Original Paper

Water-Pipe Smoke Exposure-Induced Circulatory Disturbances in Mice, and the Influence of Betaine Supplementation Thereon

Abderrahim Nemmar^a Suhail Al-Salam^b Sumaya Beegam^a Priya Yuvaraju^a
Abderrahim Oulhaj^c Badreldin H. Ali^d

^aDepartment of Physiology, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, ^bDepartment of Pathology, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, ^cInstitute of Public Health, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, UAE; ^dDepartment of Pharmacology and Clinical Pharmacy, College of Medicine & Health Sciences, Sultan Qaboos University, Al-Khod, Sultanate of Oman

Key Words

Water-pipe smoke • Nose-only exposure • Betaine • Heart • Oxidative stress • Inflammation • Thrombosis

Abstract

Background/Aims: It has been shown, both experimentally and clinically, that water-pipe smoke (WPS) exposure adversely affects the cardiovascular system (CVS) through the generation of oxidative stress and inflammation. Betaine, a naturally occurring compound in common foods, has antioxidant and anti-inflammatory actions. However, its potential to mitigate the adverse effect of WPS on the CVS has never been reported before. This is the subject of this study in mice. **Methods:** Mice were exposed daily for 30 min to either normal air (control), or to WPS for two consecutive weeks. Betaine was administered daily by gavage at a dose of 10 mg/kg, 1h before either air or WPS exposure. **Results:** Betaine mitigated the *in vivo* prothrombotic effect of WPS in pial arterioles and venules. Moreover, it reversed the WPS-induced decrease in circulating platelets. Likewise, betaine alleviated platelet aggregation *in vitro*, and the shortening of activated partial thromboplastin time and prothrombin time induced by WPS. Betaine reduced the increase of plasminogen activator inhibitor-1 and fibrinogen concentrations in plasma induced by WPS. Betaine also diminished the WPS-induced increase of plasma concentrations of interleukin 6 and tumor necrosis factor α , and attenuated the increase of lipid peroxidation and superoxide dismutase. Immunohistochemical analysis of the heart revealed an increase in the expression of inducible nitric oxide synthase and cytochrome C by cardiomyocytes of the WPS-exposed mice. These effects were averted by betaine. **Conclusion:** Our findings suggest that betaine treatment significantly mitigated WPS-induced hypercoagulability, and inflammation, as well as systemic and cardiac oxidative stress.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Professor A. Nemmar

United Arab Emirates University, College of Medicine and Health Sciences, Department of Physiology, P.O. Box 17666, Al Ain, (UAE)
Tel. +971-37137533, E-Mail anemmar@uaeu.ac.ae / anemmar@hotmail.com

Introduction

Water-pipe smoking (also termed shisha, hubble bubble or narghile) is a traditional method of tobacco smoking typically used in Eastern societies, and is now becoming increasingly popular all over the world including Europe and North America [1, 2]. Reports from the Global Tobacco Surveillance System which involved more than 100 countries showed that, unlike cigarette smoking, which is either stable or decreasing generally, water-pipe smoking is increasing in several countries and is substituting cigarette smoking in Middle Eastern youth [3, 4]. A similar trend has also been seen in adults [3, 4]. In Europe and North America the number of water-pipe smoking bars has significantly increased [5, 6]. In the United States, the popularity of water-pipe smoking has significantly increased especially between adolescents and young adults [7, 8]. Young adults in the United States reported current (preceding 30 day) water-pipe smoking rates ranging between 7.2 to 20.3 %, past-year water-pipe smoking rates between 12.1 to 43.4 % and lifetime consumption rates ranging between 10 and 48.4% [7, 8]. Moreover, it has been reported that WPS among Danish, Swedish, and German youth is a gateway for regular cigarette smoking, suggesting the emergence of a new health risk [9].

Whereas the cardiovascular toxicity associated with cigarette smoking has been widely described, reports on the circulatory impact of water-pipe smoking are very limited [10]. Clinical studies have reported that water-pipe smoking increases blood pressure, heart rate, respiratory rate and carboxyhemoglobin (COHb) levels [11, 12]. More recently, it has been shown that one session of indoor water-pipe smoking caused an increase in COHb and plasma nicotine levels and altered several cardiovascular parameters [13]. Even studies which just show similarity of adverse effects between WPS and cigarette smoking are important, because they will help dispel the commonly – held erroneous notion that WPS use is safe, or safer than cigarette smoking. It is worth mentioning, that due to the fact that water-pipe smokers are often current or former cigarette smokers, clinical studies on water-pipe smoking face problems in investigating the sole effect of water-pipe smoking [3, 10]. We recently provided experimental evidence that water-pipe smoke (WPS) exposure (for 5 days or 30 days) accelerates coagulation and causes cardiac inflammation and oxidative stress [14, 15].

Considering the difficulty of the tobacco-dependence syndrome, treatment requires a multi-faceted approach, and involves compliance to medication, behavioral and other modalities of treatment [16]. Nevertheless, no study has been reported on how to impede the cardiovascular toxicity of water-pipe smoking. Diet and nutrition play a fundamental role in cardiovascular prevention and in maintaining physiological homeostasis [17-19]. Betaine (known under various synonyms including trimethylglycine, glycine betaine, lycine and oxynurine) is a methyl derivative of the amino acid glycine which acts as a methyl donor in a reaction converting homocysteine to methionine [20]. It can be obtained from food that possesses betaine such as wheat, bran, spinach and seafood or choline-containing compounds generated in the liver and kidney from choline through the effect of choline oxidase and betaine-aldehyde dehydrogenase [20]. Experimental studies in rats have reported that betaine has a protective effect against ethanol-induced hepatic steatosis [21], CCl₄-induced kidney injury [22] and ethanol-induced oxidative stress in testes [23]. A recent study in mice has shown that betaine averts photochemically induced thrombosis [24]. However, the possible alleviating effect of betaine on WPS-induced cardiovascular toxicity has, as far as we are aware, not been studied before.

Since cardiovascular effects of WPS are mediated, at least partly, by oxidative stress [14, 15], and betaine has protective or ameliorative effects against some experimental diseases and conditions involving oxidative stress [23, 25], we thought that it was relevant to assess the possible ameliorating influence of betaine on WPS-induced cardiovascular toxicity in mice.

Therefore, we assessed in this work the subacute (2 weeks) effects of WPS on coagulation, inflammation and oxidative stress, and the possible mitigating effects of betaine thereon.

Material and Methods

Animals and treatments

This project was reviewed and approved by the Institutional Review Board of the United Arab Emirates University, College of Medicine and Health Sciences, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

WPS exposure

C57BL/6 mice (Taconic Farms Inc., Germantown, NY, USA) were housed in a conventional animal house and maintained on a 12-hour light-dark cycle (lights on at 6:00 am). The animals were placed in cages and supplied with pelleted food and water *ad libitum*. Following 1 week of acclimatization, animals were randomly divided into air (control) and WPS-exposed groups.

Mice were placed in soft restraints and connected to the exposure tower [15, 26-28]. The animals were exposed to either WPS or air through their noses using a nose-only exposure system connected to a water-pipe (InExpose System, Scireq, Canada). Animals were exposed to a commercially available honey-flavoured tobacco which is composed of tobacco, glycerin, molasses and natural flavor with nicotine and tar contents of 0.05% and 0%, respectively (Al Fakher Tobacco Trading, Ajman, UAE). Tobacco was lit with an instant light charcoal disk (Star, 3.5 cm diameter and 1 cm width). As is the case for human use, the smoke from the water-pipe passes first through the water before it was drawn into the exposure tower. The exposure regimen is controlled by a computerized system (InExpose System, Scireq, Canada). A computer-controlled puff was generated every minute, leading to 2 s puff duration of WPS exposure followed by 58 s of fresh air. The duration of an exposure session was 30 min/day [15, 26-28]. The latter was selected from a recently published study that has assessed the cardiorespiratory effects of WPS in human subjects [29]. Mice were exposed for 2 weeks. Either betaine (10 mg/kg) (betaine hydrochloride; Sigma-Aldrich Co., St Louis, MO, USA) or water was given by gavage to mice 1h before exposure to air or WPS.

Blood collection and cell count

At the end of the exposure period, animals were anesthetized intraperitoneally with sodium pentobarbital (45 mg/kg), and then blood was drawn from the inferior vena cava in EDTA (4 %). A sample was used for platelets and white blood cells (WBC) counts using an ABX VET ABC HEMATOLOGY ANALYZER with a mouse card (ABX Diagnostics, Montpellier, France). The remaining blood was centrifuged for 15 min at 4°C at 900 g, and the plasma samples obtained were stored at -80°C pending analysis.

Experimental pial arterioles and venules thrombosis model

In a separate experiment, *in vivo* pial arteriolar and venular thrombogenesis was assessed at the end of the 2 week exposure period to WPS or air with or without betaine treatment, according to a previously described technique [30-32]. Briefly, the trachea was intubated after induction of anesthesia with urethane (1mg/g body weight, i.p.), and a 2F venous catheter (Portex, Hythe, UK) was inserted in the right jugular vein for the administration of fluorescein (Sigma, St. Louis, MO, USA). Thereafter, a craniotomy was first performed on the left side, using a microdrill, and the dura was stripped open. Only untraumatized preparations were used, and those showing trauma to either microvessels or underlying brain tissue were discarded. The animals were then placed on the stage of a fluorescence microscope (Olympus, Melville, NY, USA) attached to a camera and DVD recorder. A heating mat was placed under the mice and body temperature was raised to 37°C, as monitored by a rectal thermoprobe connected to a temperature reader (Physitemp Instruments, NJ, USA). The cranial preparation was moistened continuously with artificial cerebrospinal fluid of the following composition (mM): NaCl 124, KCl 5, NaH₂PO₄ 3, CaCl 2.5, MgSO₄ 4, NaHCO₃ 23 and glucose 10, pH 7.3-7.4. A field containing arterioles and venules 15-20 µm in diameter was chosen. Such a field was taped prior to and during the photochemical insult. The photochemical insult was carried out by injecting fluorescein (0.1ml/mouse of 5% solution) via the jugular vein, which was allowed to circulate for 30-40 sec. The cranial preparation was then exposed to stabilized mercury light. The combination produces endothelium injury of the arterioles and venules. This, in turn, causes platelets to adhere at the site of endothelial damage and then aggregate. Platelet aggregates and thrombus formation grow in size until complete arteriolar or venular occlusion. The time from the photochemical injury until full vascular occlusion (time to flow stop) in arterioles and venules was measured in seconds. At the end of the experiments, the animals were euthanized by an overdose of urethane.

Platelet aggregation in mouse whole blood

In mice exposed to WPS or air, with or without betaine treatment for 2 weeks, the platelet aggregation assay in whole blood was performed with slight modification as described before [33, 34]. After anesthesia, blood from separate animals was withdrawn from the inferior vena cava and placed in citrate (3.2%), and 100- μ l aliquots were added to the well of a coagulometer (MC 1 VET, Merlin, Lemgo, Germany). The blood samples were incubated at 37.2°C with adenosine diphosphate (1 μ M) for 3 min, and then stirred for another 3 min. At the end of this period, 25- μ l samples were removed and fixed on ice in 225 ml cellFix (Becton Dickinson, Franklin Lakes, NJ, USA). After fixation, single platelets were counted in a VET ABX Micros with mouse card (ABX, Montpellier, France). The degree of platelet aggregation, assessed as a fall in single platelets counted in the presence of ADP, was determined in whole blood collected from mice exposed to either WPS or air with or without betaine treatment. These results were compared with that obtained from untreated (without ADP but with saline) whole blood obtained from control (unexposed) mice.

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) measurement in plasma in vitro

At the end of exposure to WPS or air with or without betaine treatment, mice were anesthetized, blood was withdrawn from the inferior vena cava and placed in citrate solution (3.8%) (ratio of blood to anticoagulant: 9:1). The PT was measured [15, 31, 35] on freshly collected platelet-poor plasma with human relipidated recombinant thromboplastin (Recombiplastin; Instrumentation Laboratory, Orangeburg, NY, USA) in combination with a coagulometer (MC 1 VET, Merlin, Lemgo, Germany). The aPTT was measured [31, 35] with the automated aPTT reagent from bioMerieux (Durham, NC, USA) with the same coagulometer.

Measurement of fibrinogen and plasminogen activator inhibitor-1 (PAI-1) concentrations in plasma

The plasma concentrations of fibrinogen and PAI-1 were measured using ELISA kits (Molecular Innovation, Southfield, MI, USA).

Measurement of interleukin 6 (IL-6), tumor necrosis factor α (TNF α), superoxide dismutase (SOD) and lipid peroxidation levels in plasma

The plasma concentrations of IL-6 and TNF α were determined using ELISA Kits (Duo Set, R & D Systems, Minneapolis, MN, USA). The activity of SOD was measured according to the vendor's protocol (Cayman Chemicals, Michigan, USA). NADPH-dependent membrane lipid peroxidation was measured as thiobarbituric acid reactive substance using malonaldehyde as standard (Sigma-Aldrich Fine Chemicals, St Louis, MO, USA) [27, 36].

Histopathology

Hearts were excised, washed with ice-cold saline, blotted with filter paper and weighed. They were fixed in 10% neutral formalin. Each heart was coronally sectioned into 4 pieces, casseted and dehydrated in increasing concentrations of ethanol, cleared with xylene and embedded with paraffin. Three- μ m sections were prepared from paraffin blocks and stained with haematoxylin and eosin. The stained sections were evaluated blindly using light microscopy by a histopathologist who participates in this project (SA).

Immunohistochemistry

Five- μ m sections were cut, de-waxed with xylene and rehydrated with graded alcohol. The slides were then placed in a 0.01 M citrate buffer solution (pH=6.0) and pre-treatment procedures to unmask the antigens were performed in a water bath for 60 minutes. Sections were treated with peroxidase and protein block for 15 min each and then incubated with primary antibodies iNOS (Rabbit Polyclonal antibody, Thermo Scientific™, Waltham, MA, USA) and cytochrome C (D18 C7) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) for one hour. After conjugation with primary antibodies, sections were incubated with biotin-labeled secondary antibody (Thermo Scientific, Waltham, MA, USA) for 20 minutes at room temperature. Finally, sections were incubated with streptavidin-peroxidase complex for 20 minutes at room temperature (Thermo Scientific, Waltham, MA, USA), DAB chromogen (Thermo Scientific, Waltham, MA, USA) was added and counter staining made with haematoxylin. Appropriate positive controls were used. For negative control, the primary antibody was not added to sections and the whole procedure was carried out in the same manner as mentioned above.

The immunohistochemical staining of heart tissue for iNOS and cytochrome C was scored semi quantitatively and blindly by our histopathologist (S. A.) on a scale of 0–4 according to the percentage of staining of heart muscles in four slides of each specimen, and each slide contained 4 equal coronal slices of the heart. A score of 0 was assigned if the expression was between 0–10%, 1 for 11–25%, 2 for 26–50%, 3 for 51–75% and 4 for more than 75%.

Statistics

One-way analysis of variance (ANOVA) and all graphs were produced using GraphPad Prism Version 5 for Windows software (Graphpad Software Inc., San Diego, USA). Data were expressed as means $\pm$ SEM. To adjust the *p* values obtained from multiple comparisons, we used the Bonferroni-Holm method. This method is based on a sequential approach whose goal is to increase the power of the statistical tests while keeping under control the family-wise Type I error [37, 38]. The R software version 3.0.3 (R Development Core Team, 2011) was used for this analysis. The Bonferroni-Holm method was carried out using the *p.adjust* function from the R package stats. *P* values less than 0.05 were considered significant.

Results

Effect of WPS on WBC and platelet numbers and influence of betaine treatment

Figure 1 shows the effect of WPS on WBC and platelet numbers (*n* = 6). WPS exposure for 2 weeks caused a slight but insignificant increase in WBC numbers compared with the control group (Fig. 1A). However, the number of platelets was significantly decreased in the WPS-exposed group compared with the air exposed mice. The latter effect was significantly reversed by betaine treatment (Fig. 1B).

Effect of WPS on thrombosis and influence of betaine treatment

Compared with the air-exposed group, WPS exposure for 2 weeks induced a quickening of the occlusion time in pial arterioles and venules in photochemically-injured vessels (*n* =

Fig. 1. Circulating numbers of white blood cells (WBC, A) and platelets (B) at the end of the 2 weeks exposure period to air (control) or water pipe smoke (WPS) with or without betaine treatment. Data are mean $\pm$ SEM (*n* = 6).

Fig. 2. Thrombotic occlusion time in pial arterioles (A) and venules (B) at the end of the 2 weeks exposure period to air (control) or water pipe smoke (WPS) with or without betaine treatment. Data are mean $\pm$ SEM (*n* = 6).

Fig. 3. *In vitro* platelet aggregation in whole blood in the presence or absence of adenosine diphosphate (ADP), at the end of the 2 weeks exposure period to either air (control) or water pipe smoke (WPS) with or without betaine treatment. Data are mean $\pm$ SEM (n = 5-7).

Fig. 4. Activated partial thromboplastin time (aPTT, A) and Prothrombin time (PT, B) at the end of the 2 weeks exposure period to air (control) or water pipe smoke (WPS) with or without betaine treatment. Data are mean $\pm$ SEM (n = 5).

Fig. 5. Fibrinogen (A) and plasminogen activator inhibitor-1 (PAI-1, B) concentrations in the plasma at the end of the 2 weeks exposure period to air (control) or water pipe smoke (WPS) with or without betaine treatment. Data are mean $\pm$ SEM (n = 7-8).

6) (Fig. 2A-B). The thrombotic occlusion time was significantly increased in the betaine+air group compared with the air group suggesting the anti-thrombotic effect of betaine. Likewise, the shortening of the thrombotic occlusion time in arterioles and venules induced by WPS was significantly alleviated by betaine treatment (Fig. 2).

Fig. 6. Tumor necrosis factor α (TNF α , A) and interleukin-6 (IL-6, B) concentrations in the plasma at the end of the 2 weeks exposure period to air (control) or water pipe smoke (WPS) with or without betaine treatment. Data are mean $\pm$ SEM (n = 5-6).

Fig. 7. Lipid peroxidation (LPO, A) and superoxide dismutase (SOD, B) levels in the plasma at the end of the 2 weeks exposure period to air (control) or water pipe smoke (WPS) with or without betaine treatment. Data are mean $\pm$ SEM (n = 6).

Effect of WPS on platelet aggregation in whole blood *in vitro* and influence of betaine treatment

Following the *in vitro* addition of ADP (1 μ M), whole blood collected from mice exposed to WPS showed a significant ($P < 0.00001$) difference in platelet aggregation compared with that obtained from mice exposed to air (n = 5-7) (Fig. 3).

Similar to our *in vivo* observations, in the presence of ADP, platelet aggregation *in vitro* was significantly reversed in the betaine+air group compared with the air group. Moreover, platelet aggregation *in vitro* was significantly mitigated in the betaine+WPS group compared with the WPS group (Fig. 3).

Effect of WPS on aPTT and PT *in vitro* and influence of betaine treatment

Figure 4 illustrates the effect of 2 week nose-only exposure to either WPS or air with or without betaine on aPTT and PT in mouse plasma (n = 5). The shortening of aPTT and PT reveals hypercoagulability. Exposure to WPS induced a significant quickening of aPTT compared with plasma obtained from air-exposed mice (Fig. 4A). Correspondingly, the PT was also significantly decreased in plasma collected from the WPS-exposed group versus that collected from animals exposed to air (Fig. 4B). Betaine treatment has significantly ameliorated the shortening of both aPTT and PT induced by WPS exposure (Fig. 4).

Effect of WPS on fibrinogen and PAI-1 concentrations in plasma and influence of betaine treatment

Figure 5 illustrates the effect of air or WPS on fibrinogen and PAI-1 plasma concentrations and the influence of betaine thereon (n = 7-8). Compared with air-exposed mice, exposure to WPS for 2 weeks induced a statistically insignificant increase of plasma fibrinogen ($P = 0.07$)

Fig. 8. Representative light microscopy sections of H and E stained heart tissues of mice at the end of the 2 weeks exposure period to air (control) or water pipe smoke (WPS) with or without betaine treatment. (A and B) Representative heart sections of the left ventricle obtained from air-exposed mice. (C and D) Representative heart sections of the left ventricle obtained from air-exposed mice treated with betaine. (E and F) Representative heart sections of the left ventricle obtained from WPS-exposed mice. (G and H) Representative heart sections of the left ventricle obtained from WPS-exposed mice treated with betaine.

and a significant increase in PAI-1 ($P < 0.00001$) (Fig. 5). These effects were significantly reversed by betaine treatment (Fig. 5).

Effect of WPS on TNF α and IL-6 concentrations in plasma and the influence of betaine treatment

Figure 6 shows the effect of WPS or air exposure on the concentrations of TNF α and IL-6 in the plasma ($n = 5-6$). Following the exposure to WPS, the concentrations of TNF α and IL-6 in the plasma were significantly increased compared with the control group (Fig. 6). Betaine treatment did not affect the concentrations of either TNF α or IL-6 in air-exposed mice, but it significantly prevented the increase of both pro-inflammatory cytokines in WPS-exposed mice.

Effect of WPS on LPO and SOD activity in the plasma and influence of betaine treatment

Following exposure to WPS, LPO concentration and SOD activity in the plasma were significantly increased compared with the air-exposed group ($n = 6$) (Fig. 7). The treatment of mice with betaine significantly reduced the effects induced by WPS exposure.

Effect of WPS on heart histology and expression of iNOS and cytochrome C, and influence of betaine treatment

There were no significant morphologic changes in H&E stained sections between the air, air+betaine, WPS and betaine+WPS groups (Fig. 8).

There was a clear increase in the expression of inducible iNOS by cardiomyocytes and non-cardiomyocytes in the hearts of WPS-exposed mice (score 3; Fig. 9E-F) when compared with the air-exposed group (score 1; Fig. 9A-B). Moreover, the extent and the pattern of

Fig. 9. Immunohistochemical analysis of the heart tissue sections for the detection of inducible nitric oxide synthase (iNOS) at the end of the 2 weeks exposure period to air (control) or water pipe smoke (WPS) with or without betaine treatment. (A) Representative section of the heart of air-exposed mice showing focal expression of iNOS in the left ventricle (thick arrow). (B) Representative section of the left ventricle of air-exposed mice showing expression of iNOS by few non-cardiomyocytes (arrow head) and a very few scattered cardiac myocytes (thin arrow). (C and D) Representative sections showing no expression of iNOS in the heart of air-exposed mice treated with betaine. (E) Representative section of the heart of WPS-exposed mice showing diffuse expression of iNOS in the left ventricle (thick arrow). (F) Representative section of the left ventricle of WPS-exposed group showing very high expression of iNOS in cardiac myocytes (thin arrow) and non-cardiomyocytes (arrow head). (G and H) Representative sections showing marked reduction in the expression of iNOS in the heart of WPS-exposed mice treated with betaine. Immunoperoxidase streptavidin biotin method.

Fig. 10. Immunohistochemical analysis of the heart tissue sections for the detection of cytochrome C at end of the 2 weeks exposure period to air (control) or water pipe smoke (WPS) with or without betaine treatment. (A) Representative section of the the left ventricle of air-exposed mice showing focal expression of cytochrome C in the left ventricle (thick arrow). (B) Representative section of the left ventricle of air-exposed mice showing focal expression of cytochrome C by few scattered cardiac myocytes (arrow head). (C and D) Representative sections showing less expression of cytochrome C in the heart of air exposed mice treated with betaine when compared with air-exposed heart of mice without betaine treatment (A and B). (E) Representative section of the left ventricle of WPS-exposed mice showing diffuse expression of cytochrome C (thick arrow). (F) Representative section of the left ventricle of WPS-exposed group showing high expression of cytochrome C by cardiac myocytes (arrow head). (G and H) Representative sections showing marked reduction in the expression of cytochrome C in cardiac myocytes in WPS+betaine group when compared with WPS group (E and F). Immunoperoxidase streptavidin biotin method.

expression of iNOS is also different as its expression in the normal air exposed group was focal in the left ventricle while in the WPS group, the expression involved both left and right ventricles. Interestingly, there was a clear decrease in the expression of iNOS in the heart following treatment with betaine both in air-exposed (score 0; Fig. 9C-D) and WPS-exposed (score 1; Fig. 9G-H) groups.

There was also an increase in the expression of cytochrome C by cardiac myocytes in the hearts of WPS-exposed mice (score 2; Fig. 10E-F) when compared with the air-exposed group (score 1; Fig. 10A-B). There was a clear decrease in the expression of cytochrome C in the heart following treatment with betaine both in air (score 0; Fig. 10C-D)- and WPS (score 1; Fig. 10G-H)-exposed groups.

Discussion

The present study provided experimental evidence that nose-only exposure to WPS for 2 weeks induces circulatory disturbances, systemic inflammation and oxidative stress. The immunohistochemical analysis of the heart also confirmed the occurrence of oxidative stress. Importantly, we showed that treatment with the food additive betaine alleviated the circulatory disturbances, systemic inflammation and oxidative stress. It also reduced cardiac oxidative stress.

Recent literature emphasizes the potential therapeutic effects of micronutrients found in natural products, indicating positive applications for controlling the pathogenesis of CVS disease driven by cardiovascular risk factors and oxidative stress [17-19]. Consequently, nutritional compounds that display anti-inflammatory and antioxidant effects have specific applications in preventing oxidative stress-related injury, which characterizes the pathogenesis of CVS diseases. Betaine is a naturally occurring antioxidant agent which is found in a range of foods including wheat, shellfish, spinach, and sugar beets [20]. We have recently demonstrated that exposure to WPS for 5 days and one month increased blood pressure and thrombosis and induced inflammation and oxidative stress in the heart [14, 15]. Therefore, in the present study we have assessed the subacute (2 weeks) effects of WPS on a comprehensive set of circulatory parameters and the possible palliative effect of betaine thereon.

We have recently shown that betaine given i.p. (10 and 40 mg/kg) averted photochemically-induced thrombosis in pial microvessels *in vivo* [24]. Here, we assessed, probably for the first time, a more relevant way of administration of betaine, i.e., by gavage, and evaluated the interaction between betaine (10 mg/kg) and WPS-induced cardiovascular toxicity. The dose of betaine used in the present study in mice (10 mg/kg) would correspond, for an average person of 70 kg, to 0.7 g. This dose is probably close to the dietary intake of betaine in humans. In fact, it has been estimated that dietary intake of betaine ranges from an average of 1 g/day to a high of 2.5 g/day (for a diet high in whole wheat and seafood) [20]. However, an accurate extrapolation to human dosage requires additional work. In the present study, we showed that oral administration of betaine significantly mitigated the decrease in circulating platelet numbers and shortening of thrombotic occlusion time in pial venules and arterioles. The reduction in circulating platelets is suggestive of platelet activation *in vivo*, and shortening of the thrombotic occlusion time is indicative of a prothrombotic effect of WPS at the studied time point. A decrease in platelet numbers following pulmonary exposure to particulate air pollution has been described in animal and clinical reports [30, 39]. A decrease in platelet count and augmented plasma levels of soluble CD40 ligand (sCD40L), a glycoprotein released from activated platelets which promotes inflammation, thrombosis and the formation of homotypic aggregates, has been reported in patients with coronary heart disease exposed to particulate air pollution [39]. CD40L can potentially activate CD40 bearing cells such as leukocytes, endothelial cells and platelets themselves [40]. The decrease in platelets has been explained by either a direct activation of the platelets or via an increase in adhesion molecules which allow them to adhere to various tissues including

in the lung, spleen and vascular cells [39]. In line with our findings observed at 2 weeks of exposure to WPS, it has been shown that 5 days exposure to WPS induced a decrease in platelet count and the occurrence of thrombotic events in mice [27]. More importantly, we, presently, showed that betaine treatment attenuated WPS-induced thrombotic events. To verify our *in vivo* findings, we assessed the palliative effect of betaine on platelet aggregation in whole blood of mice exposed to either air or WPS with or without betaine. Our data show that, in the presence of ADP, WPS induced *in vitro* platelet aggregation, suggesting a priming of platelet activation and their contribution to the development of a thrombotic predisposition, when such primed platelets come across a (mildly) injured vessel wall, as observed *in vivo* in the current study. Interestingly, and in line with our *in vivo* findings, betaine alleviated platelet aggregation induced by WPS.

Cigarette smoking and particulate air pollution exposures have been showed to be associated with changes in global coagulation function, indicating a propensity to hypercoagulability [41, 42]. We have recently demonstrated that exposure to WPS for 1 month induces shortening of PT and aPTT [15]. In the present study, we demonstrate the occurrence of quickening of PT and aPTT after 2 weeks of exposure to WPS, and that betaine administration significantly alleviated this effect. The latter finding confirms the antithrombotic effect of betaine against WPS-induced coagulation disturbances.

Furthermore, our data show that betaine treatment reversed the increase in PAI-1 and the statistically insignificant increase in fibrinogen caused by WPS exposure for 2 weeks. PAI-1 is an effective inhibitor of fibrinolysis and its increase has been documented as an independent risk factor for the development of ischemic cardiovascular events and has been associated with inflammation and atherosclerosis, whereas fibrinogen is an acute-phase protein that enhances blood viscosity and stimulates thrombosis [43, 44]. These findings corroborate the observed alleviating effect of betaine on thrombosis *in vivo*, and platelet aggregation and PT and aPTT *in vitro* and suggest that betaine is effective in mitigating the impairment of the fibrinolytic system and activation of blood coagulation accompanying WPS exposure. It has been recently reported that pretreatment with another natural product, namely curcumin, with antioxidant and anti-inflammatory properties prevented PAI-1 increase in plasma and platelet aggregation both *in vivo* and *in vitro* [45].

To gain more insights into the mechanism underlying the palliative effect of betaine on WPS induced circulatory disturbances, we measured, in plasma, markers of oxidative stress comprising LPO and the antioxidant enzyme SOD and markers of inflammation including TNF α and IL-6. Here, we found that WPS caused a significant increase in lipid peroxidation and SOD, indicating that WPS could trigger adaptive responses that counterbalance the potentially damaging activity of oxygen radicals induced by WPS exposure. Interestingly, betaine significantly alleviated the increase of both LPO and SOD. Our study corroborates the recent findings of Hagar et al. [46] who showed that betaine mitigated the increase of LPO and total antioxidant status caused by cisplatin-induced nephrotoxicity. Reactive oxygen species are able to actuate redox-sensitive transcription factors, nuclear factor- κ B, and activator protein-1, initiating the genes of pro-inflammatory cytokines TNF α , IL-1 β , and IL-6 [47]. These proinflammatory cytokines play a key role in the pathogenesis of atherosclerosis and their concentrations were elevated in plasma and the myocardium of patients with heart failure [48, 49]. In the present study, we also found that betaine averted the increase in plasma concentrations of IL-6 and TNF α caused by a 2-week exposure to WPS, suggesting the systemic anti-inflammatory effects of betaine. Recent studies showed that betaine mitigates the increase of TNF α caused by cisplatin-induced nephrotoxicity [46] and recovery from hypothalamic neural injury by inhibiting astrogliosis and inflammation in fructose-fed rats [50].

We have recently showed the occurrence of oxidative stress in heart homogenate at 5 days and 1 month WPS exposure [15, 27]. However, no immunohistochemical analysis has been reported following exposure to WPS, and no study has reported the possible mitigating effect of betaine thereon. While we found no morphological changes in the heart following WPS exposure, there was a clear increase in the expression of iNOS by cardiomyocytes and

non-cardiomyocytes cells in the heart of mice exposed to WPS compared with air-exposed group. Moreover, the extent and the pattern of expression of iNOS was also different as its expression in the normal air exposed group was focal in the left ventricle while in WPS group, the expression involved both left and right ventricles. The activation of iNOS generates a large amount of NO which is associated with the elevation of production of reactive oxygen species, which are detrimental to various organs [32]. An increased iNOS protein and mRNA expression in the myocardium has been reported in mice with doxorubicin-induced cardiotoxicity [51]. Also, a marked cytoplasmic staining for iNOS in pancreatic islet cells and some acinar cells was seen in diabetic mice exposed to particulate air pollution [52]. Moreover, iNOS knockout mice had a significant reduction of cytokines in the lung after exposure to ambient particles [53]. Interestingly, here, we show a clear decrease in the expression of iNOS in the heart following treatment with betaine both in air-exposed and WPS-exposed groups. This finding, along with the decrease of markers of oxidative stress in plasma, illustrates the important antioxidant effect of betaine, which mitigates the cardiovascular toxicity of WPS. Along with iNOS, there was also a marked increase in the expression of cytochrome C by cardiomyocytes in the hearts of WPS-exposed mice. Cytochrome C is an important part of the electron transport chain in mitochondria. It has been recently reported that exposure of mice to side-stream smoke causes oxidative stress leading to an increase in cytochrome C release in hearts indicating mitochondrial damage [54]. Moreover, it has been demonstrated that TNF- α increases the release of cytochrome C from mitochondria in cytoplasm, which is known to bind with the adaptor apoptotic protease activator factor-1 and recruit caspase-9/3 that ultimately lead to cell apoptosis [55]. In our study, remarkably, we found a clear decrease in the expression of cytochrome C in the heart following treatment with betaine both in air and WPS-exposed groups. Our findings suggest that betaine mitigated the mitochondrial damage by reducing oxidative stress in the heart, illustrated by the decrease in the expression of iNOS and systemic inflammation and oxidative stress. A previous report showed that the use of antioxidant N-acetylcysteine was effective in alleviating myocardial dysfunction, Ca²⁺ mishandling and mitochondrial damage in mice exposed to second-hand smoke [54].

In the present study, betaine was given only prophylactically, thus, additional studies are needed to assess its therapeutic effects (i.e., after the induction of cardiovascular events by WPS), and the effects of betaine following chronic exposure to WPS. Moreover, the possible interaction of betaine with WPS, and the effects of betaine's metabolites remain to be established.

In conclusion, the current experiments have shown that WPS exposure for two weeks induced coagulation disturbances, systemic inflammation and oxidative stress. Moreover, immunohistochemical analysis of the heart revealed the occurrence of oxidative stress and mitochondrial damage. Importantly, we showed that betaine treatment mitigated WPS-induced cardiovascular toxicity. Our study provided experimental evidence that the use of betaine, pending additional pharmacological and toxicological studies, can be considered a useful agent, and may have the potential to alleviate the cardiovascular toxicity induced by WPS.

Acknowledgments

This work was supported by funds of the College of Medicine and Health Sciences grant (31M215), and United Arab Emirates University UPAR (31M189) and center-based interdisciplinary (31R052) grants.

The authors would like to thank Prof Gerald Blunden (University of Portsmouth, UK) for critically reading the manuscript.

Disclosure Statement

None.

References

- 1 Chaouachi K: Hookah (Shisha, Narghile) Smoking and Environmental Tobacco Smoke (ETS). A critical review of the relevant literature and the public health consequences. *Int J Environ Res Public Health* 2009;6:798-843.
- 2 Maziak W: The global epidemic of waterpipe smoking. *Addict Behav* 2011;36:1-5.
- 3 Maziak W: Rise of waterpipe smoking. *BMJ* 2015;350:h1991.
- 4 Agaku IT, Filippidis FT, Vardavas CI, Odukoya OO, Awopegba AJ, Ayo-Yusuf OA, Connolly GN: Poly-tobacco use among adults in 44 countries during 2008-2012: evidence for an integrative and comprehensive approach in tobacco control. *Drug Alcohol Depend* 2014;139:60-70.
- 5 Warren CW, Lea V, Lee J, Jones NR, Asma S, McKenna M: Change in tobacco use among 13-15 year olds between 1999 and 2008: findings from the Global Youth Tobacco Survey. *Glob Health Promot* 2009;16:38-90.
- 6 Jackson D, Aveyard P: Waterpipe smoking in students: prevalence, risk factors, symptoms of addiction, and smoke intake. Evidence from one British university. *BMC Public Health* 2008;8:174.
- 7 Soule EK, Lipato T, Eissenberg T: Waterpipe tobacco smoking: A new smoking epidemic among the young? *Curr Pulmonol Rep* 2015;4:163-172.
- 8 Maziak W, Ben TZ, Jawad M, Afifi R, Nakkash R, Akl EA, Ward KD, Salloum RG, Barnett TE, Primack BA, Sherman S, Cobb CO, Sutfin EL, Eissenberg T: Consensus statement on assessment of waterpipe smoking in epidemiological studies. *Tob Control* DOI:10.1136/tobaccocontrol-2016-052958.
- 9 Jensen PD, Cortes R, Engholm G, Kremers S, Gislum M: Waterpipe use predicts progression to regular cigarette smoking among Danish youth. *Subst Use Misuse* 2010;45:1245-1261.
- 10 Knishkowsky B, Amitai Y: Water-pipe (narghile) smoking: an emerging health risk behavior. *Pediatrics* 2005;116:e113-e119.
- 11 Al Kubati M, Al Kubati AS, al'Absi M, Fiser B: The short-term effect of water-pipe smoking on the baroreflex control of heart rate in normotensives. *Auton Neurosci* 2006;126-127:146-149.
- 12 Hakim F, Hellou E, Goldbart A, Katz R, Bentur Y, Bentur L: The acute effects of water pipe smoking on the cardio- respiratory system. *Chest* 2011;139:775-781.
- 13 Bentur L, Hellou E, Goldbart A, Pillar G, Monovich E, Salameh M, Scherb I, Bentur Y: Laboratory and clinical acute effects of active and passive indoor group water-pipe (narghile) smoking. *Chest* 2014;145:803-809.
- 14 Nemmar A, Yuvaraju P, Beegam S, Ali BH: Short-term nose-only water-pipe (shisha) smoking exposure accelerates coagulation and causes cardiac inflammation and oxidative stress in mice. *Cell Physiol Biochem* 2015;35:829-840.
- 15 Nemmar A, Yuvaraju P, Beegam S, John A, Raza H, Ali BH: Cardiovascular effects of nose-only water-pipe smoking exposure in mice. *Am J Physiol Heart Circ Physiol* 2013;305:H740-H746.
- 16 Klein JW: Pharmacotherapy for Substance Use Disorders. *Med Clin North Am* 2016;100:891-910.
- 17 Grassi D, Desideri G, Di GP, De FM, Fellini E, Cheli P, Ferri L, Ferri C: Tea, flavonoids, and cardiovascular health: endothelial protection. *Am J Clin Nutr* 2013;98:1660S-1666S.
- 18 Grassi D, Desideri G, Ferri C: Flavonoids: antioxidants against atherosclerosis. *Nutrients* 2010;2:889-902.
- 19 Hooper L, Kay C, Abdelhamid A, Kroon PA, Cohn JS, Rimm EB, Cassidy A: Effects of chocolate, cocoa, and flavan-3-ols on cardiovascular health: a systematic review and meta-analysis of randomized trials. *Am J Clin Nutr* 2012;95:740-751.
- 20 Craig SA: Betaine in human nutrition. *Am J Clin Nutr* 2004;80:539-549.
- 21 Kharbanda KK, Todero SL, King AL, Osna NA, McVicker BL, Tuma DJ, Wisecarver JL, Bailey SM: Betaine treatment attenuates chronic ethanol-induced hepatic steatosis and alterations to the mitochondrial respiratory chain proteome. *Int J Hepatol* 2012;2012:962183.
- 22 Ozturk F, Ucar M, Ozturk IC, Vardi N, Batcioglu K: Carbon tetrachloride-induced nephrotoxicity and protective effect of betaine in Sprague-Dawley rats. *Urology* 2003;62:353-356.
- 23 Alirezai M, Jelodar G, Niknam P, Ghayemi Z, Nazifi S: Betaine prevents ethanol-induced oxidative stress and reduces total homocysteine in the rat cerebellum. *J Physiol Biochem* 2011;67:605-612.
- 24 Nemmar A, Yuvaraju P, Beegam S, Ali BH: Betaine (N,N,N-trimethylglycine) averts photochemically-induced thrombosis in pial microvessels *in vivo* and platelet aggregation *in vitro*. *Exp Biol Med (Maywood)* 2015;240:955-960.

- 25 Purohit V, Abdelmalek MF, Barve S, Benevenga NJ, Halsted CH, Kaplowitz N, Kharbanda KK, Liu QY, Lu SC, McClain CJ, Swanson C, Zakhari S: Role of S-adenosylmethionine, folate, and betaine in the treatment of alcoholic liver disease: summary of a symposium. *Am J Clin Nutr* 2007;86:14-24.
- 26 Nemmar A, Raza H, Yuvaraju P, Beegam S, John A, Yasin J, Hameed RS, Adeghate E, Ali BH: Nose-only water-pipe smoking effects on airway resistance, inflammation and oxidative stress in mice. *J Appl Physiol* 2013;115:1316-1323.
- 27 Nemmar A, Yuvaraju P, Beegam S, Ali BH: Short-term nose-only water-pipe (shisha) smoking exposure accelerates coagulation and causes cardiac inflammation and oxidative stress in mice. *Cell Physiol Biochem* 2015;35:829-840.
- 28 Nemmar A, Al-Salam S, Yuvaraju P, Beegam S, Yasin J, Ali BH: Chronic Exposure to Water-Pipe Smoke Induces Alveolar Enlargement, DNA Damage and Impairment of Lung Function. *Cell Physiol Biochem* 2016;38:982-992.
- 29 Hakim F, Hellou E, Goldbart A, Katz R, Bentur Y, Bentur L: The acute effects of water-pipe smoking on the cardiorespiratory system. *Chest* 2011;139:775-781.
- 30 Nemmar A, Al Salam S, Dhanasekaran S, Sudhadevi M, Ali BH: Pulmonary exposure to diesel exhaust particles promotes cerebral microvessel thrombosis: protective effect of a cysteine prodrug l-2-oxothiazolidine-4-carboxylic acid. *Toxicology* 2009;263:84-92.
- 31 Nemmar A, Zia S, Subramaniyan D, Fahim MA, Ali BH: Exacerbation of thrombotic events by diesel exhaust particle in mouse model of hypertension. *Toxicology* 2011;285:39-45.
- 32 Nemmar A, Raza H, Subramaniyan D, Yasin J, John A, Ali BH, Kazzam EE: Short-Term Systemic Effects of Nose-Only Cigarette Smoke Exposure in Mice: Role of Oxidative Stress. *Cell Physiol Biochem* 2013;31:15-24.
- 33 Nemmar A, Melghit K, Ali BH: The Acute Proinflammatory and Prothrombotic Effects of Pulmonary Exposure to Rutile TiO₂ Nanorods in Rats. *Exp Biol Med (Maywood)* 2008;233:610-619.
- 34 Nemmar A, Albarwani S, Beegam S, Yuvaraju P, Yasin J, Attoub S, Ali BH: Amorphous silica nanoparticles impair vascular homeostasis and induce systemic inflammation. *Int J Nanomedicine* 2014;9:2779-89.
- 35 Nemmar A, Al Salam S, Zia S, Dhanasekaran S, Shudadevi M, Ali BH: Time-course effects of systemically administered diesel exhaust particles in rats. *Toxicol Lett* 2010;194:58-65.
- 36 Nemmar A, Al HA, Al HN, Yuvaraju P, Beegam S, Yasin J, Elwasila M, Ali BH, Adeghate E: Early pulmonary events of nose-only water pipe (shisha) smoking exposure in mice. *Physiol Rep* 2015;3.
- 37 Holm S: A simple sequentially rejective multiple test procedure. *Scand J Statist* 1979;6:65-70.
- 38 Aickin M, Gensler H: Adjusting for multiple testing when reporting research results: the Bonferroni vs Holm methods. *Am J Public Health* 1996;86:726-728.
- 39 Ruckerl R, Phipps RP, Schneider A, Frampton M, Cyrus J, Oberdorster G, Wichmann HE, Peters A: Ultrafine particles and platelet activation in patients with coronary heart disease - results from a prospective panel study. *Part Fibre Toxicol* 2007;4:1.
- 40 Kaufman J, Sime PJ, Phipps RP: Expression of CD154 (CD40 ligand) by human lung fibroblasts: differential regulation by IFN-gamma and IL-13, and implications for fibrosis. *J Immunol* 2004;172:1862-1871.
- 41 Mitsiakos G, Giougi E, Papaioannou G, Karagianni P, Papadakis E, Nikolaidis N: Influence of smoking during pregnancy on haemostasis in healthy full term neonates. *Thromb Res* 2009;123:476-481.
- 42 Vermynen J, Nemmar A, Nemery B, Hoylaerts MF: Ambient air pollution and acute myocardial infarction. *J Thromb Haemost* 2005;3:1955-1961.
- 43 Cesari M, Pahor M, Incalzi RA: Plasminogen activator inhibitor-1 (PAI-1): a key factor linking fibrinolysis and age-related subclinical and clinical conditions. *Cardiovasc Ther* 2010;28:e72-e91.
- 44 Kamath S, Lip GY: Fibrinogen: biochemistry, epidemiology and determinants. *QJM* 2003;96:711-729.
- 45 Nemmar A, Subramaniyan D, Ali BH: Protective effect of curcumin on pulmonary and cardiovascular effects induced by repeated exposure to diesel exhaust particles in mice. *PLoS One* 2012;7:e39554.
- 46 Hagar H, Medany AE, Salam R, Medany GE, Nayal OA: Betaine supplementation mitigates cisplatin-induced nephrotoxicity by abrogation of oxidative/nitrosative stress and suppression of inflammation and apoptosis in rats. *Exp Toxicol Pathol* 2015;67:133-141.
- 47 MacNee W: Pulmonary and systemic oxidant/antioxidant imbalance in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005;2:50-60.
- 48 Libby P, Ridker PM, Hansson GK: Progress and challenges in translating the biology of atherosclerosis. *Nature* 2011;473:317-325.

- 49 Azzawi M, Hasleton PS, Hutchinson IV: TNF-alpha in acute cardiac transplant rejection. *Cytokines Cell Mol Ther* 1999;5:41-49.
- 50 Li JM, Ge CX, Xu MX, Wang W, Yu R, Fan CY, Kong LD: Betaine recovers hypothalamic neural injury by inhibiting astrogliosis and inflammation in fructose-fed rats. *Mol Nutr Food Res* 2015;59:189-202.
- 51 Mukhopadhyay P, Rajesh M, Batkai S, Kashiwaya Y, Hasko G, Liaudet L, Szabo C, Pacher P: Role of superoxide, nitric oxide, and peroxynitrite in doxorubicin-induced cell death *in vivo* and *in vitro*. *Am J Physiol Heart Circ Physiol* 2009;296:H1466-H1483.
- 52 Nemmar A, Al-Salam S, Beegam S, Yuvaraju P, Yasin J, Ali BH: Pancreatic effects of diesel exhaust particles in mice with type 1 diabetes mellitus. *Cell Physiol Biochem* 2014;33:413-422.
- 53 Becher R, Bucht A, Ovrevik J, Hongslo JK, Dahlman HJ, Samuelsen JT, Schwarze PE: Involvement of NADPH oxidase and iNOS in rodent pulmonary cytokine responses to urban air and mineral particles. *Inhal Toxicol* 2007;19:645-655.
- 54 Hu N, Han X, Lane EK, Gao F, Zhang Y, Ren J: Cardiac-specific overexpression of metallothionein rescues against cigarette smoking exposure-induced myocardial contractile and mitochondrial damage. *PLoS One* 2013;8:e57151.
- 55 Yu L, Zhao Y, Xu S, Jin C, Wang M, Fu G: Leptin confers protection against TNF-alpha-induced apoptosis in rat cardiomyocytes. *Biochem Biophys Res Commun* 2014;455:126-132.