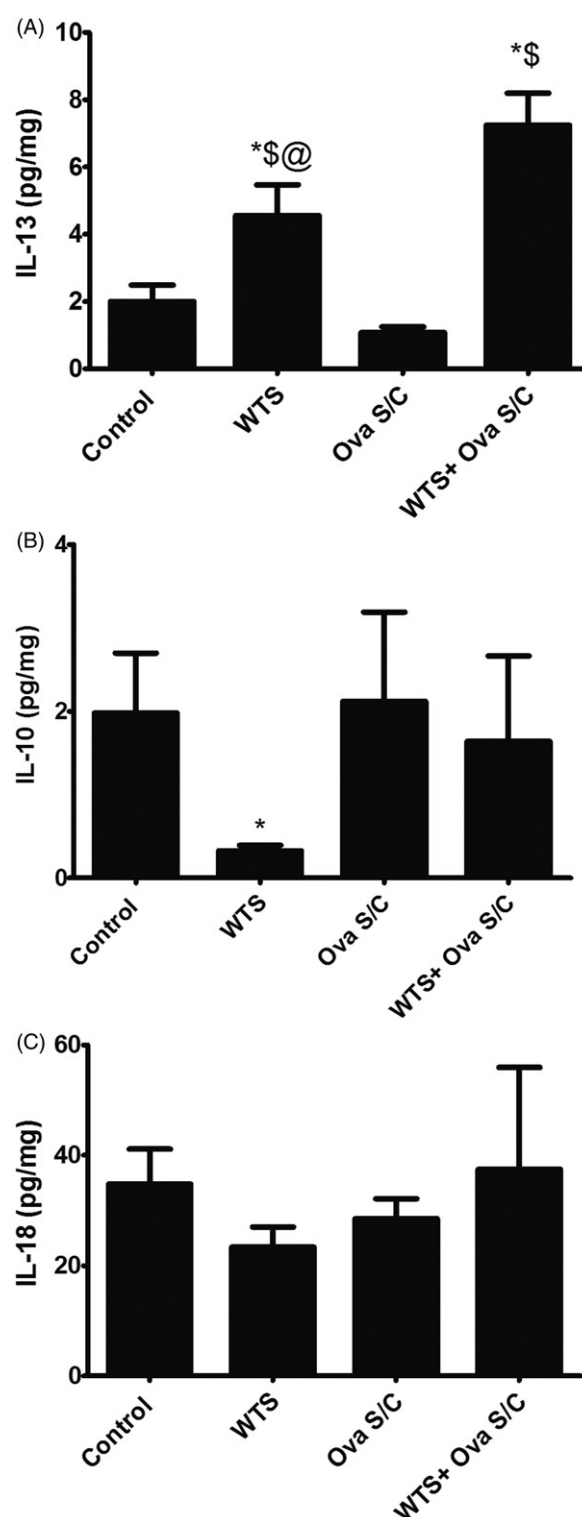


Figure 3. Effect of waterpipe smoke (WTS) exposure on level of inflammatory cytokines in mice lungs. Lungs were collected from mice that were exposed to fresh air and saline challenge (control group), 1 h of WTS daily for 4 weeks and saline challenge (WTS group), fresh air and ovalbumin challenge (Ova S/C group) or, WTS and ovalbumin challenge (WTS and Ova S/C group). Lungs were collected 24 h after the last challenge and last WTS exposure. Lung tissue homogenates were analyzed for (A) interleukin-13, (B) interleukin-10 and (C) interleukin-18. * indicates significant difference from control group, # indicates significant difference as compared to WTS exposure, \$ indicates significant difference as compared to Ova S/C, @ indicates significant difference as compared to Ova S/C and WTS exposure. Values represent the mean $\pm$ SEM of data from 6–8 mice in each group. $p < 0.05$ was considered significant.

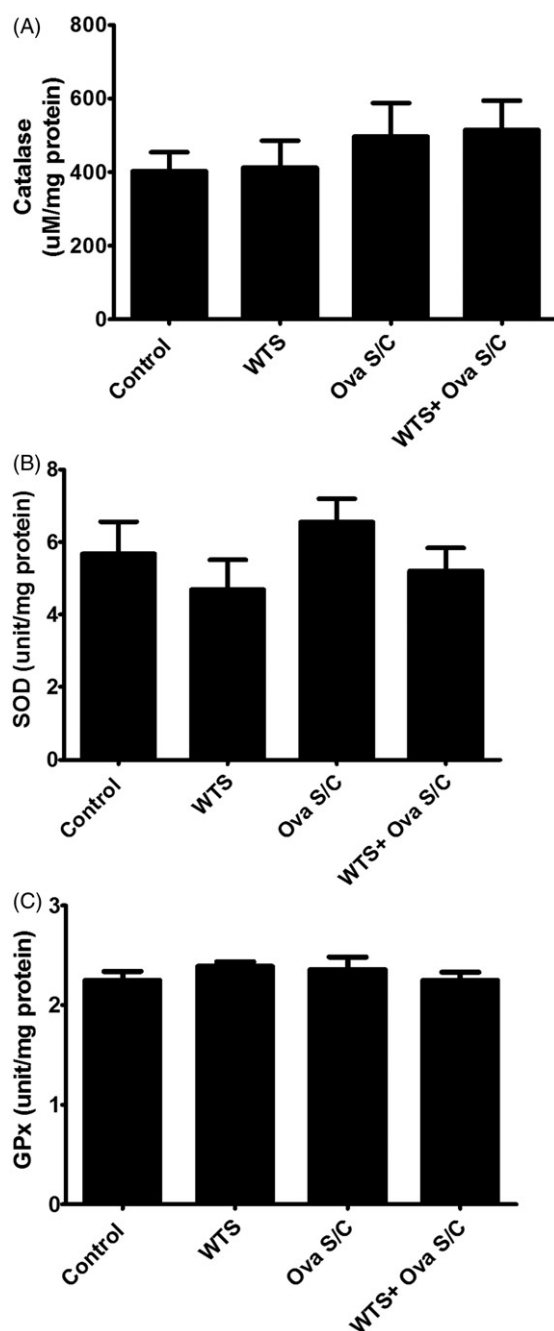


Figure 4. Effect of waterpipe smoke (WTS) exposure on level of oxidative stress markers in mice lungs. Lungs were collected from mice that were exposed to fresh air and saline challenge (control group), 1 h of WTS daily for 4 weeks and saline challenge (WTS group), fresh air and ovalbumin challenge (Ova S/C group) or, WTS and ovalbumin challenge (WTS and Ova S/C group). Lungs were collected 24 h after the last challenge and last WTS exposure. Lung tissue homogenates were analyzed for (A) catalase, (B) superoxide dismutase (SOD) and (C) glutathione peroxidase (GPx). Values represent the mean $\pm$ SEM of data from 6–8 mice in each group. $p < 0.05$ was considered significant.

Ova S/C, did not affect the level of anti-oxidative enzymes; SOD, catalase and GPx in mice lung.

In this study, we used whole-body exposure system to study the effect of WTS on airway inflammation. In inhalational experiments, the animals were either restrained and the exposure was localized to the nose (nose-only exposure) or the animals were unstrained and exposed to the atmosphere (whole-body exposure) (Cheng et al., 2010). In the

nose-only exposure system, the animals are under the restrain stress, which could affect airway inflammation (Kumlien Georen et al., 2008), and are unable to thermo-regulate (Cheng et al., 2010). Whole-body exposure system eliminates the effect of stressful restraint. However, some concerns are raised as oral exposures (ingestion) could occur which affect the extent of absorption (Cheng et al., 2010). In fact, whole-body exposure is commonly used in evaluating the effect of cigarette tobacco smoke in small animals (Anderson et al., 2004; Simons et al., 2005; Small et al., 2010). The developed model of waterpipe exposure is useful in studying the harmful consequences of waterpipe tobacco smoke inhalation in experimental animals.

The effect of acute or chronic cigarette smoking on airway inflammation has received a lot of attention in the recent years (Guatura et al., 2000; Marwick et al., 2004; Moerloose et al., 2005; Sato et al., 2008; Valenca et al., 2008). The popularity of WTS has increased in the recent years due to its sweet taste and the misconception that it is less harmful and addictive as compared to cigarette smoking (Tee et al., 2015). Though smoking is a known trigger for asthma, the prevalence of WTS has increased among asthma patients (Al-Sheyab et al., 2014; Martinasek et al., 2014). Thus, it was essential to examine the effect of WTS on airway inflammation in an animal model of asthma. Here, for the first time, we showed that simultaneous WTS exposure; 1 h for 28 days, along with Ova S/C resulted in a significant increase in airway recruitment of inflammatory cells, specifically; eosinophils, neutrophils and macrophages as compared to control and Ova S/C mice. Asthma is characterized by an eosinophilic type of airway inflammation (Watt et al., 2005) and the level of eosinophils in blood is correlated with the disease severity (Griffin et al., 1991). Eosinophils participate in airway inflammation by releasing several pro-inflammatory mediators (Rosenberg et al., 2013) and leukotrienes that contribute to airway smooth muscle contraction and vascular permeability (Broadley, 2006). The increased numbers of neutrophils in the airway by WTS indicates its role in intensifying the severity of airway inflammation (Jatakanon et al., 1999) as well as participation in the development of corticosteroid-resistant asthma (Wenzel et al., 1999). Mirsadraee and colleagues found that WTS exposure, five doses for 10 min, increased airway influx of eosinophils and reduced neutrophils and macrophages recruitment in Ova sensitized mice as compared to control non-sensitized mice (Mirsadraee et al., 2010). This inconsistency in results could be due to differences in WTS exposure duration. Hence, we predict that the observed changes in our study were due to the cumulative effect of chronic WTS as compared to an acute exposure. However, evaluating the effect of WTS on asthma exacerbation, after initiating the asthma model, is essential for our future studies.

WTS exposure increased the level of IL-13 in the lungs as compared to control. In addition, simultaneous WTS exposure and Ova S/C resulted in higher level of lung IL-13 as compared to Ova S/C mice. IL-13 is a pleiotropic T-helper (TH) 2 cytokine that is crucial for asthma pathogenesis (Corren, 2013). IL-13 transgenic mice, that overexpress

IL-13 in their airways, developed airway inflammation, airway hyperresponsiveness and airway remodeling (Zhu et al., 1999). Therefore, WTS plays a role in skewing airway inflammation toward the TH2 response. Evaluating the level of other TH2 cytokines such as IL-4 and IL-5, and TH1 cytokines such as IL-2, are of great importance for future studies. The lung level of IL-10 was reduced by WTS as compared to control mice. IL-10 is an anti-inflammatory cytokine that reduces antigen presentation (Pretolani & Goldman, 1997) and the secretion of different cytokines that are involved in asthma (Fiorentino et al., 1991). Khabour and colleagues found that acute WTS did not affect IL-10 level in tissue homogenates (Khabour et al., 2012). This inconsistency in results could be explained by the effects of chronic duration of administration of WTS. The noticed reduction of IL-10 and increased IL-13 levels by WTS could be the reason of the observed increased airway recruitment of inflammatory cells. IL-18 level was not changed by WTS, consistent with Wanger and colleagues finding (Wagner et al., 2015). IL-18, an inhibitory cytokine, induces the release of IFN- γ (Ushio et al., 1996) and enhances airway neutrophils recruitment and activation (Nocker et al., 1996). Further, the levels of IL-10 and IL-18 in mice lungs were not affected by simultaneous exposure to WTS and Ova S/C as compared to Ova S/C. In this study, the effect of WTS on other parameters of asthma phenotype; mucus metaplasia and airway hyperresponsiveness, was not evaluated and this of an outstanding importance in future studies.

Earlier reports found that WTS produces high amount of free radicals (Fahn et al., 1998) and activates tissue inflammation (D'Hulst et al., 2005). Free radicals are highly reactive molecules that randomly attack and adversely affect organs' function including the lungs (Frei et al., 1991). In this study, the level of anti-oxidative enzymes; SOD, catalase and GPx, were not changed by WTS. However, these results are inconclusive and more studies are needed to evaluate the effect of WTS on other oxidative stress markers such as hydrogen peroxide, thiobarbituric acid reactive products and 8-isoprostanes among others. It has been shown previously that a single session of WTS decreased the level of fractional exhaled nitric oxide in healthy volunteers (Hakim et al., 2011) and this could be due to the reduction in the level of inducible nitric oxide synthase (iNOS) (Hoyt et al., 2003). iNOS is known to activate oxidative stress pathways (Prado et al., 2011). Therefore, evaluating the role of simultaneous chronic WTS exposure and Ova S/C in regulating iNOS and the cross-talk between iNOS and oxidative stress markers is an essential element to be included in future studies.

Conclusion

The results of this study highlighted the potential harmful effect of chronic waterpipe smoking on potentiating airway inflammation in asthma. Chronic waterpipe smoking induced airway inflammation in control mice and potentiated airway inflammation in antigen-driven murine model of asthma through modulating the level of inflammatory cytokines.

Acknowledgments

The authors thank the Deanship of Research in Jordan University of Science and Technology (grant number 165/2015) for funding this work. The authors thank Vaidehi Thanawala, PhD, Rhadika Josh, PhD, Weam Alyacoub, BSc, Hanadi Boul, MSc, Yehya Almahmoud, BSc for their technical assistance.

Disclosure statement

The authors report no conflicts of interest.

Funding

This work was supported by Deanship of Research in Jordan University of Science and Technology (grant number 165/2015).

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