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Short-Term Exposure to Waterpipe/Hookah Smoke Triggers a Hyperactive Platelet Activation State and Increases the Risk of Thrombogenesis

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Abstract

Objective-—Cardiovascular disease (CVD) is a major public health problem. Among CVD's risk factors, tobacco smoking is considered the single most preventable cause of death, with thrombosis being the main mechanism of CVD mortality in smokers. While tobacco smoking has been on the decline, the use of waterpipes/hookah has been rising, mainly due to the perception that they are "less harmful" than regular cigarettes. Strikingly, there are few studies on the negative effects of waterpipes on the cardiovascular system, and none regarding their direct contribution to thrombus formation.

Approach and results-—We employed a waterpipe whole body exposure protocol that mimics real-life human exposure scenarios, and investigated its effects, relative to clean air, on platelet function, hemostasis and thrombogenesis. We found that, waterpipe smoke (WPS) exposed mice exhibited both shortened thrombus occlusion and bleeding times. Further, our results show that platelets from WPS exposed mice are hyperactive, with enhanced agonist-induced aggregation, dense and α-granule secretion, αIIbβ3 integrin activation, phosphatidylserine expression and platelet spreading, when compared with clean air exposed platelets. Finally, at the molecular level, it was found that Akt and ERK phosphorylation are enhanced in the WPS, and in nicotine-treated platelets.

Conclusion-—Our findings demonstrate, for the first time, that WPS exposure directly modulates hemostasis and increases the risk of thrombosis, and that this is mediated, in part, via a state of platelet hyperactivity. The negative health impact of WPS/hookah, therefore, should not be underestimated. Moreover, this study should also help in raising public awareness of the toxic effects of waterpipe/hookah.

Graphical Abstract

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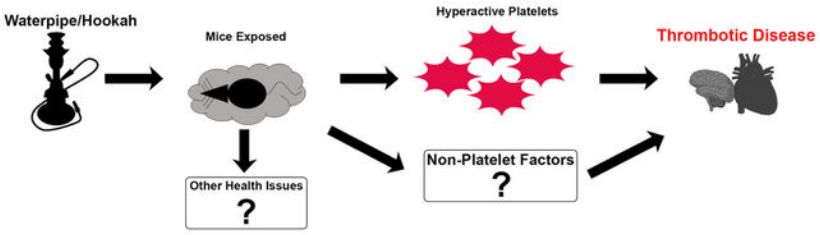
Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors



Keywords

Waterpipe; hookah; cardiovascular disease; platelets; thrombosis

Introduction

Cardiovascular disease (CVD) is a major public health problem, not only in the US, but also all over the world, as it accounts for more than 17 million deaths worldwide[1]. In the US, CVD is the number one leading cause of deaths accounting for 610,000 deaths each year, mainly as a result of heart attack and stroke[2]. Among CVD risk factors, tobacco smoking is the single most preventable cause of death and it is responsible for 33% of CVD-linked deaths[3]. Although reports have been showing a decline in traditional cigarette smoking in the western world[4], waterpipe (also known as hookah) tobacco smoking is an emerging trend in the US and Europe[5] especially among the youth[6–9]. It is to be noted that waterpipes have been in existence for a millennium, and thought to have emerged in the North Western provinces of India, before they spread to Iran, and the Arab world[10]. One of the reasons for such a surge in the popularity of waterpipes is in part due to: the wide spread misperception that waterpipe smoke is “filtered” via its passage through water, and therefore is less harmful than cigarette smoke[5]. Further, the type of tobacco used in the waterpipe, called “ma’assel”, which is honeyed and available in numerous flavors is another appealing reason for smoking waterpipes. Other reasons for use include social acceptability and accessibility[11], curiosity[12], and peer pressure[13]. While waterpipe users and those in close proximity are exposed to many of the potentially dangerous toxicants, the health risks associated with its use- including its impact in the context of the cardiovascular system- continue to be under debate. This is unlike the effects of tobacco smoking on the cardiovascular system, which are well-established[14–19]. Thus, cigarette smoking predisposes to cardiovascular events and contributes significantly to cardiovascular related mortality and morbidity[16, 20]. In addition, smoking causes a prothrombotic state through disrupting the hemostatic balance[21], enhanced aggregation[22], amongst others[23]. However, whether waterpipe use/WPS exerts similar effects remains ill defined, but warrants investigation. It is to be noted that waterpipes’ toxicant/chemical profile overlaps with that of regular cigarettes[24–26].

While studies on the impact of waterpipe usage on the cardiovascular system are limited, they have shown that it is associated with increased heart rate (HR) and blood pressure (BP)[27, 28], endothelial dysfunction[29], as well as oxidative stress[30]. These studies would suggest that waterpipe use/smoke would also increase the risk of thrombotic disease. Therefore, the current study examined the impact of waterpipes/hookah/WPS on platelet function, hemostasis and thrombogenesis, in mice, by employing a whole-body exposure

method. Moreover, we employed the widely adopted "Beirut protocol"[31] that resembles real-life waterpipe exposure scenarios. Our findings revealed that "short-term" WPS exposure triggers a hyperactive platelet state, both functionally and biochemically, that seems to increase the risk of thrombus formation, and to enhance hemostasis. Taken together, our data support the conclusion that waterpipes/WPS, contrary to many held beliefs, do exert negative health effects, and predispose users to thrombosis-related CVD.

Materials and Methods

The data that support the findings of this study are available from the corresponding authors upon reasonable requests.

Reagents and Materials

Thrombin was purchased from Chronolog Corporation (Havertown, PA), whereas ADP, nicotine and cotinine were from Sigma Aldrich (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated anti-P-selectin and FITC-conjugated Annexin V were purchased from Cell Signaling Technology, Inc (Danvers, MA). The JON/A antibody was obtained from Emfret analytics (Würzburg, Germany). The phycoerythrin-conjugated anti-CD69 and FITC-conjugated anti-CD45 were obtained from BD Biosciences (San Jose, CA). Stir bars and other disposables were purchased from Chrono-Log Corporation (Havertown, PA). Akt, phospho-Akt (Ser473), ERK and phospho-ERK antibodies were purchased from Cell signaling (Danvers, MA). The anti-PAR4 and anti-GPIIb-IIIa (αIIbβ3) antibodies were from Abcam (Cambridge, MA), whereas the anti-p85/PI3K antibody was from Proteintech (Rosemont, IL). The ELISA cotinine detection kit was purchased from Calbiotech (El Cajon, CA). The fibrinogen plasma detection/quantification ELISA kit was purchased form Innovative Research (Novi, MI).

Animals

C57BL/6J (10-week-old male) mice (referred to hereafter as C57BL/6) were purchased from the Jackson Laboratory (Bar Harbor, ME), and were housed in groups of 1 to 4 at 24°C, under 12/12 light/dark cycles, with access to water and food ad libitum. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Texas at El Paso.

Waterpipe Smoke (WPS) Whole Body Exposure Protocol

The exposure of mice to waterpipe smoke (WPS) utilized a whole-body exposure chamber (40cm × 30cm × 25cm, LxWxH), which was connected to a programmable waterpipe smoking machine, as described in a previous report[32]. The smoking machine delivered 171 puffs of 530 mL volume, 2.6 s puff duration, and 17 s interpuff interval according to the Beirut Method[33]. This method is based on the smoking behavior in real-life waterpipe/hookah settings (Hookah café), and the protocol itself has been used worldwide and in a host of different studies[31, 34–43]. The clay bowl of the waterpipe used in this study was loaded with 12g of commercially available flavored tobacco (Al Fakher - Double Apple Tobacco Trading, Ajman, UAE), whose ingredients include tobacco, glycerin, molasses and natural flavor with nicotine and tar. We also conducted exposures using a second tobacco brand,

namely the Starbuzz (Dale Ave Stanton, CA), and performed specific platelet function experiments (i.e., aggregation and dense granule secretion). After covering the clay bowl with Aluminum foil, quick-light charcoal briquettes (Holland Hookah Charcoal) were used to burn the tobacco. The bowl of the waterpipe was filled with about 700 mL of tap water, prior to each exposure session. Along with the smoke puff delivered to the exposure chamber by the smoking machine, fresh/clean air was continuously pumped into the chamber, resulting in an air change rate of approximately 1.5 changes per hour. Carbon monoxide (CO) concentration (average levels were 925 ppm) was continuously monitored in the chamber using an electrochemical sensor (Bacharach Monoxor Plus) drawing a flow rate of 0.3 LPM through a 25 mm glass fiber filter (Pall Type A/E). The WPS mice were exposed, as described above, for one exposure session 60 min/day for seven days, and all experiments performed on the day of the last exposure session.

Cotinine Assay

The serum levels of cotinine, a metabolite of nicotine, were measured in both WPS and clean air exposed mice, using the Cotinine Direct ELISA kit as per the manufacturer's instructions.

Tail Bleeding Time

After a complete cycle of exposure, we performed the tail bleeding time assay as we described before[44, 45]. Briefly, anesthesia was induced and WPS and clean air exposed mice were placed on a homeothermic blanket at 37°C, after that the tail was transected 5 mm from the tip using a sterile scalpel. After transection, the tail was immediately immersed in saline (37°C constant temperature), and the time to bleeding stoppage was measured. Bleeding stoppage was not considered complete until bleeding had stopped for 1 minute. If bleeding did not stop in 10 minutes, the experiment was stopped to prevent excessive blood loss from the mice, and 10 minutes was considered the cutoff bleeding time.

In vivo $FeCl_3$ Carotid Artery Injury Induced Thrombosis Model

Thrombosis studies were performed as described previously[44, 45]. Briefly, WPS and clean air exposed mice (8–10 weeks old) were anesthetized with avertin (2.5%), and the left carotid artery was exposed and cleaned with normal saline (37°C), before baseline carotid artery blood flow was measured with Transonic Micro-Flowprobe (Transonic Systems Inc, Ithaca, NY). After stabilizing blood flow, 7.5% ferric chloride was applied to a filter paper disc (1-mm diameter) that was immediately placed on top of the artery for 3 minutes. Blood flow was continuously monitored for 20 minutes or until blood flow reached stable occlusion (no blood flow for 2 minutes). Data were recorded, and time to vessel occlusion was calculated as the difference in time between stable occlusion and removal of the filter paper (with ferric chloride). An occlusion time of 20 minutes was considered as the cutoff time for statistical analysis.

Peripheral Blood Cell/Platelet Counts

The hematology profile, including platelets and white blood cells, of the WPS/clean air exposed mice was performed on whole blood using a HEMAVET® 950FS Multi-species Hematology System from Erba® Diagnostics (Miami Lakes, FL).

Murine Platelet Rich Plasma Preparation

Mice were anesthetized before blood was collected from the heart, with 0.38% sodium citrate solution (Fisher Scientific, Hampton, NH) added to inhibit coagulation. Then blood was centrifuged (237*g* for 15 minutes) at room temperature, and the platelet-rich plasma was then collected. Platelets were counted with the HEMAVET® 950FS Multi-species Hematology System, and their count adjusted to 7×10^7 platelets/mL before each experiment.

Washed Platelet Preparation

Blood was collected form each mice as described above and were pooled in each group by diluted phosphate-buffered saline, pH 7.4, in 1:1 ratio, incubated with PGI_2 (10 ng/mL; 5 minutes), and centrifuged at 237*g* for 10 minutes at room temperature. Platelet-rich plasma was recovered, and incubated with 0.37 U/mL apyrase, and 10 ng/mL PGI_2, platelets were pelleted at 483×g for 10 minutes at room temperature. The pellets were resuspended in HEPES/Tyrode buffer (20 mmol/L HEPES/KOH, pH 6.5, 128 mmol/L NaCl, 2.8 mmol/L KCl, 1 mmol/L MgCl2, 0.4 mmol/L NaH2PO4, 12 mmol/L NaHCO3, 5 mmol/L D-glucose) supplemented with 1 mmol/L EGTA, 0.37 U/mL apyrase, and 10 ng/mL PGI_2. Platelets were then washed and resuspended in HEPES/Tyrodes (pH 7.4). Platelets were counted using the HEMAVET® 950FS Multi-species Hematology System and adjusted to the indicated concentrations.

In vitro Platelet Aggregation

The platelet-rich plasma (PRP) from WPS exposed and clean air mice was activated with thrombin (0.1 U/mL) or ADP (5 μmol/L). Platelet aggregation was measured by the turbidometric method using a model 700 aggregometer (Chrono-Log Corporation, Havertown, PA). Each experiment was repeated at least 3 times with blood pooled from at least 3 different groups (i.e., at least 8 mice each) that were exposed to either WPS or clean air. The aggregation assay was conducted after exposure to the two different tobacco brands.

Dense Granule Release

Platelet-rich plasma (250 μL; 7×10^7/mL) were placed into siliconized cuvettes and stirred for 5 minutes at 37°C at 1200 rpm, followed by the addition of luciferase substrate/luciferase mixture (12.5 μL, Chrono-Log). PRP was then activated using the agonists thrombin (0.1 U/mL) or ADP (5 μmol/L). Release of ATP were measured using a model 700 aggregometer (Chrono-Log Corporation, Havertown, PA). This assay was conducted after exposure to the two different tobacco brands.

Flow Cytometric Analysis

Flow cytometric analysis was carried out as described[44, 45]. Briefly, washed platelets (2×10^7/mL) from clean air- or WPS–exposed mice were prepared as described above and

stimulated with thrombin (0.1 U/ml) or ADP (5 μmol/L) for 5 minutes. Platelets were then fixed with 2% formaldehyde for 30 minutes at room temperature, followed by incubated with FITC-conjugated CD62P (P-selectin), Annexin V or phycoerythrin-conjugated rat anti-mouse JON/A antibodies at room temperature for 30 minutes in the dark. Finally, platelets were diluted 2.5-fold with HEPES/Tyrode buffer (pH 7.4). The samples were transferred to FACS-tubes and fluorescent intensities were measured using a BD Accuri C6 flow cytometer and analyzed using CFlow Plus (BD Biosciences, Franklin Lakes, NJ). Each experiment was repeated at least 3 times with blood pooled from at least 3 different groups (at least 8 mice each) that were exposed to either clean air or WPS.

Platelet Spreading

Platelet spreading assay was carried out as we described before[46, 47]. Briefly, sterile glass coverslips were coated with 0.2 μg/mL of fibrinogen for 30 minutes at room temperature. Washed platelets were placed onto these fibrinogen-coated coverslips before they were fixed with 3.7% (vol/vol) formaldehyde for 15 minutes and quenched with 50 mmol/L ammonium chloride. Cells were rinsed with PBS and incubated with tetramethylrhodamine-conjugated phalloidin (1 μg/mL) in 10% fetal bovine serum/PBS with 0.2% saponin. Coverslips were mounted and examined and imaged using a Leica DMi8 inverted widefield fluorescence microscope with integrated high-precision focus drive. Images were processed using LAS X Wizard imaging software. Objective lenses used were a ×63/×100 numeric aperture. Type A immersion oil (Fryer) was used for the ×63 objective. This experiment was repeated at least 3 times, with blood pooled from a group of 8 mice each time. Comparison is based on difference in the level of spreading.

Immunoblotting

Immunoblot was carried out as described before[44, 48]. Briefly, clean air– or WPS–exposed washed platelets were stimulated with thrombin (0.1 U/ml) for 3 minutes followed by lysis with 1X sample buffer. Next, proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to Immobilon-P PVDF membranes (Bio-Rad, Hercules, CA). Membranes were then probed with the primary antibodies (ERK, pERK, Akt, and pAkt) and visualized with horseradish peroxidase–labeled antirabbit or antimouse immunoglobulin G as required. The antibody binding was detected using enhanced chemiluminescence substrate (Thermo Scientific, Rockford, IL). Images were obtained with ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA), and quantified using the Image Lab Software 6.0.1 (Bio-Rad, Hercules, CA). For expression levels of GPIIb-IIIa, PI3K (p85) and PAR4, platelet proteins were separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Bio-Rad, Hercules, CA). They were then probed with anti-GPIIb-IIIa (αIIbβ3), anti-PI3K/p85 or anti- PAR4 primary antibodies and visualized with an appropriate alkaline phosphatase–coupled secondary antibody using enhanced chemifluorescent substrate (Thermo Scientific, Rockford, IL).

Mouse Treatment with Nicotine and Cotinine

Mice were injected with 1 μM cotinine (a “real-life” dose selected based on waterpipe literature)[49–52] or vehicle once daily for one week. Experiments (specifically platelet aggregation and dense granule secretion) on cotinine treated animals were performed on the

day of the last injection. As for nicotine, mice were injected with 1 mg/kg nicotine, as previously used in the literature[53], and the blood collected after one hour post injection, before Akt and ERK phosphorylation were assessed as described above.

Fibrinogen Assay

The plasma concentration of fibrinogen was determined using an ELISA kit as per the manufacturer's instructions.

Statistical Analysis

All experiments were performed at least 3 times with blood pooled from 3 groups of at least 8 mice each time, as applicable. Data analysis was performed using GraphPad PRISM. V7 statistical software and presented as mean ± standard deviation/SD, but the normality and variance were not tested to determine whether the applied parametric tests were appropriate. The Mann-Whitney test was used for the evaluation of differences in mean occlusion and bleeding times, whereas the one-way ANOVA with Tukey's multiple comparisons test as post hoc were used for the analysis of the flow cytometry data, as applicable (per number of groups). Significance was accepted at $P<0.05$, unless stated otherwise.

Results

Waterpipe/hookah exposure systemically delivers nicotine in exposed mice

As means of validating the WPS exposure protocol, the serum levels of the nicotine metabolite cotinine[54] are typically measured in exposed animals and/or "tobacco users". Hence, our results showed significant increases of cotinine levels in the WPS exposed mice (15.2 ± 0.72 ng/ml; Fig 1A), in serum obtained immediately after the conclusion of the exposures, whereas it was undetectable in that from the clean air controls. These levels overlap with those found in human waterpipe users (5.5–77.8 ng/ml)[49–51, 55, 56]. Our data support the notion that our WPS exposure protocol results in the systemic delivery of nicotine into the exposed mice.

WPS Exposure Modulate Hemostasis and Thrombosis Development

Numerous studies showed that cigarette smoking causes disease states that are linked to thrombosis[22, 57]. On contrast, it is yet to be determined whether waterpipe smoking carries a similar effect. Therefore, we initially investigated the effects of waterpipe smoke (WPS) exposure on both hemostasis and thrombogenesis. As for its effect on hemostasis, the tail bleeding time assay revealed that the time to bleeding stoppage was significantly shortened in the WPS-exposed mice, when compared with those exposed to clean air, specifically 11.43 ± 2.15 seconds to 300.7 ± 7.32 seconds, respectively (Figure 1B). This line of evidence indicates a prothrombtic phenotype in the WPS-exposed mice. Thus, we next sought to examine if the WPS mice are more prone to thrombosis. Using the ferric chloride carotid artery injury–induced thrombosis model, we indeed found that the WPS-exposed mice had a shortened time for occlusion, in comparison with the controls, specifically, 72 ± 33.7 seconds to 370 ± 62.9 seconds, respectively (Figure 1C). These data document, for the first time, that WPS exposure directly modulates hemostasis and thrombus occlusion.

Acute WPS Exposure Does Not Affect Peripheral Blood Cell Counts

To exclude the possibility that changes in platelet number may have contributed to the hemostasis and thrombosis phenotype we observed, platelets from the WPS and clear air mice were counted. We did not observe any significant difference in the platelet count (Table 1) between the WPS-, and clean air-exposed mice, namely (556 ± 24.3 to 573 ± 31.2 (thousand/μL; P=0.40). These results indicate that under the present experimental conditions, the prothrombotic phenotype is independent of changes in platelet count. Moreover, we did not observe any detectable effects for WPS on the counts of white/ peripheral blood cells (Table 1).

WPS Exposure Enhances Agonist-Induced Platelet Aggregation

Previous studies have shown that cigarette smoking enhances platelet function[58]. Given that our data showed a robust prothrombotic phenotype in WPS-exposed mice, we hypothesize that this will derive, in part, from platelet hyperactivity as a result of WPS exposure. Thus, we sought to examine the effects of WPS exposure on agonist–induced platelet aggregation. It was found that platelets from WPS-exposed mice exhibited an enhanced platelets aggregation in comparison to those from clean air controls, in response to thrombin or ADP (Figure 2A & 2B; inset shows quantification of data). Together, the hyperactive platelet state is consistent with shortened bleeding and occlusion times phenotype observed in the WPS mice. Similar result was obtained when the same experiment was repeated with a different waterpipe tobacco brand (see Methods for details; Figure 2C & 2D; inset shows quantification of data).

WPS Exposure Enhances Agonist-Induced Platelet Secretion

We next sought to investigate the impact of WPS exposure on agonist-induced platelet secretion, given the importance of the platelet granule release in amplifying the initial platelet response[59, 60]. Our results demonstrated dense granule/ATP secretion is enhanced in the WPS platelets, in response to thrombin or ADP, even with exposure to a different tobacco brand (Figure 2E–2H), which is consistent with the aggregaion data. Similarly, platelets from mice exposed to WPS had significantly higher expression of P-selectin on their surface in response to 0.1 U/ml of the agonist thrombin or 5 μM ADP (Figure 3A & 3B). These findings indicate that whole-body exposure to WPS enhances both dense and α-granule secretion, which is in line with the notion that these platelets are hyperactive.

WPS Exposure Enhances Agonist-Induced Integrin αIIbβ3 Activation

In light of the notion that WPS exposure is responsible for the hyperactive platelet phenotype, including enhanced aggregation, we next determined if the integrin GPIIb-IIIa (αIIbβ3) activation would also be potentiated. Indeed, our data revealed that activation of the αIIbβ3 integrin is enhanced in the WPS platelets, in contrast with the clean air controls, in response to 0.1 U/ml thrombin or 5 μM ADP (Figure 3C & 3D). These results are consistent with the enhanced aggregation response in the WPS-exposed platelets.

WPS Exposure Enhances Agonist-Induced Phosphatidylserine Expression

Phosphatidylserine (PS) exposure provides the essential platelets "platform" upon which the assembly of coagulation factor complexes takes place[61]. Therefore, it is important to assess any changes in PS expression that may result from WPS exposure. Our data showed that PS expression was significantly higher in the WPS-exposed platelets, compared to those from the clean air-exposed mice, upon stimulation with 0.1 U/ml thrombin or 5 μM ADP (Figure 4A & 4B).

Taken together, our data thus far support the notion that exposure of platelets to WPS produces a hyperactive state, in comparison to clean air. This hyperactive state (enhanced function) was in the form of enhanced aggregation, secretion, integrin activation, and phosphatidylserine exposure.

WPS Exposure Enhances Platelet Spreading

Platelet spreading is a vital aspect of hemostatic plug formation and thrombosis[62]. To this end, and upon vessel injury, platelet activation mediated via a host of receptor-induced cascades, triggers rapid reorganization of the actin cytoskeleton, thereby resulting in platelet rounding, and adhesion to the surface. Consequently, platelet morphological changes occur, e.g. filopodia and lamellipodia formation, which ultimately strengthens contact with the surface as well as to other platelets. As for the impact of WPS on spreading, filopodia and lamellipidia formation were found to be higher/potentiated in the WPS-exposed platelets, compared to those from the clean air-exposed mice, upon stimulation with 0.1 U/ml thrombin (Figure 5). This finding further supports the notion that WPS results in a hyper-state of platelet activity.

WPS Exposure and Nicotine Treatment Enhance Agonist-Induced Akt and ERK Phosphorylation

A critical signaling mechanism in platelet function and thrombus formation is the phosphorylation of Akt and/or ERK proteins[63–66]. Therefore, we investigated whether WPS exposure would enhance phosphorylation of Akt and/or ERK in platelets. Our data showed that Akt and ERK phosphorylation are indeed enhanced in WPS-exposed platelets, relative to controls after stimulation with 0.1 U/ml thrombin (Figure 6A; data quantification is shown in Figure 6B). These results provide biochemical evidence and indicate that Akt and/or ERK are an essential component in the WPS-mediated modulation of platelets toward a hyperactive state. We next determined whether nicotine, the WPS chemical, has the capacity to trigger Akt and/or ERK activation. Indeed, platelets from mice that were injected with nicotine (1 mg/kg IV) exhibited enhancement of Akt and ERK phosphorylation in response to 5 μM ADP (Figure 6C; data quantification is shown in Figure 6D); which is consistent with previous reports[67–69]. This data suggests that nicotine plays a role- "at least in part"- in the priming of platelets into a hyperactive state.

WPS Exposure Does Not Modulate Plasma Fibrinogen Levels

To determine if the coagulation system is impacted by WPS, we sought to investigate if there is any change in the plasma concentration of fibrinogen. Our results showed that the plasma

concentration of fibrinogen was not affected in WPS exposed mice in comparison to clean air, at least under the present experimental conditions (Figure 7A).

Cotinine Enhance Platelets Aggregation and Secretion

Previous studies suggested that cotinine, a main metabolite of nicotine (with a long/er half-life) can induce thrombus formation[70]. Therefore, we sought to investigate the effect of cotinine on platelets function (aggregation and secretion *ex vivo*). Our data revealed that cotinine (1 μM injection for one week) did in fact enhance thrombin-induced platelet aggregation and dense granule secretion, in comparison to the vehicle control (Figure 7B & 7C). These data indicate that cotinine is a likely key contributor to WPS's "negative" effects on platelets.

WPS Exposure Does Not Modulate Expression of GPIIb-IIIa, PAR4 or PI3K.

We next sought to investigate of the aforementioned effects of WPS may involve changes in the expression levels of some of platelet proteins, namely GPIIb-IIIa (αIIbβ3), PAR4 and PI3K/p85. To this end, we did not observe any detectable changes in the expression levels of PAR4 or PI3K/p85, between WPS and clean air platelets (Figure 7D; data quantification is shown in Figure 7E). These data suggest that, at least under the current experimental conditions, short-term exposure to WPS does not alter expression of platelet proteins.

Discussion & Conclusion

In this study, we investigated the effect of exposure to hookah/waterpipe smoke (WPS) on platelet function, hemostasis, and thrombogenesis. Similar to cigarette smoking, our results indicates that, even when the exposure to WPS is short-term, namely in this case seven/7 days long, mice are under an increased risk for thrombosis with a shortened time for physiological hemostasis, which seems to derive from a hyperactive platelet function phenotype.

Many studies have undisputedly established that cigarette smoking is a risk factor for cardiovascular disease, including unstable angina[71, 72], myocardial infarction[73], and stroke[74]. On the other hand, despite its widespread use and being a rising trend in the west[5–9]- driven by its perceived "less harm"- studies on the effects of waterpipe smoking/WPS on cardiovascular disease are limited. Moreover, the direct consequences of WPS exposure in the context of platelets and thrombosis are yet to be investigated. Waterpipes carry a toxic profile that is thought to be comparable or to even exceed that of cigarette smoking[32, 75–77], which would suggest that they have "similar" negative health effects. In fact, according to some studies, the smoke emitted from a single waterpipe tobacco smoking episode contains eight times the CO, three times the nitric oxides, an average of 11.5 times the acrolien, an average of 18.2 times the formaldehyde, and up to 245 times the polycyclic aromatic hydrocarbons (PAHs)[26, 41, 77, 78], relative to a single regular tobacco cigarette.

Based on the aforementioned considerations, and limitations of previous studies, we sought to "uncover" the effects of WPS on platelet function by employing a "novel" whole body exposure protocol that simulates real life exposure scenarios[31]. An important aspect of our

system is the fact that it involves minimal animal handling, with the animals unrestrained and less stressed. Thus, the animal model we used in our study (mice) should provide translational results that are clinically applicable to humans, especially in the context of tobacco exposure[79, 80], especially since the number of puffs, puff interval, etc., are based on human exposure data. Of note, this model gives us flexibility in mimicking many aspects of human exposure that cannot be directly obtained from humans, due to ethical considerations and other challenges[81]. Nonetheless, one limitation is the fact that the exposure parameters (number of puffs, puffs interval, etc.) do not consider the concentration of the smoke relative to the size of a mouse.

The primary aim of this study was to determine the effects of short-term WPS exposure on platelet activation, and the genesis of thrombosis. Our findings revealed- for the first time- that short term exposure to WPS significantly reduced the bleeding time in exposed mice, and increased their tendency to carotid artery thrombosis. These findings provide evidence that WPS is not safe as the common misperception implies, even with a short-term exposure of only seven/7 days. In addition, our data are consistent with other reports that short-term use of waterpipes disrupts normal cardiovascular function[82–89], and that exposure to other forms of tobacco impacts hemostasis and thrombosis[45, 90]. Notably, patients who have a history of cardiovascular disease (e.g. atherosclerotic disease) are probably at a higher risk of having negative outcomes from waterpipe use.

We next investigated the mechanism by which enhanced hemostasis and thrombosis may have manifested in the WPS exposed mice. Importantly, since it has been shown that an increase in platelets activity plays a major role in the pathogenesis of acute myocardial infarction (MI)[91] and acute stroke[92–94], and since thrombosis is the main mechanism of smoking related cardiovascular mortality[21], this notion was investigated herein. Our results demonstrated that agonist-induced platelet aggregation, secretion (dense and α-granules), integrin activation, phosphatidylserine expression, and platelet spreading were all enhanced as a result of WPS exposure. Moreover, similar enhancement of platelet aggregation and dense granule secretion was observed when mice were exposed to a different waterpipe/hookah tobacco brand (see Methods for details). It is noteworthy that one study in humans reported a significant increase in TXB_2 levels - a metabolite of the biologically active TXA_2- after a single waterpipe smoking session[95]. This increase in TXB_2 levels would suggest an increase in platelet activity[96], which is consistent with our findings. Together, these findings support our initial hypothesis that WPS exposure (similar to cigarette smoking) modulates platelets to a state of hyperactivity, which eventually leads to the prothrombotic phenotype observed in the WPS-exposed mice. Notably, there were no changes in platelet count under our exposure protocol, which indicates that short-term (7 days) exposure to WPS does not result in any change in platelet formation. This data is consistent with a report that showed insignificant difference in platelet count in mice exposed to waterpipe smoke[97]. However, whether platelet number would change if waterpipe exposure is repeated over longer periods of time, as was also reported previously with waterpipe exposure[98], remains to be determined.

Given the “functional” evidence we have obtained thus far, we next examined whether platelet activation-mediated downstream signaling, namely Akt and ERK activation, would

also be impacted by WPS. Our data showed an increase in the levels of phosphorylated Akt and ERK in the WPS exposed platelets in comparison to the clean air controls. Interestingly, we previously observed similar results with exposure to e-cigarette[44]. This might suggest overlap between waterpipes and e-cigarettes with regard to the mechanism by which they enhance platelet activation, which is presumably through nicotine. The latter notion is consistent with previous findings that nicotine upregulates Akt and ERK pathways[67–69], a notion we were able to verify under our experimental conditions, i.e., platelets from nicotine treated mice showed an increase in phosphorylation of Akt and ERK. Given the short half life of nicotine (6–7 minutes), one cannot exclude the fact that one of its metabolites could be responsible for the enhanced Akt and ERK activation.

As far as what toxic ingredient(s) in waterpipe smoke that might be responsible for the hyperactive state of platelets, several toxicants have been found including nicotine/cotinine[41, 78, 99–101], aldehydes[26, 101], particulate matter[102] as well as furanic and phenolic compounds[76, 103]. To this end, we found that treatment with cotinine- one of nicotine's main metabolites- is capable of priming platelets into a hyperactive state. These data support the notion that cotinine is a key toxic waterpipe ingredient, and is a likely contributor to their prothrombotic phenotype, as well as to their harmful effects on the cardiovascular system. It is noteworthy that the amounts of these toxicants vary between waterpipe compared with cigarette smoke (per cigarette/and per pack/day) due to different heating process and charcoal combustion[104–107]. Although, the effect of each one of these chemicals on platelets is not fully studied, particulate matter[108, 109] and the aldehyde acrolein[90] were indeed found to enhance platelet function. Of note, waterpipes may in fact result in higher levels of nicotine given the longer duration of a session, and which involves 100–200 times the volume of smoke from a single cigarette. This also applies to other toxins in tobacco[110]. In regards to carbon monoxide (CO), which is one of the main toxic ingredients of WPS, there is a debate regarding its impact on hemostasis and platelets function. On the one hand, studies reported an inhibitory effect on hemostasis and platelets function, whereas on the other hand, separate studies reported an enhancing effect on hemostasis and platelets function[111]. This controversy may be related to the variability in the exposure protocols, as well as the methods used. Nonetheless, CO may have contributed to the negative health effects we observed with WPS.

We also examined whether the WPS phenotype observed thus far may involve modulation of coagulation, or changes in the basal expression of receptors and/or signaling molecules/proteins critically involved in platelet activation. Our data showed no detectable differences in the plasma levels of the clotting factor fibrinogen between the WPS and control mice. In terms of changes in the basal expression levels of proteins, our data revealed that there was no difference in the levels of the GPIIb-IIIa, PAR4 and p85 (PI3K) proteins between the control and the WPS platelets. However, one cannot exclude the possibility that other aspects of coagulation and/or that the expression of other proteins may have changed. It is also possible that different real-life exposure scenarios may lead to changes in the plasma levels of fibrinogen and/or the expression levels of platelet proteins. Of note, higher fibrinogen plasma levels were reported in long term waterpipe smokers[112], and it was previously shown that the levels of the P2Y12 receptor protein do change in megakaryoblasts and other cells[113], in response to nicotine.

Notably, it is well documented that regular cigarette smoking and (more recently) e-cigarettes enhance platelet function and increase the risk of thrombosis[44, 114], and our present studies show “similar” negative effects from WPS. However, comparative studies regarding differences in the magnitude or potency of each form of tobacco are lacking, but are clearly warranted to better understand and appreciate the relative negative health risk associated with each form.

The present study did not address the role of sex in the negative health consequences of waterpipe, as it employed exclusively male mice. The main purpose of this experimental design is/was to minimize the variability that would be caused by hormonal fluctuations associated with the female’s reproductive cycle[115]. Moreover, it is highly likely that “non-platelet” factors are contributing to the prothrombotic phenotype associated with WPS/hookah. Both of these issues will be the subject of future investigations.

In summary, by employing a validated animal exposure model, we are the first to investigate the impact of short-term whole-body WPS/waterpipe exposure on platelet function, homeostasis, and thrombosis. We document that WPS increases the risk of thrombosis, due to platelet hyperactivity. In conclusion, our findings provide evidence to argue against the common/currently held beliefs regarding the perceived safety of waterpipe smoking, which is expected to raise awareness regarding the negative health consequences of this increasingly popular form of tobacco.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

CVD	Cardiovascular disease
WPS	Waterpipe smoke
HR	Heart rate
BP	Blood pressure
ADP	Adenosine diphosphate
CO	Carbon monoxide
GPCRs	G-protein coupled receptors
ERK	Extracellular signal regulated kinases

AKT	Protein kinase B
MPV	Mean platelet volume
HCT	Hematocrit
PS	Phosphatidylserine

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Highlights

- Whole body exposure to hookah/waterpipe smoke/WPS modulates physiological hemostasis and increases the risk of thrombotic disease.
- WPS enhances a host of platelet functional responses, namely aggregation, secretion, PS exposure, GPIIb-IIIa activation, and spreading, as well as Akt and ERK phosphorylation.
- These findings are expected to help clinicians educate their patients and the public regarding the evidence that waterpipes are apparently not as safe as one might think, as well as regarding the their negative cardiovascular health effects.

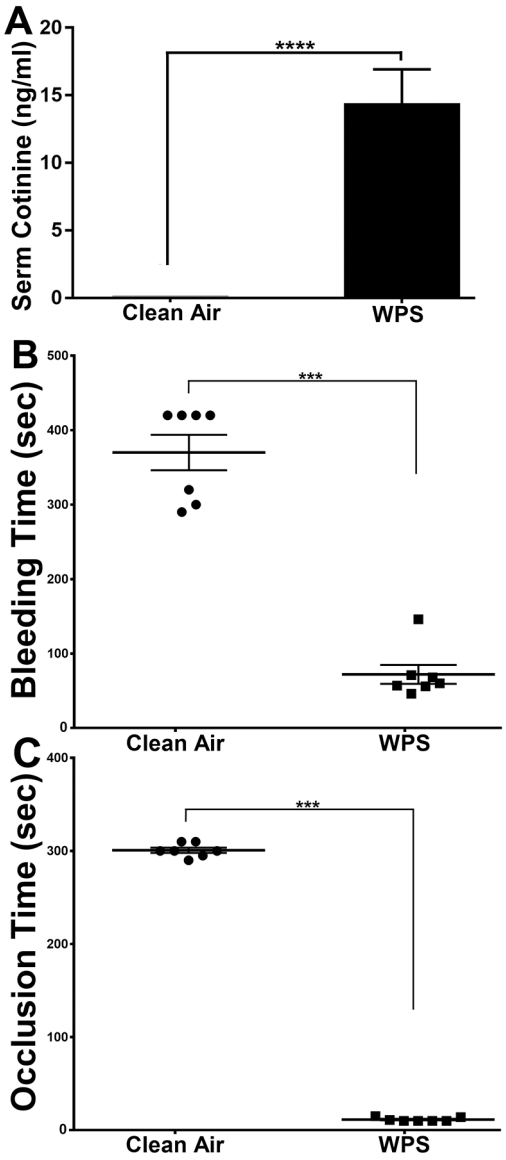

Figure 1:

WPS exposure results in systemic delivery of nicotine (cotinine) and shortens the bleeding time in the tail bleeding time assay, and the time to occlusion in the ferric chloride *in vivo* thrombosis model. **(A)** The concentrations of cotinine were measured in serum from WPS and clean air exposed mice. Each bar represents the mean ± SD (n = 10; ****P<0.0001). **(B)** This data illustrates the results of tail bleeding time assay (as described in the “Methods”) comparing WPS and clean air exposed mice. Each point represents the tail bleeding time of a single animal (clean air, n=7; and WTS, n=7; ***P<0.001). **(C)** This data illustrates the results of the ferric chloride–induced thrombosis model (as described in the “Methods”) comparing WPS and clean air exposed mice time to occlusion. Each point represents the occlusion time of a single animal (clean air, n=7; and WPS, n=7; ***P<0.001). WPS indicates Waterpipe Smoke.

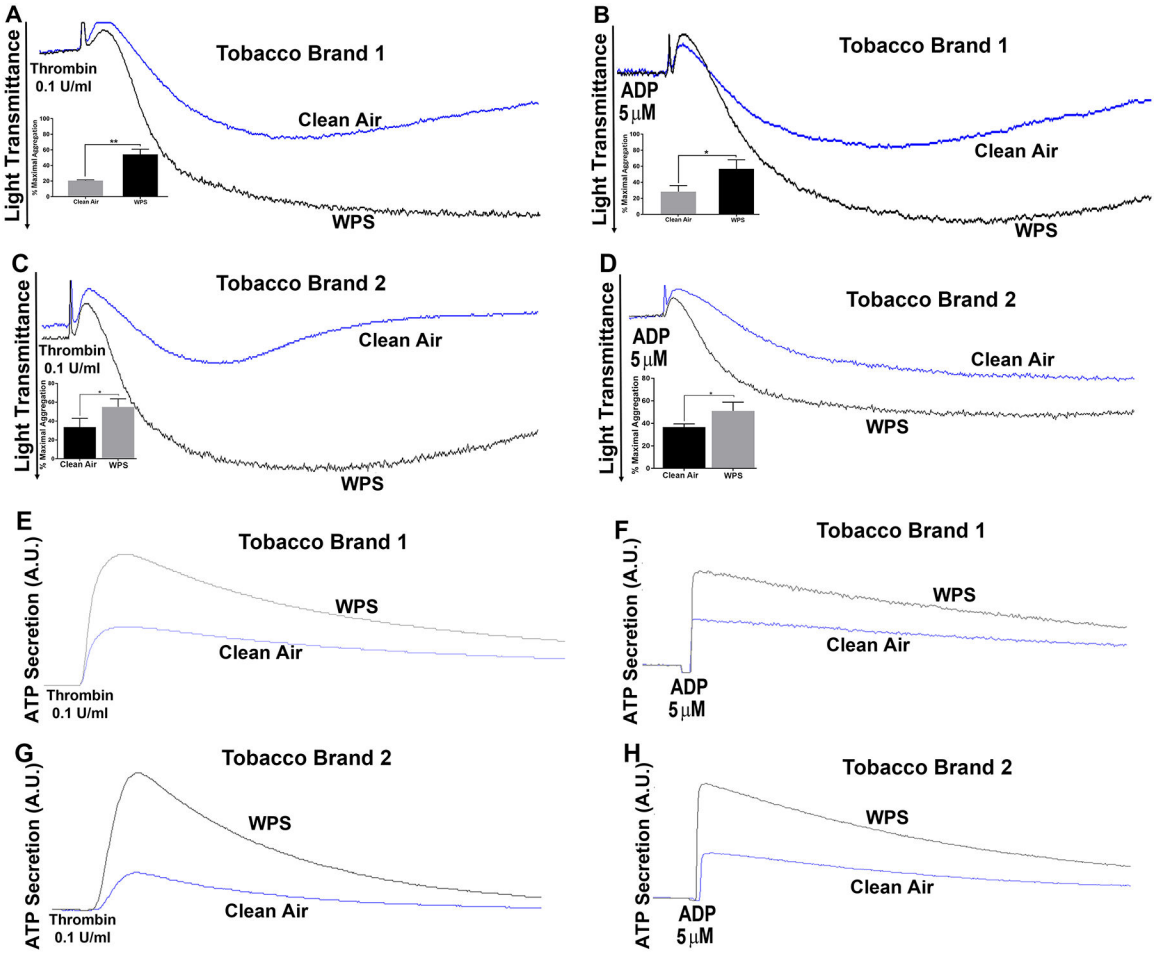

Figure 2:

Platelet aggregation and dense granule secretion are enhanced in WPS exposed mice, using two different brands. Platelets from WPS (two separate brands) and clean air exposed mice were stimulated with 0.1 U/ml thrombin, or 5 μmol/L ADP, before their aggregation **(A-D)** and dense granule secretion **(E-H)** responses were measured in a lumi-aggregometer. Platelets were incubated with luciferase/luciferin (12.5 μL) for the dense granules measurements. The experiment was repeated 3 times, with blood pooled from at least 8 mice each time (*P <0.05; **P <0.01). WPS indicates Waterpipe Smoke.

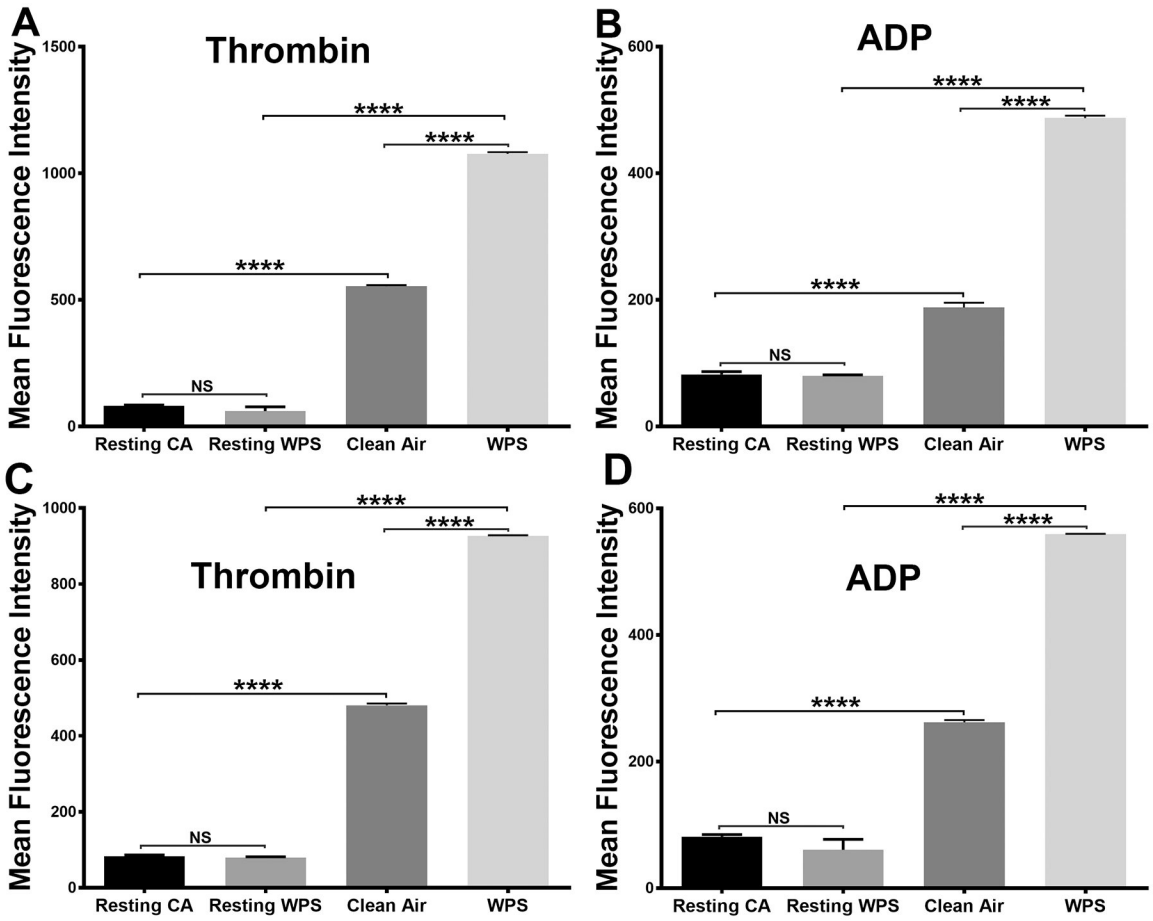

Figure 3:

Platelets alpha granule secretion and integrin GPIIb-IIIa activation are increased in WPS exposed mice. Platelets from WPS and clean air exposed mice were prepared and washed. **(A, B)** Platelets were incubated with fluorescein isothiocyanate–conjugated CD62P antibody (for α granules), and the fluorescent intensities were measured by flow cytometry after stimulation with 0.1 U/ml thrombin or 5 μmol/L ADP. **(C, D)** Platelets were incubated with fluorescein isothiocyanate–conjugated JON/A antibody, and the fluorescent intensities were measured by flow cytometry after stimulation with 0.1 U/ml thrombin or 5 μmol/L ADP. Average mean fluorescence intensities shown (**** $P < 0.0001$; NS. nonsignificant). Each experiment was repeated 3 times, with blood pooled from at least 8 mice each time. WPS indicates Waterpipe Smoke.

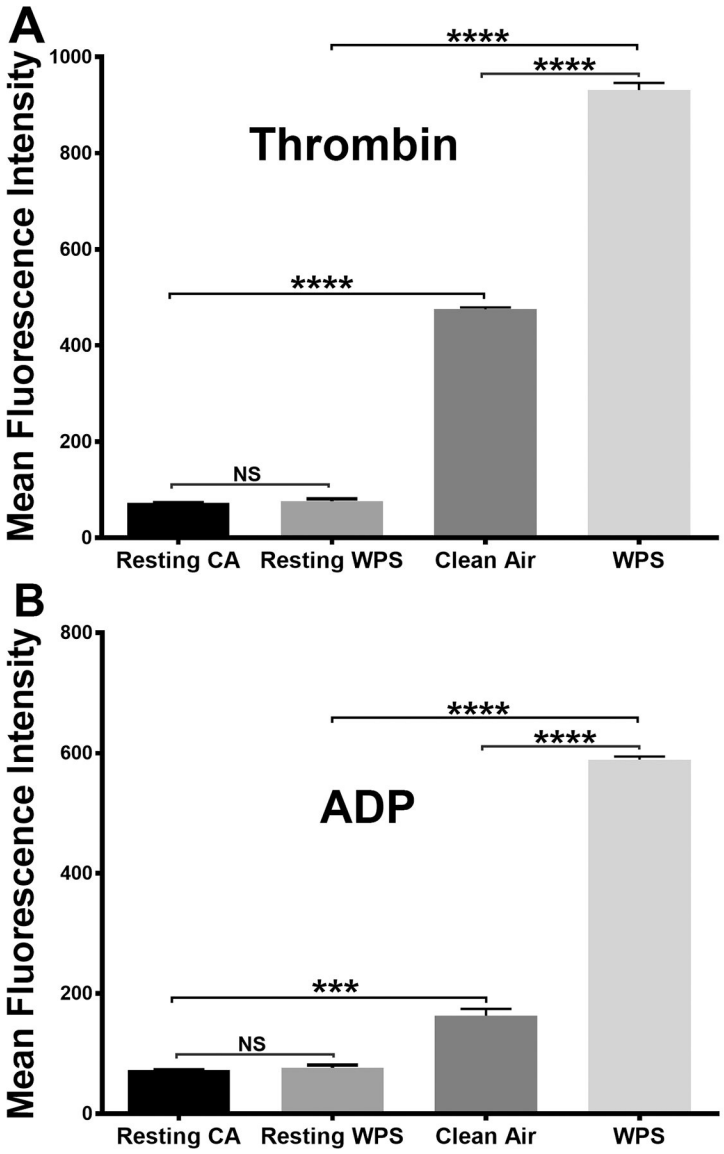

Figure 4:

Platelet phosphatidylserine (PS) exposure is enhanced in WPS exposed mice. Platelets from WPS and clean air exposed mice were prepared and washed. Platelets were incubated with fluorescein isothiocyanate-conjugated Annexin V antibody and the fluorescent intensities were measured by flow cytometry after stimulation with 0.1 U/ml thrombin **(A)** or 5 μmol/L ADP **(B)**. Average mean fluorescence intensities shown (**** $P < 0.0001$; NS. nonsignificant). Each experiment was repeated 3 times, with blood pooled from at least 8 mice each time. WPS indicates Waterpipe Smoke.

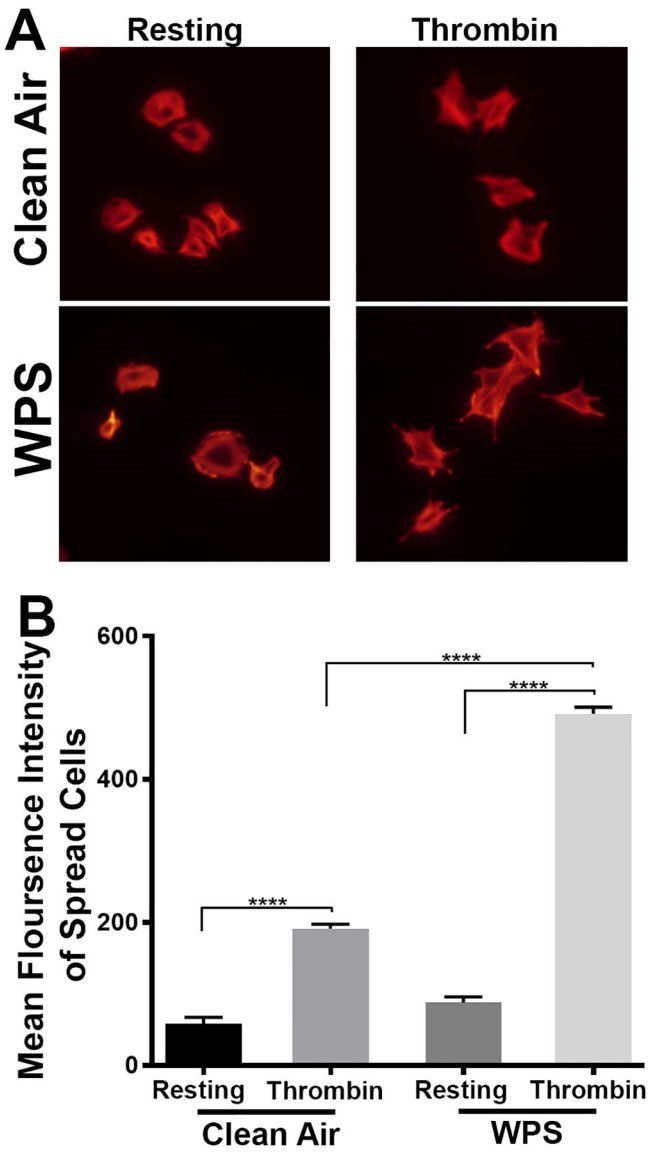

Figure 5:

Platelet spreading is enhanced in WPS exposed mice. Platelets from WPS and clean air exposed mice were allowed to adhere to fibrinogen-coated coverslips for 5 minutes after stimulation with thrombin (0.1 U/mL). (**A**) Tetramethylrhodamine-conjugated phalloidin was used to stain for F-actin and imaged using Leica DMi8 inverted widefield fluorescence microscope with integrated high precision focus drive. Images were processed using LAS X Wizard imaging software. (**B**) Quantification of the spreading data. Data are representative of 3 independent experiments. Each experiment was repeated at least 3 times, with blood pooled from a group of 8 mice each time. WPS indicates Waterpipe Smoke.

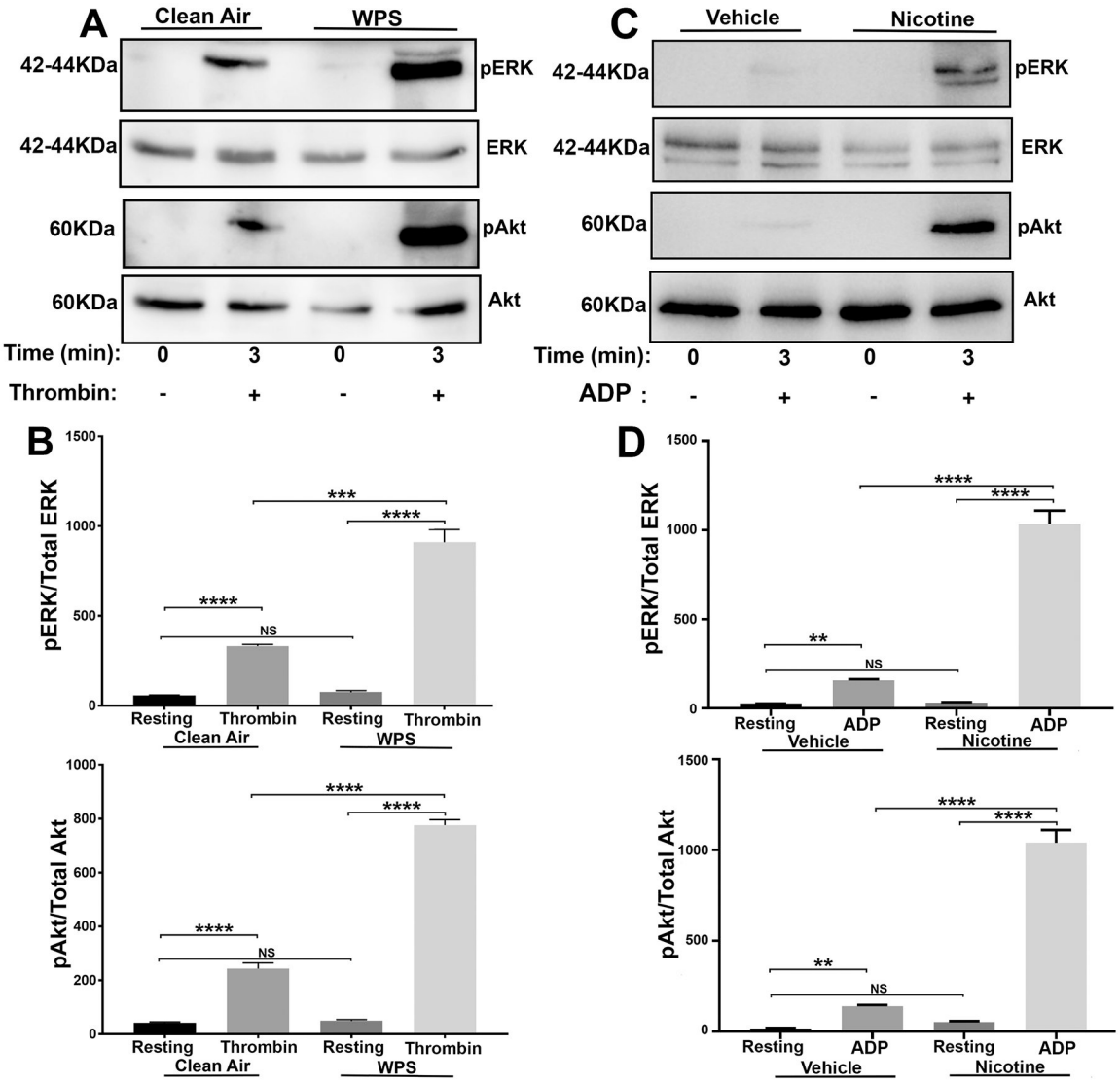

Figure 6:

Platelet Akt and ERK activation (phosphorylation) are enhanced in WPS exposed and nicotine treated mice. Platelets from WPS and clean air exposed mice as well as from nicotine and vehicle injected mice were prepared and washed. Platelets were stimulated with 0.1 U/ml thrombin **(A)** or 5 μmol/L ADP **(C)** for 3 minutes, and proteins were lysed using 1X sample buffer. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis before being subjected to immunoblotting with anti-Akt, anti-pAkt (Ser473), anti-ERK, and anti-pERK antibodies. **(B. D)** Data quantification of agonist-induced ERK and Akt phospgorylation (****$P < 0.0001$; ***$P < 0.001$; NS. nonsignificant). Each experiment was repeated at least 3 times, with blood pooled from a group of 8 mice each time. WPS indicates Waterpipe Smoke.

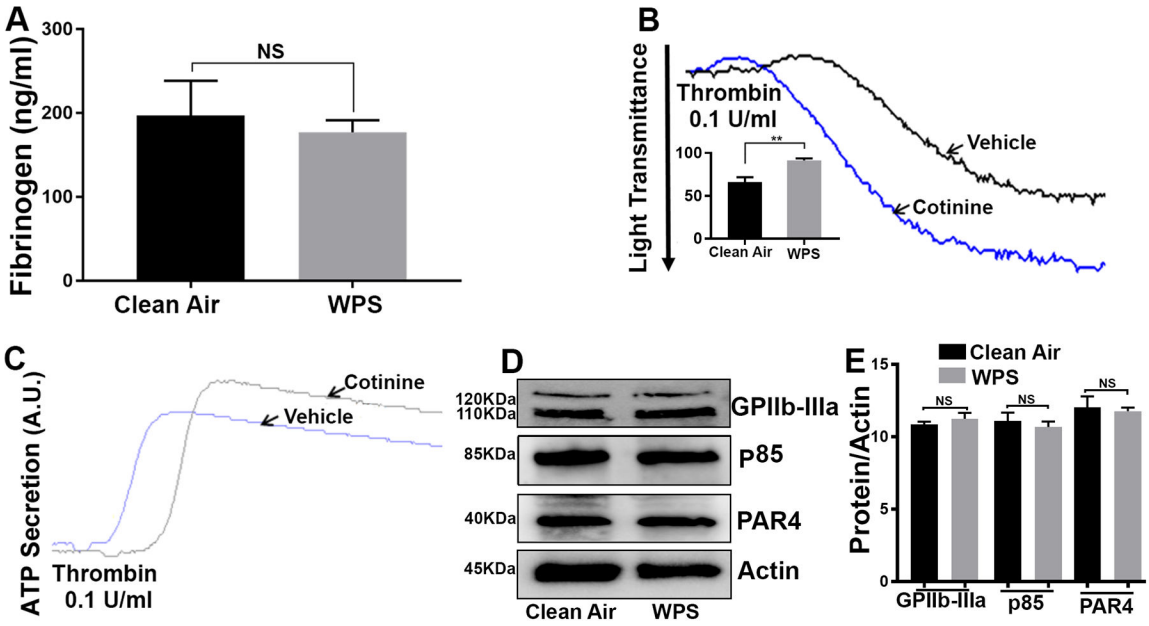

Figure 7:

WPS does not affect plasma fibrinogen or protein expression levels of GPIIb-IIIa, PAR4 or p85 (PI3K), whereas continine enhances aggregation and dense granule secretion. Plasma was collected from WPS and clean air controls before the levels of fibrinogen were measured **(A)**; data is presented as mean ± SD (n = 5). Mice were injected with 1 μM cotinine or vehicle, once daily for 1 week before platelets were harvested, and stimulated with thrombin (0.1 U/ml), and aggregation **(B)** and ATP release **(C)** were monitored using Lumi-aggregometer. **(D)** Platelets from WPS and clean air exposed mice were prepared and washed. Proteins were lysed using 1X sample buffer, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis before being subjected to immunoblotting with anti-GPIIb-IIIa (αIIbβ3), anti-PAR4 or anti-p85/PI3K antibodies; **(E)** data quantification of GPIIb-IIIa,PAR4 and p85/PI3K expression; NS: nonsignificant. Each experiment was repeated at least 3 times, with blood pooled from a group of 8 mice each time. WPS indicates Waterpipe Smoke.

Table 1:

Blood was collected from the heart and counted as described in the Methods section. All counts are expressed as thousands per microliter, except for red blood cells, which are expressed as millions per microliter. Data are presented as mean ± SD. WPS indicates Waterpipe Smoke; MPV, mean platelet volume.

Cell Type	WPS	Clean Air	P Values
Platelets	556 ± 24.3	573 ± 31.2	0.40
MPV	4.8 ± 0.26	4.9 ± 0.1	0.54
Red blood cells	8.15 ± 0.85	8.05 ± 0.90	0.88
Lymphocytes	1.77 ± 0.05	1.83 ± 0.37	0.76
Monocytes	0.07 ± 0.01	0.10 ± 0.02	0.17
Granulocytes	2.29 ± 0.10	2.50 ± 0.30	0.3
HCT	40.4 ± 1.45	39.45 ± 2.11	0.72