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The effect of volatile organic compounds on endothelial function and atherogenesis.

Samantha McFall
University of Louisville

THE EFFECT OF VOLATILE ORGANIC COMPOUNDS ON ENDOTHELIAL FUNCTION AND ATHEROGENESIS

By

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ABSTRACT

EFFECT OF VOLATILE ORGANIC COMPOUNDS ON ENDOTHELIAL FUNCTION AND ATHEROGENESIS

Samantha A. McFall

August 23, 2022

Volatile organic compounds (VOCs) are a group of pervasive air pollutants that are released into the atmosphere from both anthropogenic and environmental sources. Recent epidemiological studies suggest that exposure to various VOCs is associated with an increased risk of cardiovascular disease. However, the relationship between VOCs and cardiovascular disease is not well-studied. Using animal models and three select VOCs, benzene, xylene, and trichloroethylene, we studied the effects of VOCs on endothelial injury and benzene-induced atherogenesis. We found that exposure to benzene and xylene increased circulating endothelial microparticles, depleted progenitor cells, and increased platelet activation. Following the exposure to the representative VOC, trichloroethylene (TCE), we saw a decrease in circulating endothelial microparticles, modest changes in platelet activation, and modest changes in progenitor cell populations. This suggests that petroleum products such as benzene and xylene may be playing a more deleterious role in endothelial injury and atherogenesis than chlorinated VOCs like TCE.

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INTRODUCTION

Cardiovascular Disease

Cardiovascular disease is the number one cause of death worldwide and are a group of disorders that affect the heart and the blood vessels (57). According to the Center for Disease Control (CDC) as of 2020, roughly 91 to 220 people per 100,000 die due to cardiovascular disease in the United States, but in the southeastern region of the US the rates can be as high as 1,170 people per 100,000. These diseases can include coronary heart disease, peripheral arterial disease, rheumatic heart disease, and ischemic stroke. In areas like Eastern Asia many of the cardiovascular disease-related deaths are attributed to ischemic stroke while in the Americas most cardiovascular disease related-deaths result from peripheral arterial disease and coronary heart disease (37). However, these diseases all occur through the development of atherosclerosis within the arteries leading to the brain, the peripheral arteries, or the coronary artery, respectively. The blockage of these blood vessels by the atherosclerotic plaques is what can lead to acute cardiovascular events and death. According to the American Heart Association, on average, people over the age of 40 have a fifty percent chance of developing serious atherosclerosis, and the risk continues to increase with age (53). Atherosclerosis is referring specifically to the development of fatty, fibrous

plaques within arterial walls. As these plaques increase in size they can obstruct the arterial lumen, reduce blood flow, and lead to ischemia.

The initial stages of atherogenesis begins with fatty streak formation, and the fatty streaks occur through progressive stages. These stages are comprised of largely of endothelial injury, LDL-C trapping, activation of endothelial cells, leukocyte activation, and formation of foam cells (9). Chronic endothelial injury can occur in various conditions that can also be present as risk factors of atherogenesis and CVD, including hypertension, hyperlipidemia, smoking, exposure to toxicants, but also things like immune reactions or viral infections (10,17, 24, 47). In normal conditions, low density lipoprotein exists in an equilibrium between plasma and intracellular concentrations but under chronic endothelial injury this equilibrium is altered (9). Additionally, following endothelial injury, the endothelial cells are activated. The activated endothelial cells have increased permeability, increased expression of adhesion molecules, and increased transmigration. This leads to an enhanced leukocyte and monocyte adhesion and transmigration into the intima layer. Within the intima, macrophages are activated and begin to take up the increased lipids, die, and become foam cells (21). This progression then furthers into the later stages of atherosclerosis, atheroma, and atherosclerotic plaque formation where the blood vessels can become blocked or injured. Following vessel injury, platelets are activated (21). Activation of platelets can increase coagulation to inhibit excess blood loss resulting from vessel injury. A deleterious role of platelets is through

aberrant activation, where they can lead to increased thrombus formation and acceleration of atherogenesis (21).

In the later stages of atherosclerosis, the endothelial cells and nearby smooth muscle cells release cytokines and growth factors like interleukins and tumor necrosis factors. The secretion of these factors results in the smooth muscles cells (SMCs) migrating into the luminal side of the vessel wall (21). This migration allows for the formation of the fibrous cap. The fibrous cap is composed of collagen-rich fiber tissues, SMCs, macrophages, and T lymphocytes. As this fibrous cap forms into the mature atherosclerotic plaque, it bulges into the channel leading to reduced blood flow and the potential for acute events (21).

The Endothelium and Biomarkers of Injury

The endothelial system is composed of the arteries, veins, and capillaries within the brain, skin, lungs, heart, and muscles. Because of the vast localization of the endothelium, it is involved in the physiology of multiple organ systems. The initial role of the endothelium was believed to be a type of selectively permeable wall of the vasculature (50). As the understanding of the endothelium increased it was found that it functions as a more complex endocrine organ. The functions of the endothelial cells include fluid filtration, blood vessel tone, hemostasis, immune cell recruitment, hormone trafficking, sensory, and regulating blood flow (50). When the endothelial layer is injured or dysfunctional it can promote pathophysiological conditions. Endothelial dysfunction is associated with diseases like diabetes, peripheral vascular disease, coronary artery disease,

chronic renal failure, severe viral infection, hypertension, nearly all cardiovascular diseases, and it plays a major role in the development of atherosclerosis, tumor angiogenesis, vascular leakage, infectious disease, and stroke (50). The apparent critical role of the endothelium in a variety of disorders necessitates measures of endothelial function as a method of early detection and potential treatment. There are both invasive and noninvasive approaches to measuring endothelial function that are used in clinical and research settings.

One invasive approach utilizes vasoactive agents delivered through intra-arterial infusion, and the response is measured using high resolution ultrasound. The other major invasive method used is intravascular infusions of vasoactive stimulants and intravascular ultrasound commonly known as intravascular coronary flow mediated dilation (FMD), (3). The noninvasive methods include ultrasound FMD, pulse wave or pulse counter analysis, Flow-mediated Magnetic Resonance Imaging (MRI), Laser Doppler Flowmetry, and Flow Mediated Pulse Amplitude Tonometry (3,5). The most commonly used noninvasive approach is ultrasound FMD of the brachial artery as it is more widely available and less costly than FMD measured by MRI and has less variability than pulse wave and pulse counter analyses (3).

In addition to measures used in the clinical setting it has become increasingly informative to measures indices of subclinical endothelial dysfunction. One of these subclinical markers are circulating endothelial microparticles. These microparticles are shed from activated, or apoptotic endothelial cells, and they have been shown to be a sensitive marker for vascular

injury and a predictor for cardiac events (45,34). An increase in these microparticles has been associated with cardiovascular events and with endothelial dysfunction in patients with diabetes mellitus, obesity, end stage renal failure, and coronary artery disease (4,19,29,66). An increase in these circulating microparticles has also been associated with exposure to fine particulate matter, suggesting they may be useful in identifying air pollutant induced endothelial dysfunction (46). Other subclinical biomarkers that have been identified in recent studies as useful in measuring endothelial dysfunction are levels of soluble adhesion molecules, markers of angiogenesis, cytokines, chemokines, circulating immune cells, endothelial progenitor cells, and platelet leukocyte aggregates (21). Soluble adhesion molecules are released by the activated, injured endothelial cell layer signaling the early stages of atherogenesis. The other plasma markers like cytokines and chemokines are induced as the later stages of atherosclerotic development progress but also are signals of a dysfunctional endothelium (31). The immune cells can also act as another marker of progressive or later stage atherosclerotic plaques, as these lesions often contain macrophages, monocytes, T lymphocytes, and B lymphocytes (24). In addition, any modulation in the recruitment of immune cells could impact the progression of endothelial injury and atherosclerosis, as immune cells play a role in recruitment of angiogenic cells. Angiogenic cells include several types of progenitor cells. These progenitor cells derive from stem cells and are involved in several aspects of endothelial repair. Therefore, a depletion of angiogenic cells can be another marker of a dysfunctional endothelium. Specifically, endothelial

progenitor cells (EPCs) play a vital role in endothelial repair and changes in circulating EPCs are a predictor of arterial stiffness and cardiovascular disease (51,63). Another important marker of atherogenesis is platelet activation. Under normal conditions, platelets are important in maintaining vascular integrity and prevent spontaneous hemorrhaging. In the context of atherogenesis or endothelial injury, the platelets are recruited to the site of injury and adhere to the endothelial cell layer, owing in part to the increased expression of adhesion molecules. This increased adhesion leads to an accumulation of platelets on the arterial wall which promotes further platelet activation and aggregation. Platelet activation and the presence of platelet leukocyte aggregates are markers of thrombosis and are central in the progression of atherogenesis and an index of endothelial dysfunction (52).

Air Pollutants and Risk Factors of CVD

One of the important advances in addressing CVD has been the identification of risk factors which have been identified in large cohort studies like the Framingham Heart Study. There are various risk factors of atherosclerosis found through these studies including age, high LDL cholesterol, elevated blood pressure, diabetes mellitus, and smoking (36, 49, 53). These classic risk factors are well established and are used to assess cardiovascular risk within the general population. In addition to these classical risk factors, there are emerging risk factors. These factors are not as broadly established but have emerging evidence which suggests a strong association with cardiovascular disease in population-based studies and are involved in the physiological development of

CVD. Emerging risk factors include exposure to certain pollutants in the air, food, and drinking water (6,37, 48, 49).

Air pollution is an intricate mixture of gases and particles that permeate the indoor and outdoor atmosphere leading to inevitable exposure. The sources of air pollution range from natural sources like wildfires, to exhaust from motor vehicles, industrial processes, and the use of tobacco products. To reduce the increased prevalence of air pollution, several countries have passed legislation to reduce air pollutants and regulate specific air toxicants. Specifically, the United States have regulated more than 180 air toxins through the Clean Air Act and through government agencies like the Environmental Protection Agency (EPA). Air pollutants have been broadly separated by the EPA into two groups, criteria air pollutants and hazardous air pollutants. Criteria air pollutants include things like particulate matter, ground level ozone, and carbon monoxide. They are closely regulated and monitored by the EPA and other government agencies which, according to the EPA, has led to a 78% decrease in the emissions of the 6 most common air pollutants between 1990 and 2020. Hazardous air pollutants are not as strictly monitored or regulated but include various toxic substances like dioxins, asbestos, some metals that can be carried in the air (e.g. cadmium and mercury), and several volatile organic compounds (VOCs). Despite these ongoing attempts to decrease air pollution, the health impacts of breathing polluted air remain. According to the World Health Organization (WHO), air pollution is believed to cause an estimated seven million premature deaths per year globally (72). These deaths are attributed largely to cardiovascular diseases

with 33% and 37% being due to ischemic heart disease and stroke, respectively (72). Recent studies show an association between air pollution and endothelial injury, systemic inflammation, ischemic heart disease and increased hospitalization for heart failure, and even death (6, 7, 46, 62, 70).

Volatile Organic Compounds

Volatile organic compounds are a group of chemicals that are ubiquitous in indoor and outdoor air. VOCs derive from both natural sources and anthropogenic processes. High levels of VOCs are also associated with Superfund waste sites (39). These are sites annotated by the EPA as contaminated sites due to hazardous waste being improperly managed and include manufacturing, processing, or mining locations. According to the EPA, these sites have historically posed a risk to human health and through the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) the EPA has begun to execute cleanup of these sites, identify parties responsible for the hazardous waste, reach out to communities impacted, and return the sites to productive use. It is well known that levels of VOCs can be 2 to 5 times higher in indoor air compared with outdoor air and after the use of certain household or commercial products like paint strippers, VOCs can be nearly 1,000 times the outdoor levels (35). Presently, there are no current standards in place for VOCs levels in non-industrial settings. However, of the thousands of VOC chemicals, several have been identified as specific health risks and, in some cases, have developed guidelines for exposure levels.

VOC Toxicity and CVD

As a class, VOCs encompass a broad set of chemicals that exhibit different toxic effects and can induce these effects at different severities. Previous studies have associated VOC exposures to respiratory illness, leukemia, birth defects, neurocognitive impairments, and cancer (35). Some VOCs such as, benzene, 1,3-butadiene, and trichloroethylene are classified as known carcinogens by the International Agency for Research on Cancer (IARC). Specifically, exposure to 1,3-butadiene, which is abundant in tobacco smoke, increased the risk of lung cancer (35). Acrylamide, acrylonitrile, N,N-dimethylformamide, ethylbenzene, isoprene and styrene are also classified as probable IARC carcinogens. Notably, acrylamide exposure was not only linked to breast cancer but also to central and peripheral nervous system damage (35). Acrolein, crotonaldehyde, toluene, and xylene have been classified as group 3 carcinogens, meaning in this instance, they are unclassifiable as to their carcinogenicity in humans. Exposure to benzene, toluene, ethylbenzene, and xylene (BTEX) affects the central nervous system as well as immune and reproductive systems in humans (35). Tetrachloroethylene exposure during pregnancy was linked to increased risk of stillbirth and placental diseases (35). Prenatal VOC exposure was associated with elevated risk of neural tube defects, congenital male genital abnormalities, and wheezing/asthma in infants (39). The extent of toxicity of a given VOC can be attributed to its chemical property, the duration and exposure level.

Evidence of the role that VOCs may play in air pollution induced cardiovascular disease can be seen through studies that link VOCs to CVD risk

factors. Recent studies show that acrolein can increase human susceptibility to vasospasm, a risk factor of atherosclerosis (15). Additionally, it has been shown that benzene is associated with cardiovascular disease risk in humans as assessed through Framingham Risk Score (1). In addition to human studies, animal studies have also linked VOCs to CVD risk. In a mouse model, exposure to benzene was shown to cause a decrease in circulating angiogenic cells and increase in lipoprotein levels (1). Similarly, chronic exposure to acrolein was shown to decrease EPCs, B-cells, and monocytes (16). Acute acrolein exposure in mice also induced platelet activation, thrombosis, diminished vascular repair, and promoted arrythmias and cardiac dysfunction (59). As these pathogenic changes occur following VOC exposure, it suggests that endothelial dysfunction may be playing an important role, and there is a need to explore the plausibility that VOC exposure is enough to induce endothelial dysfunction and CVD. In these studies, we systematically examine the effect of three major VOCs, benzene, xylene, and trichloroethylene, on surrogate markers of cardiovascular disease within a well-controlled mouse model.

MATERIALS AND METHODS

Housing and Treatment of Mice:

All mice were obtained from Jackson Laboratories and maintained under standard laboratory conditions and treated humanely according to American Physiological Society Guiding Principles in the Care and Use of Animals. All protocols were approved by University of Louisville Institutional Animal Care and Use Committee. Mice were housed under pathogen-free conditions in the University of Louisville vivarium under controlled temperature, humidity (70-72°F, 30-70% humidity), 12h light/12h dark cycle and maintained on standard chow (Rodent Diet 5010, LabDiet, St. Louis, MO) containing 4.5% fat by weight). Inhalation exposures were performed as described in previous publications (2,16).

Benzene exposure:

At eight weeks of age, C57BL/6 male mice were exposed to either 50ppm aerosolized benzene or filtered (charcoal and HEPA) air (n=24/group) for 6 hours a day, 5 days a week for 6 weeks (**Schematic 1a, Schematic 2**). Additionally, a subset (n=10/group) of the benzene and air-exposed mice were treated with 0.5 mg/kg lipopolysaccharide (LPS, Sigma Cat# 2630, Lot# 028M4022V; intraperitoneal injection) to assess the susceptibility to inflammation. Animals were euthanized at the end of the final six-hour exposure period by sodium

pentobarbital overdose and blood and tissues were collected. Blood was obtained through cardiac puncture using 0.2mM EDTA as an anticoagulant. Blood was either centrifuged to collect plasma or prepared for use in flow cytometry.

Xylene exposure:

At eight weeks of age, C57BL/6 male mice were exposed to either 50 ppm aerosolized xylene or (charcoal and HEPA)-filtered air for 6 hours a day, 5 days a week for 12 weeks (n=24/group) (**Schematic 1a, Schematic 2**). Animals were euthanized at the end of the final six-hour exposure period through the use of sodium pentobarbital overdose and blood and tissues were collected. Blood was obtained through cardiac puncture using 0.2mM EDTA as an anticoagulant. Blood was either centrifuged to collect plasma or prepared for use in flow cytometry.

TCE exposure:

At eight weeks of age, both male and female C57BL/6 mice were exposed to either 0.5 milligrams per milliliter trichloroethylene (TCE) + alkamuls EL620 (1% w/v) in drinking water or alkamuls EL620 (1% w/v) in drinking water for one year (**Schematic 1b**). Animals were euthanized following an overnight fasting period, using sodium pentobarbital overdose and blood and tissues were collected. Blood was obtained through cardiac puncture using 0.2mM EDTA as an anticoagulant. Blood was either centrifuged to collect plasma or prepared for use in flow cytometry.

Flow Cytometry:

Microparticles (MPs)

Microparticles in the peripheral blood were measured using flow cytometry (**Schematic 3**). Plasma was centrifuged for 2 min (11,000xg at 4°C) to remove residual cells and debris, and the supernatant was collected and centrifuged for 45 min (17,000xg at 4°C). The resulting microparticle pellet was resuspended in Annexin V Buffer pre-filtered through 0.22μm syringe filter and incubated with the anti-mouse FcBlock (CD32/CD16) for 10 minutes. Endothelial microparticles were stained with the antibody cocktail containing Annexin V-Pacific Blue, Flk-APC, Sca-PECy7, CD62E-PE and CD143-FITC for 30 min. Identical samples with no antibodies were utilized as controls for the gating. Counting beads, added to individual samples were used for data normalization. Samples were collected on a BD LSR II flow cytometer for 5 min at low speed. Microparticle numbers were quantified in gated populations <1μm in size and positive for Annexin V staining using the FlowJo software. Microparticle subpopulations were further identified based on expression of various surface markers.

Circulating endothelial progenitor cells

Endothelial progenitor cells were quantified from the blood plasma. Murine blood was collected by cardiac puncture after euthanasia. Circulating levels of Flk-1+/Sca-1+ cells were measured by flow cytometry and analyzed using FlowJo software.

Bone marrow derived progenitor cells

Bone marrow cells were collected from the femur and tibia of exposed mice and separated by Ficoll gradient centrifugation. The cells were then washed twice with PBS containing 1% BSA (PBS/BSA) and incubated with Fc Block (anti-mouse CD32/CD16) for 10 minutes at 4°C to block non-specific binding. Samples were incubated again for 30 minutes at 4°C with antibody cocktails containing Lineage-Pacific Blue, ckit-APC-Cy7, Sca-FITC, and CD34-Alexa Fluor 700 antibodies, and events were collected on an LSR II flow cytometer for 90 seconds at high speed. Cell populations were gated using FlowJo software.

Platelet-Leukocyte aggregates

Blood was collected in Na4·EDTA (0.2M; 16 µl/ml blood). Aliquots of the whole blood were diluted with 4 times volume of HEPES-Tyrode solution and the samples were fixed in 1% formaldehyde for 30 minutes at 4 °C. Red blood cells were lysed by dilution in water. Cells were then collected by centrifugation at 400g for 8 minutes, were washed with HEPES-Tyrode solution containing 1% BSA, and then stained with FITC-labeled anti-CD41 and anti-CD45 for 30 minutes on ice. The stained cells were analyzed on an LSR II Flow Cytometer. Leukocyte aggregates were identified and quantified as events that are double positive for CD41 (platelets) and CD45 (leukocytes) and expressed as a percentage of total events.

Markers of endothelial function and inflammation:

Concentrations of soluble adhesion molecules, markers of angiogenesis, and cytokines and chemokines in plasma were measured by multiplex arrays at Eve Technologies (SKU: MD31, Calgary, Alberta, Canada).

Clinical chemistry parameters:

Plasma levels of cholesterol, high density- and low density-lipoproteins (HDL and LDL), creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured on Roche Cobas MIRA Plus chemistry analyzer.

Complete blood counts:

Complete blood counts were measured using the Hemavat 950FS hematology analyzer calibrated with multispecies hematology reference controls (Drew Scientific, INC)

***In vitro* experiments:**

To examine the effect of benzene metabolites on endothelial cell apoptosis and microparticle formation *in vitro*, Human aortic endothelial cells (HAEC) were incubated with hydroquinone (HQ), catechol (Cat), and t,t-mucondialdehyde (MA) (10 μM each) for 18h, and the extent of apoptosis was examined by Western blotting for cleaved-caspase-7, caspase-3, caspase-8, and caspase-9 antibodies. Microparticles (<1mm, Annexin V+) released into the cell culture medium were analyzed by flow cytometry *as described above*.

Statistics:

For all *in vivo* experiments, data are expressed as mean ± standard error of mean (SEM). Statistical significance was accepted at $P<0.05$ level. Student's two-tailed t test was used to compare two sets of data. Two-way analysis of variance, followed by Bonferroni post-tests was used to compare more than two experimental groups.

RESULTS

VOC exposure and endothelial activation and injury: The endothelium plays a critical role in regulating vascular homeostasis. Circulating endothelial microparticles are subclinical markers of endothelial activation and injury. These microparticles are sub 1 μm vesicles that are shed from activated and injured cells. Increased levels of blood endothelial microparticles have been previously observed in patients with coronary artery disease and stroke.

We observed here that inhalation exposure to benzene increased levels of circulating endothelial microparticles (EMP; <1μm, AnnexinV+/Flk+), activated endothelial microparticles (AEMP; <1μm, AnnexinV+/CD62E+), endothelial progenitor cell microparticles (EPCMP; <1μm, AnnexinV+/Flk+ /Sca+), lung endothelial microparticles (LEMP; <1μm, AnnexinV+/Flk+ /CD143+), and activated lung endothelial microparticles (ALEMP; <1μm, AnnexinV+/CD62E+/CD143+) by 1.8 to 3.8 fold (**Fig. 1**).

To further assess the effect that benzene exposure had on endothelial activation we measured the levels of soluble adhesion molecules. We saw a slight decrease in levels of soluble intra cellular adhesion molecule-1 (sICAM-1;

Table 1), while other soluble adhesion molecules including, platelet endothelial cell adhesion molecule-1 (sPECAM-1), endothelial selectin (sE-selectin), and platelet selectin (sP-selectin) had levels that were comparable to the controls (**Table 1**). In addition, another study has shown that benzene inhalation depletes angiogenic cells in mice (1). Therefore. we measured circulating angiogenesis markers, but our data showed that under our experimental conditions, the levels of these angiogenesis markers in benzene-exposed mice were comparable to air controls (**Table 1**).

To evaluate the potential of xylene to induce endothelial activation and injury we again measured circulating endothelial microparticles in the peripheral blood of xylene- and air-exposed mice. We observed that exposure to xylene increased the levels of circulating endothelial microparticles, activated endothelial microparticles, and EPC-derived microparticles by 2.4-3.6-fold (**Fig 2**).

In agreement with the increase of circulating endothelial microparticles we saw an increase in the plasma levels of the adhesion molecule, sICAM-1. Taken together, these data suggest that inhalation exposure to xylene induces endothelial activation in mice (**Table 2**).

Lastly to address the potential of trichloroethylene to induce endothelial activation and injury we measured circulating endothelial microparticles. Our results show that TCE exposure in male mice reduced levels of circulating endothelial microparticles, activated endothelial microparticles, and endothelial progenitor cell microparticles to 43%-47% of the levels seen in air-control mice

(**Fig 3**). In female mice, the levels of circulating endothelial microparticles were comparable to the air control (**Fig 3**).

To support *in vivo* data provided from these three studies, we also utilized *in vitro* experiments. Because the toxicity of benzene is mediated by its metabolism to reactive metabolites, we directly examined the effect of benzene metabolites hydroquinone (HQ), catechol (Cat), and t,t-mucondialdehyde (t,t-MA)on human aortic endothelial cell (HAEC) apoptosis. Hydroquinone and catechol only modestly increased the activation of the apoptosis marker caspase-7 in HAEC, whereas t,t-mucondialdehyde profoundly increased caspase-7 cleavage (**Fig 4a**). To further examine the pathway of benzene metabolite-induced apoptosis, we assessed the cleavage of caspase-3, caspase-8, and caspase-9. Our results show that exposure to t,t-MA increased activation of caspase-3 and caspase-9, but had no effect on caspase-8 (**Fig 4b**). This suggests that t,t-MA is selectively activating the intrinsic pathway of apoptosis. Next, we examined the effect of t,t-mucondialdehyde on endothelial microparticle formation *in vitro*. We found that incubation of HAEC with t,t-mucondialdehyde increased the microparticle formation by 2.4-fold (**Fig 4c**).

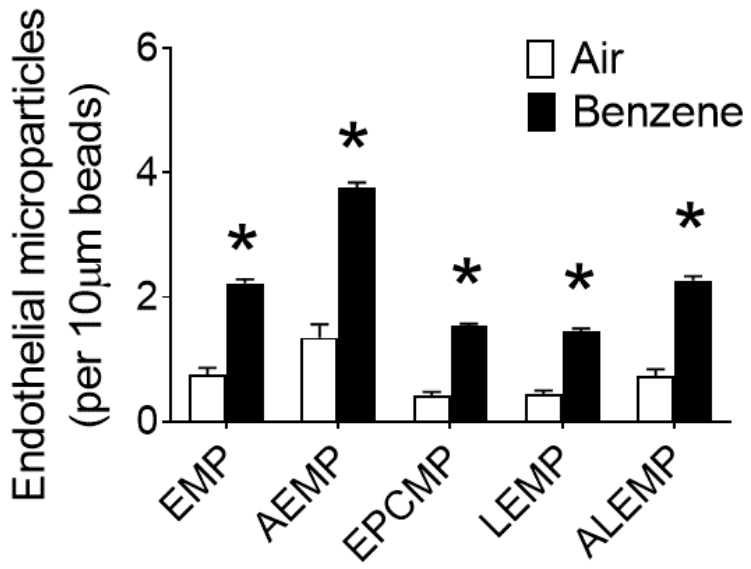


Figure 1: ***Benzene exposure increases circulating endothelial microparticles***. The measurements of endothelial microparticles (EMP; <1μm, AnnexinV+/Flk+), activated endothelial microparticles (AEMP; <1μm, AnnexinV+/CD62E+), endothelial progenitor cell microparticles (EPCMP; <1μm, AnnexinV+/Flk+ /Sca+), lung endothelial microparticles (LEMP; <1μm, AnnexinV+/Flk+ /CD143+), and activated lung endothelial microparticles (ALEMP; <1μm, AnnexinV+/CD62E+/CD143+) in the plasma of benzene (50 ppm, 6hr/d. 5d/wk, 6wks)- or filtered (HEPA and charcoal) air-exposed male mice were determined through flow cytometry (n=10/ group). Data previously published in *Toxicology and Applied Pharmacology* (73).

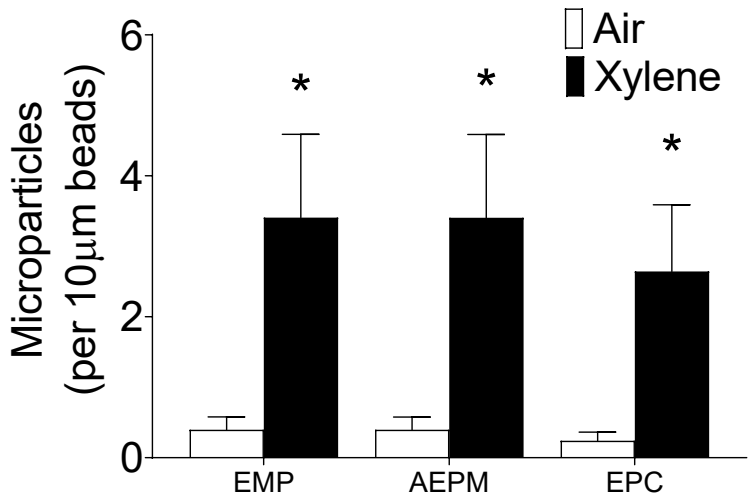


Figure 2: ***Xylene exposure increases circulating endothelial microparticles.*** The measurements of endothelial microparticles (EMP; <1μm, AnnexinV+/Flk+), activated endothelial microparticles (AEMP; <1μm, AnnexinV+/CD62E+), and endothelial progenitor cell microparticles (EPCMP; <1μm, AnnexinV+/Flk+ /Sca+) in the plasma of xylene- (50ppm, 6 hrs/d, 5d/wk, 12 weeks) or filtered (HEPA and charcoal) air-exposed male mice were determined through flow cytometry (n=10/ group).

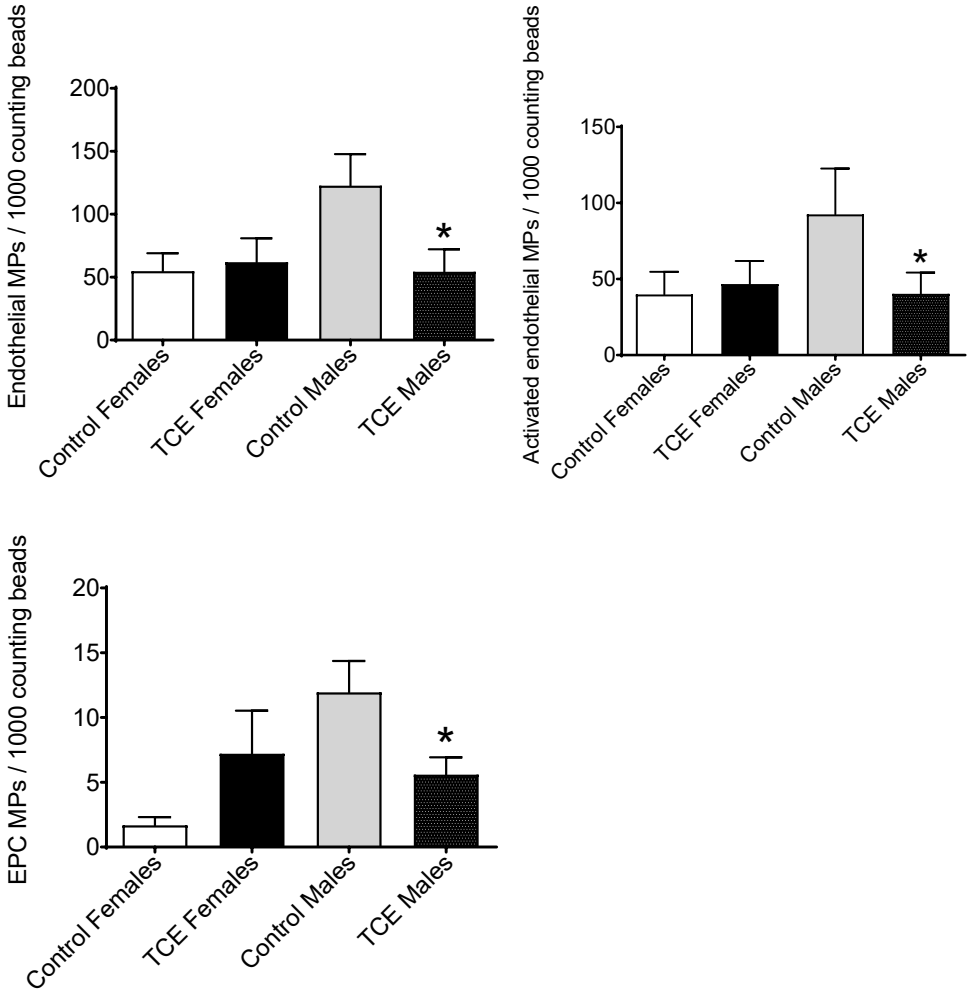
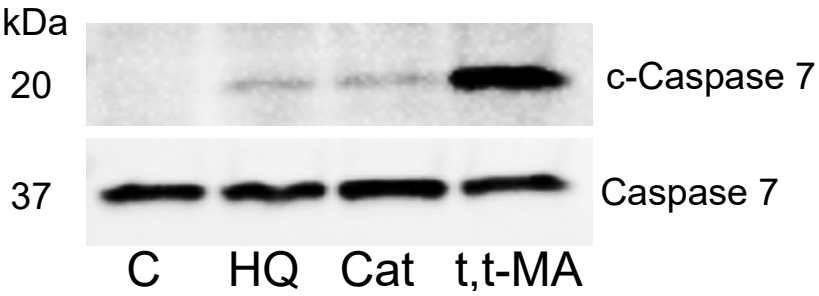


Figure 3: ***TCE exposure alters levels of circulating endothelial microparticles.*** The measurements of endothelial microparticles (EMP; <1µm, AnnexinV+/Flk+), activated endothelial microparticles (AEMP; <1µm, AnnexinV+/CD62E+), and endothelial progenitor cell microparticles (EPCMP; <1µm, AnnexinV+/Flk+ /Sca+) in the plasma of TCE (5mg/mL, 52 weeks)- or alkamuls (1% w/v)- exposed mice (female and male) were determined through flow cytometry (n=10/ group).

4a.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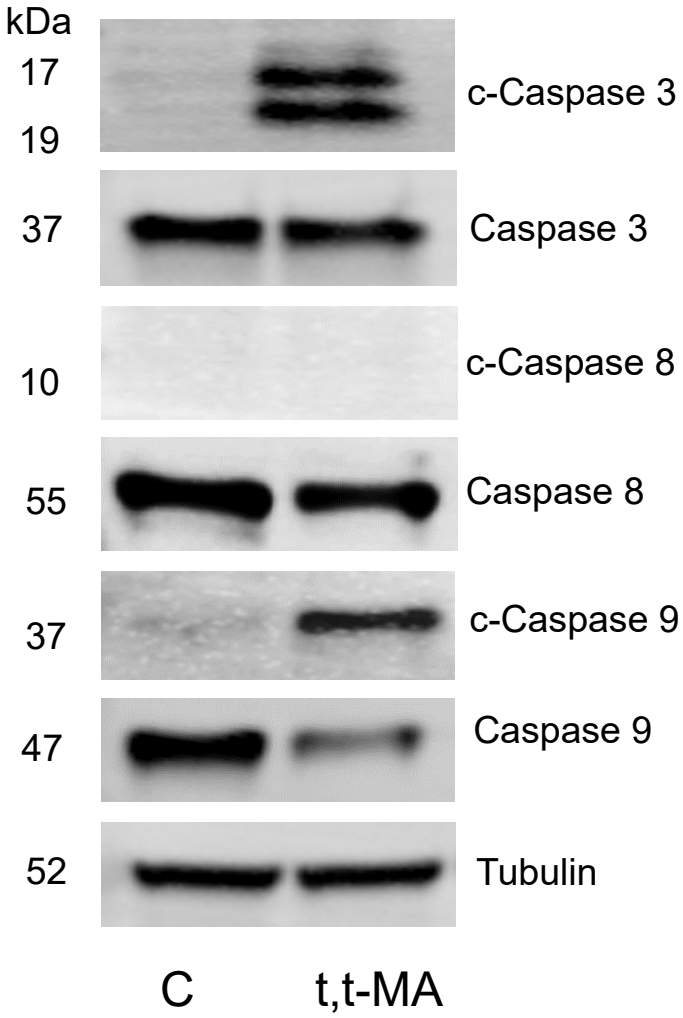
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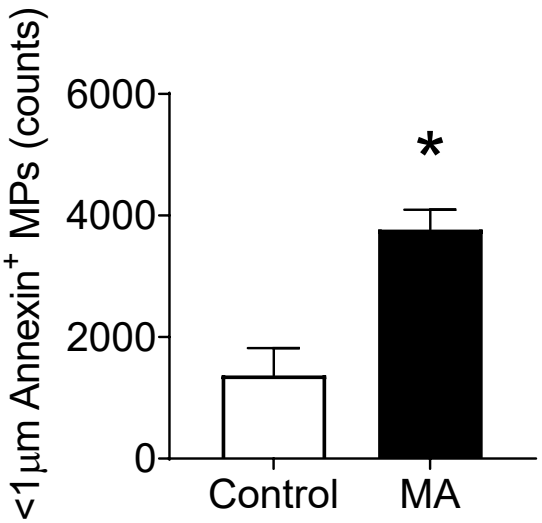


4c.

Figure 4. ***Effect of Benzene metabolites on endothelial cell apoptosis.*** **4a**. Benzene metabolites t,t-Mucondialdehyde (MA), hydroquinone (HQ), and catchol (Cat) induce activation of caspase-7 (10uM, 18hr). **4b**. Benzene metabolite, t,t-mucondialdehyde (10uM, 18hr), induces activation of caspase-3 and caspase-9. **4c**. t,t-MA exposure (10uM, 18hr) causes an increase in endothelial cell microparticles in human aortic endothelial cells. *P<0.05 vs control. Data previously published in *Toxicology and Applied Pharmacology* (73).

VOC exposure and endothelial repair: Progenitor cells are a precursor cell type that can further differentiate into unique and specialized cell types. Under normal conditions, these cells are important in maintaining vascular homeostasis. Progenitor cells play a vital role in the repair processes of the endothelium following injury. Specifically, bone marrow derived hematopoietic progenitor cells and circulating endothelial progenitor cells are directly involved in the processes of paracrine signaling, healing endothelial damage, and formation of new blood vessels in ischemic tissue.

Benzene, and other VOCs, have been previously identified as a hematopoietic toxin (11,31,42,56). We observed that inhaled benzene did not alter the levels of common myeloid progenitor (CMPC) and multipotent progenitor cells (MPPC) in the bone marrow, but it did significantly decrease the levels of hematopoietic progenitor cells (HPCs; **Fig. 5**). Hematopoietic progenitor cells play an important role in osteoblast and hematopoietic cell formation and is critical in hematopoiesis and endothelial cell development.

Xylene, unlike benzene, is not an established hematopoietic toxin. However, understanding any potential impact it could have on stem cells is important. We thus measured the levels of common myeloid progenitor (CMPC), multipotent progenitor cells (MPPC) and hematopoietic progenitor cells (HPC). However, we saw no difference between any of the levels of progenitor cells in the xylene-exposed group compared to the air control (**Fig 6**).

To further understand the effect of inhaled xylene on endothelial toxicity, we also measured circulating EPC levels. A depletion of blood EPCs has been

associated with cardiovascular disease and has been shown to be a predictor for future cardiac events (63). EPCs are also an important factor in endothelial repair. In addition, a previous study has shown that benzene exposure depleted circulating endothelial progenitor cells in mice (1). Here we saw that inhaled xylene exposure also depletes blood EPC (Flk+/Sca+) levels by 60% (**Fig. 7**)

To determine if TCE has hematopoietic toxicity, we measured the levels of hematopoietic progenitor cells (HPCs), multipotent progenitor cells (MPPC), and common myeloid progenitor cells (CMPC). Our data shows only a modest increase in common myeloid progenitor cells following TCE exposure in female mice (**Fig 8a**). All other levels of progenitor cells in TCE-exposed mice were comparable to air-exposed controls (**Fig 8a**).

Given that both benzene and xylene depleted circulating EPC levels in mice, we also measured circulating EPC levels in the TCE-exposed mice. However, we saw no statistically significant difference in either male or female mice following TCE exposure compared to the control (**Fig 8b**)

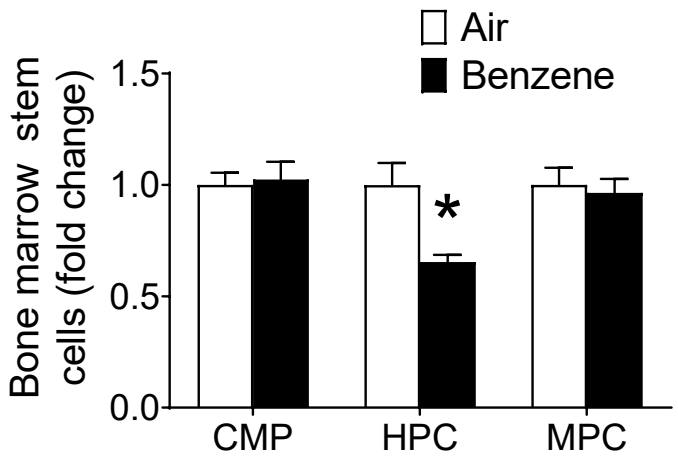


Figure 5: ***Benzene exposure reduces hematopoietic progenitor cells in bone marrow.*** Male mice were exposed to either benzene (50ppm 6hrs/d, 5d/wk, 6wks) or filtered (HEPA and charcoal) air, as described under *Methods,* and the bone marrow derived stem cells were analyzed using flow cytometry (n=10/group). Subpopulations of stem cells were evaluated through expression of surface markers. Common Myeloid Progenitor Cells (CMPC; Lin-ckit+Sca-CD34+), Hematopoietic Progenitor Cells (HPC; Lin-ckit+Sca+CD34-) and Multipotent Progenitor Cells (MPC; Lin-ckit+Sca+CD34+). Values are mean ± SEM. *P<0.05 vs control mice. Data previously published in *Toxicology and Applied Pharmacology* (73).

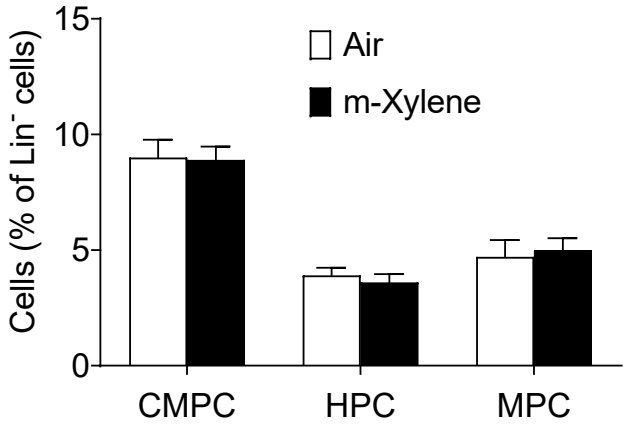


Figure 6: ***Xylene exposure effects on hematopoietic progenitor cells in bone marrow.*** Male mice were exposed to either m-xylene (50ppm, 6hrs/d, 5d/wk, 12wks) or filtered (HEPA and charcoal) air, as described under *Methods,* and the bone marrow derived stem cells were analyzed using flow cytometry (n=10/group). Subpopulations of stem cells were identified through expression of surface markers. Common Myeloid Progenitor Cells (CMPC; Lin-ckit+Sca-CD34+), Hematopoietic Progenitor Cells (HPC; Lin-ckit+Sca+CD34-) and Multipotent Progenitor Cells (MPC; Lin-ckit+Sca+CD34+). Values are mean ± SEM. *P<0.05 vs control mice.

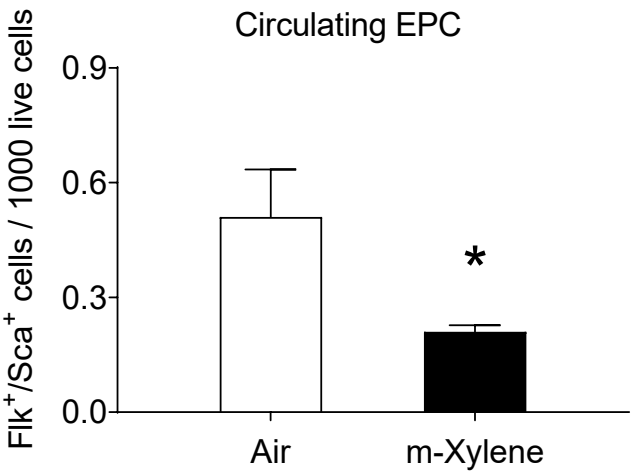
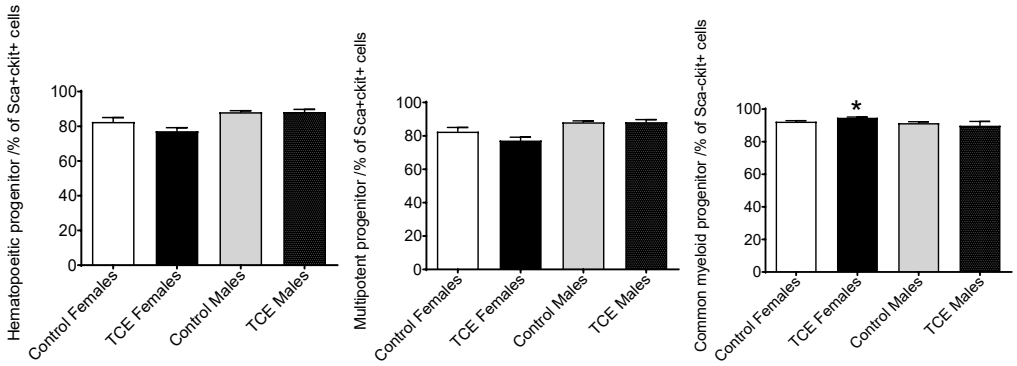
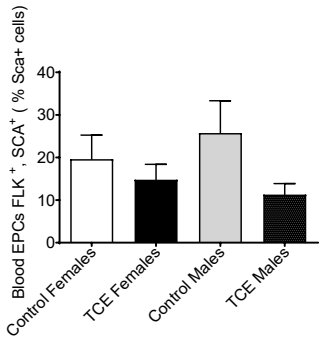


Figure 7: ***Xylene exposure depletes circulating Endothelial Progenitor Cells in mice.*** The measurements of endothelial progenitor cells (Flk+/ Sca+) in the plasma of xylene- (50ppm, 6hrs/d, 5d/wk, 12wks) or filtered (HEPA and charcoal) air- exposed mice were determined through flow cytometry (n=10/group). *P<0.05 vs control mice.



a.



b.

Figure 8: ***TCE exposure effect on progenitor cells.*** **8a**. Female and male mice were exposed to either TCE (5mg/mL, 52 weeks) or alkamuls (1% w/v) as described under *Methods,* and the bone marrow derived stem cells were analyzed using flow cytometry (n=10/group). Subpopulations of stem cells were assessed through expression of surface markers; Hematopoietic Progenitor Cells (HPC; Lin-ckit+Sca+CD34-), Multipotent Progenitor Cells (MPC; Lin-ckit+Sca+CD34+), and Common Myeloid Progenitor Cells (CMPC; Lin-ckit+Sca-CD34+). **8b**. Circulating Endothelial progenitor cells were also measured from the blood plasma using the expression of surface markers (Flk+, Sca+) and analyzed by flow cytometry. Values are mean ± SEM. *P<0.05 vs control mice.

VOC exposure and platelet activation: One function of the endothelium beyond vascular homeostasis is the modulation of platelet function and thrombosis. The luminal surface of the endothelial layer which faces the blood is normally anti-adhesive, but in conditions of endothelial injury and activation platelet adhesion is promoted for repair processes (52).

We observed that benzene exposure increases platelet leukocyte aggregate formation by 3-fold (**Fig. 9a**). This augmentation was in addition to a 1.6-fold increase in the levels of circulating platelet microparticles in benzene-exposed mice (**Fig. 9b**). These data suggest that benzene exposure is enhancing platelet activation in mice.

Our data also shows that inhaled xylene exposure increases platelet-lymphocyte and platelet-granulocyte adduct formation (**Fig 10a**). In addition, we saw a 3-fold increase in circulating platelet microparticles in xylene-exposed mice (**Fig 10b**). These data suggest that xylene exposure enhances platelet activation and that platelet-mononuclear cell adducts and that platelet-derived microparticles could be a biomarker of pro-thrombotic responses to inhaled xylene.

To assess if TCE exposure could affect platelet activation we measured platelet-leukocyte aggregates and platelet-monocyte aggregates through flow cytometry. Our data shows that in male mice, TCE exposure increased platelet-leukocyte aggregates by 30% and increased platelet-monocyte aggregates by 38% (**Fig 11a**). In female mice, exposure to TCE did not alter platelet aggregate levels in comparison to air controls (**Fig 11a**). To further understand the role of

TCE exposure on platelet activation, we measured circulating platelet microparticles by flow cytometry. Our data shows that in male mice, TCE exposure caused a 40% decrease in platelet microparticles compared to air-control and there was no change in the female mice (**Fig 11b**).

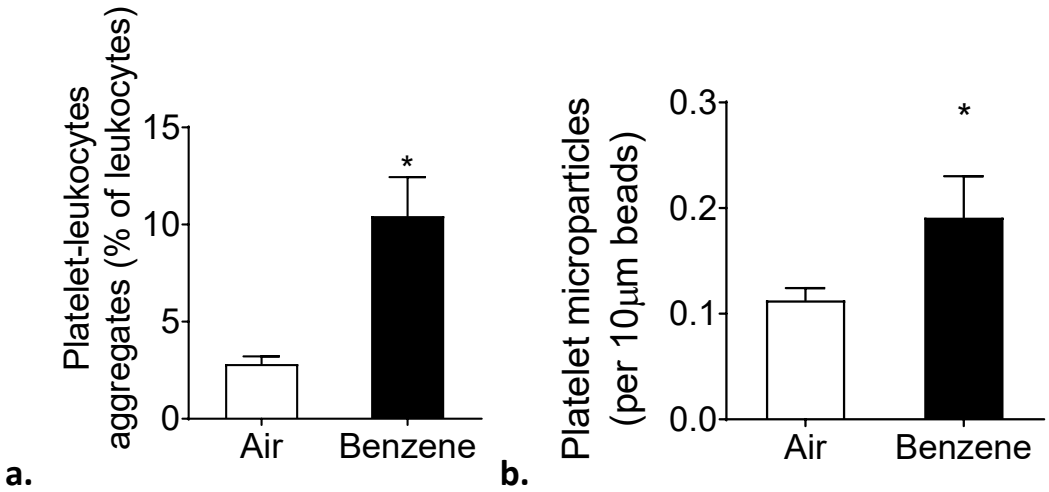


Figure 9: ***Exposure to benzene increases platelet-leukocyte adduct formation.*** Platelet-leukocyte markers were assessed in the peripheral blood of Benzene (50ppm, 6hrs/d, 5d/wk, 6wks) and filtered (HEPA and charcoal) air exposed male mice by flow cytometry *as described under methods*. **9a.** Platelet-leukocyte adduct (n=10/group) formation was tested using FITC-labeled anti-CD-41 (platelets) and APC-labeled anti-CD 45 (leukocytes) antibodies. **9b.** Platelet microparticle levels (<1um cells double positive for Annexin V and CD41). Values are mean ± SEM. *P<0.05 vs control mice. Data previously published in *Toxicology and Applied Pharmacology* (73).

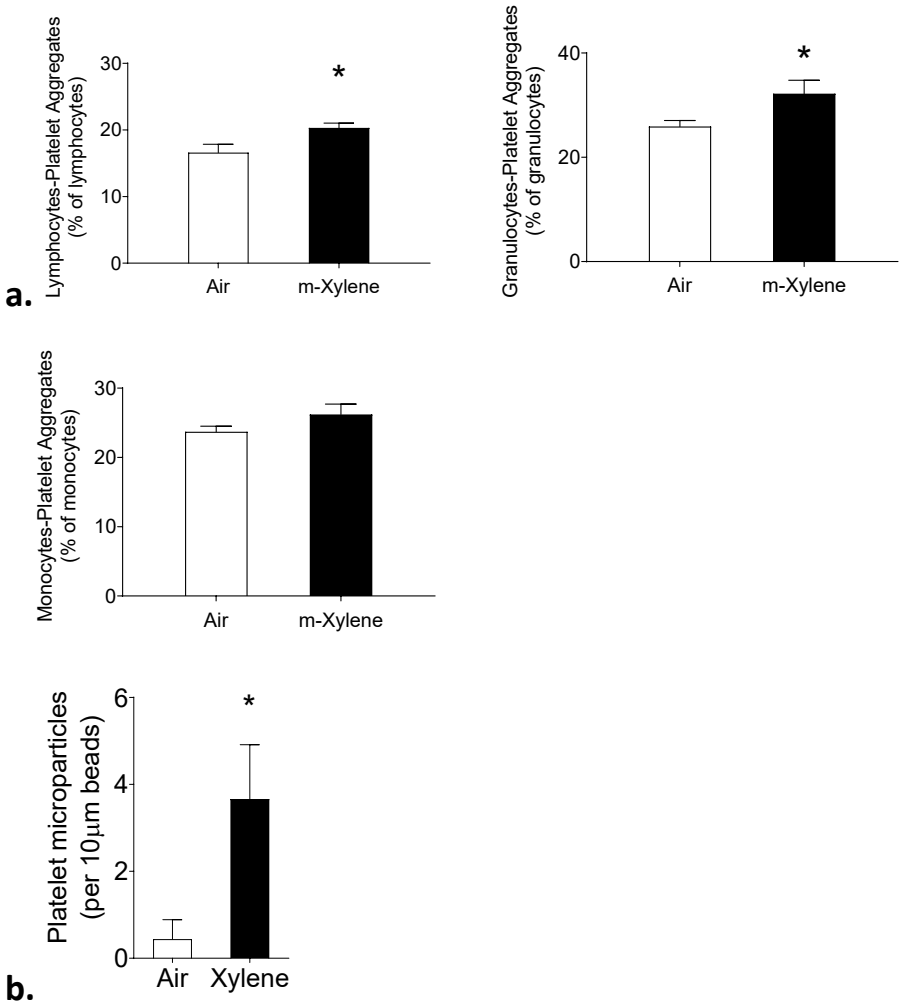


Figure 10: ***Exposure to xylene increases platelet-leukocyte adduct formation.*** Platelet-leukocyte markers were assessed in the peripheral blood of m-xylene (50ppm, 6hrs/d, 5d/wk, 12wks) and filtered (HEPA and charcoal) air exposed mice by flow cytometry *as described under methods.* **10a.** Platelet-leukocyte adduct (n=10/group) formation was tested using FITC-labeled anti-CD-41 (platelets) and APC-labeled anti-CD 45 (leukocytes) antibodies. **10b.** Platelet microparticle levels (<1um cells double positive for Annexin V and CD41). Values are mean ± SEM. *P<0.05 vs control mice.

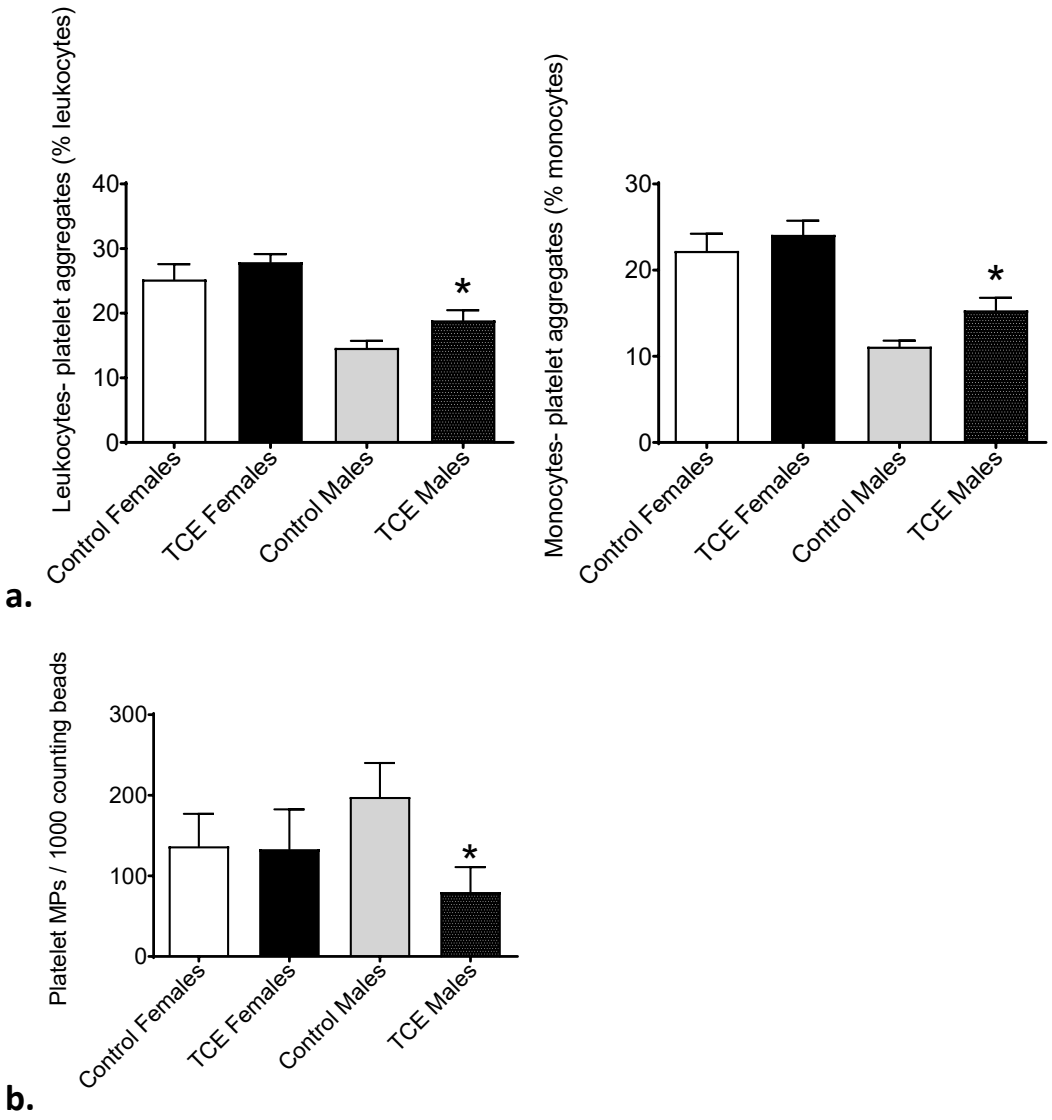


Figure 11: ***Exposure to TCE alters platelet-leukocyte adduct formation.*** Platelet-leukocyte markers were assessed in the peripheral blood of TCE (5mg/mL for 52 weeks)-and alkamuls (1% w/v)- exposed mice (female and male) by flow cytometry *as described under methods.* **11a.** Platelet-leukocyte adduct (n=10/group) formation was tested using FITC-labeled anti-CD-41 (platelets) and APC-labeled anti-CD 45 (leukocytes) antibodies. **11b.** Platelet microparticle levels (<1um cells double positive for Annexin V and CD41). Values are mean ± SEM. *P<0.05 vs control mice.

VOC exposure and plasma parameters: Markers of inflammation like cytokines have been associated with an increase in the susceptibility to benzene-induced hematopoietic toxicity in humans (31). Previous studies have shown that benzene exposure increases insulin resistance and significantly depletes levels of leukocytes, lymphocytes, monocytes, neutrophils, and endothelial progenitor cells (EPCs) in the peripheral blood of mice (1,2). We saw that plasma IL-6 levels were significantly lower in the benzene exposed mice. However, circulating cytokines in these mice were equivalent to those in air exposed controls (**Table 1**). Additionally, stimulation through low dose LPS, 18 hours prior to euthanasia, we observed a significant increase in the levels of several cytokines and chemokines including, GM -CSF, IL-6, IL-10, KC, and MCP-1. However, LPS-induced cytokine formation was not affected. Overall, these data suggest that benzene-induced inflammatory signaling is not inducing systemic inflammation.

To further understand potential mechanisms of xylene induced endothelial activation or injury, we measured the systemic levels of oxidative stress by quantifying plasma 8-iso prostaglandin F2α levels. Our data shows that inhaled m-xylene exposure increased plasma 8-iso prostaglandin F2α levels by 1.5-fold (**Table 2**).

We also showed that xylene exposure decreased the plasma total cholesterol by 13% and plasma high density lipoprotein (HDL) levels by 15%, whereas plasma triglyceride levels were increased by 31% in m-xylene-exposed mice as compared with the air controls. However, xylene exposure did not affect the plasma creatinine, AST, and ALT in mice. These data suggest that exposure

to xylene did not induce renal or hepatic toxicity but did induce dyslipidemia in mice.

Previous studies have shown that occupational exposure to low dose TCE or inhalation exposure in rats may impact cholesterol metabolism (69). To further assess systemic toxicity, lipid levels and inflammation we measured various clinical chemistry parameters in the plasma of the TCE- and air-exposed mice. Our data shows no difference in plasma albumin, creatinine, AST, and ALT in TCE exposed male mice compared with air controls (**Table 3**). However, we did observe that plasma cholesterol and HDL were decreased in the TCE-exposed male mice (**Table 3**). These data moderately support previous studies showing that low dose TCE exposure alters cholesterol metabolism (69). Similarly, in female mice there was no difference in plasma albumin or creatinine following exposure to TCE compared with air controls, but we did see a decrease in the albumin to creatinine ratio and a modest increase in the overall total protein when compared to air-exposed control female mice (**Table 3**). The decrease in total protein and albumin to creatinine ratio following exposure to TCE in females could suggest alterations in hepatic function. Changes in hepatic and renal function have been observed following TCE exposure in other animal models (32). However, no change in the other parameters suggests this may be a reversable, modest change.

Table 1: **Parameters measured in the plasma of benzene exposed male mice**. Mice were exposed to 50ppm Benzene or Air (HEPA and charcoal filtered) for 6 hours per day, 5 days per week, for 6 weeks (n=24 per group). A subset of the benzene and air-exposed mice were treated with 0.5 mg/kg lipopolysaccharides (n=10 per group). Values are mean ± SEM. *P<0.05 vs control mice

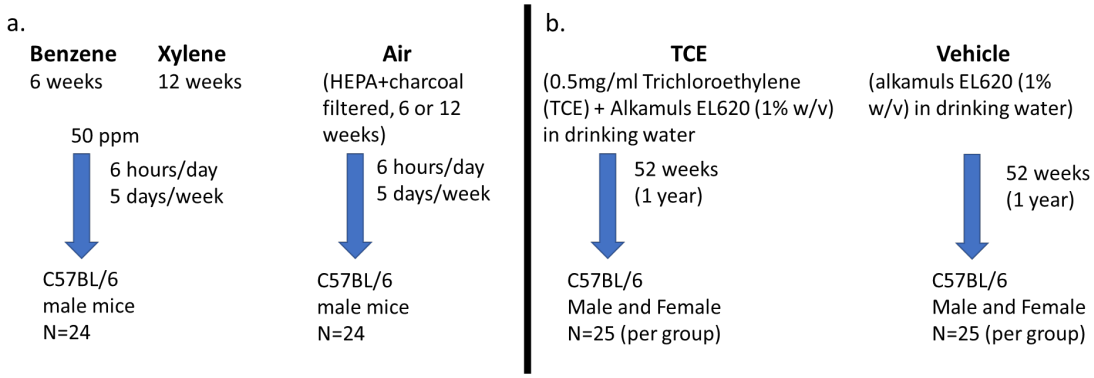
Parameters (pg/mL)	Air	Benzene	Air + LPS	Benzene + LPS
Adhesion molecules				
sE-Selectin	643±59	610±66	2154±161	2131±204
sP-Selectin	1732±254	1390±119	2427±336	2217±328
sICAM-1	128±6	**109±6***	476±25	466±23
Pecam-1	37±3	30±2	48±3	41±3
Angiogenesis markers				
Angiopoietin-2	4732±342	4872±243		
EGF	156±89	513±339		
FGF-2	ND	ND		
HGF	327±74	232±26		
Leptin	608±126	801±222		
PLGF-2	3.3±0.7	3.2±0.4		
SDF-1	ND	ND		
VEGF	1.02±0.1	1.12±0.05		
Cytokines				
GM-CSF	2±1	14±8	9±2	8±1
IFNγ	1.3±0.4	2.3±0.5	1.8±0.3	1.6±0.4
IL-1α	8±3	3±1	14±4	6±2
IL-1β	ND	ND	ND	ND
IL-2	13±6	17±11	15±8	5±1
IL-4	0.19±0.11	0.04±0.01	0.19±0.04	0.16±0.04
IL-5	6±1	5±1	13±3	9±1
IL-6	22±8	**4±1***	293±54	354±80
IL-7	22±9	9±5	11±5	23±9
IL-10	3.7±0.4	3.4±0.4	583.5±39.6	447±45.5
IL-12	6±2	6±1	17±5	11±2
IL-13	10±2	9±2	12±1	18±4
LIX	130±61	166±72	165±41	222±43
IL-17A	6±2	5±1	4±1	6±1
KC	406±153	178±34	3048±443	2675±503
MCP-1	14±3	31±22	593±133	481±56
MIP-2	75±14	53±8	192±25	210±51

Table 2: **Parameters measured in the plasma of xylene-exposed mice.** Mice were exposed to 50ppm Xylene or Air (HEPA- and charcoal-filtered) for 6 hours per day, 5 days per week, for 6 weeks. n=24 per group. Values are mean ± SEM. *P<0.05 vs control mice

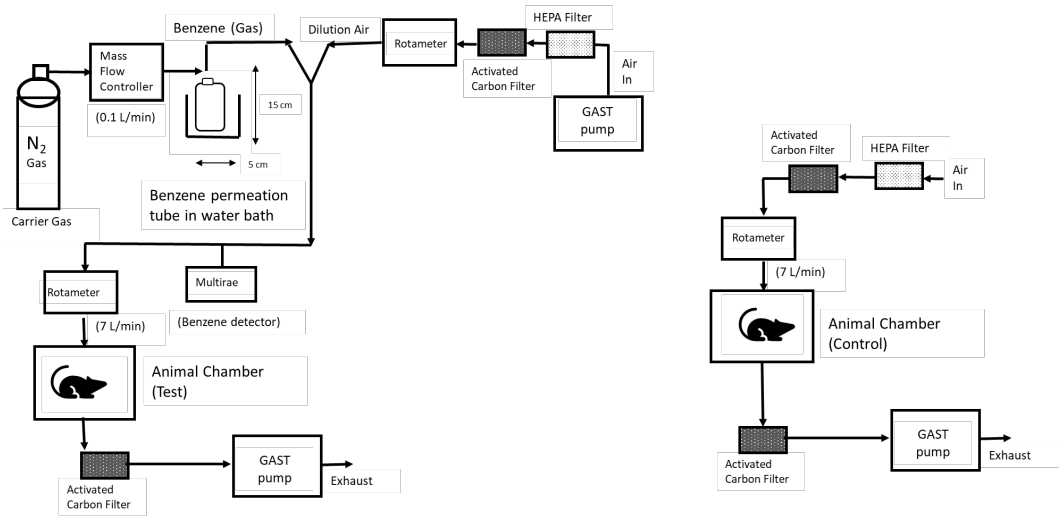
Parameters	Air	m-Xylene
Analytes in the Plasma		
sICAM (ng/mL)	5.5±0.7	**7.6±0.3***
8-Isoprostane F2a (pg/mL)	82±12	127±17
Cholesterol (mg/dL)	84±9	**71±7***
HDL (mg/dL)	42±5	**37±4***
Triglycerides (mg/dL)	57±6	**75±9***
Creatinine (mg/dL)	0.5±0.3	0.5±0.2
AST (U/L)	41±5	51±7
ALT (U/L)	17±2	20±2

Table 3: **Parameters measured in the plasma of TCE-exposed mice.** Male and female mice were exposed to 0.5 mg/mL of TCE and Alkamuls EL620 (1% w/v) or Alkamuls EL620 (1% w/v) in drinking water for 52 weeks. n= 25 per group. Values are mean ± SEM. *P<0.05 vs control mice

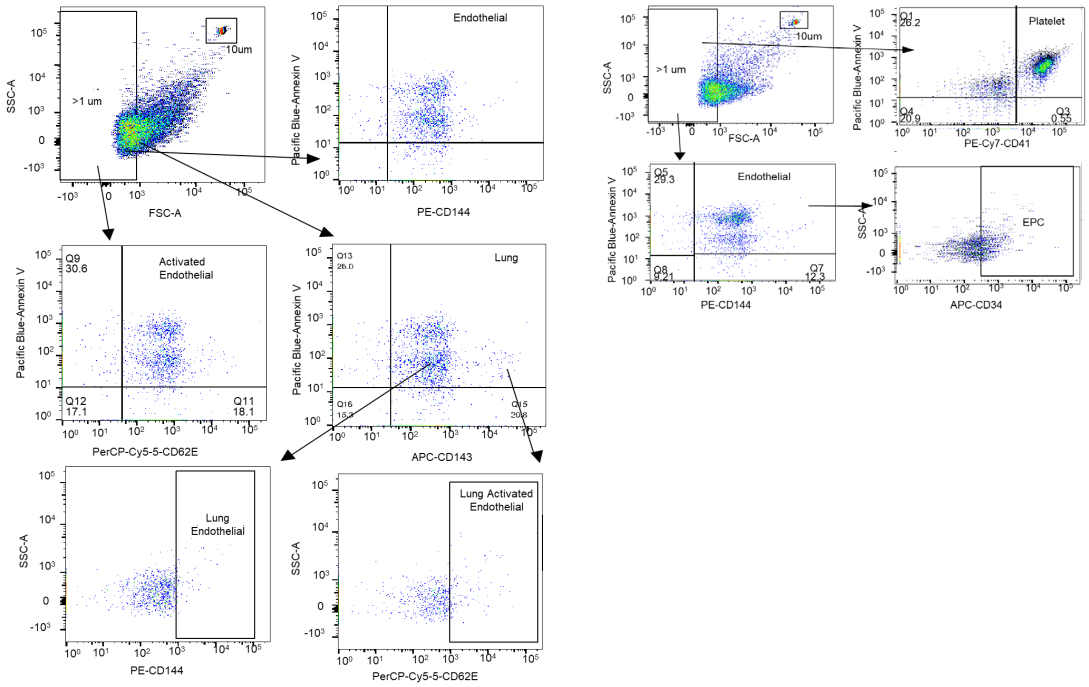
Parameter	Control females	TCE females	Control males	TCE males
Albumin (g/dL)	2.7±0.05	2.8±0.06	2.87±0.02	2.93±0.06
Creatinine (mg/dL)	0.15±0.01	0.16±0.01	0.15±0.01	0.18±0.03
Albumin/ Creatinine	185±18	**139±11***	140±9	157±21
BUN (mg/dL)	19±1	17±1	26±2	28±1
TP (g/dL)	5.15±0.1	**5.43±0.05***	4.88±0.04	4.89±0.1
ALT (U/L)	26±1	22±1	20±1	24±2
AST (U/L)	64±2	62±2	45±3	46±3
CK (U/L)	126±7	111±8	123±14	93±11
LDH (U/L)	206±11	194±8	139±7	142±7
TRIG (mg/dL)	54±3	55±4	73±3	70±6
Cholesterol (mg/dL)	73±2	77±4	90±2	**82±2***
HDL (mg/dL)	35±1	39±2	53±1	**48±1***
LDL (mg/dL) (Calculated)	26±1	27±2	23±1	20±2



Schematic 1: **Schematic of *In Vivo* Experiments. 1a.**C57BL/6 male mice were exposed to inhaled benzene (50ppm, 6 weeks), xylene (50ppm, 12 weeks), or HEPA- and charcoal- filtered air for 6 hours a day for 5 days per week. **1b.** C57BL/6 female and male mice were exposed to 0.5mg/mL TCE+ alkamuls EL620 (1% W/V) or alkamuls EL620 (1% w/v) in drinking water continuously for 52 weeks.



Schematic 2**: Schematic of Inhalation Exposure System.** Chemical atmospheres were generated from liquid benzene or liquid xylene (Sigma-Aldrich) in a KIN-TEK Analytical, Inc permeation tubes. A carrier gas (N_2) was delivered to the permeation tube at 100 ml/min and diluted with HEPA- and charcoal-filtered room air (HFA; 7 L/min) and diluted gas directed to an exposure unit. Flow was distributed through a fine mesh screen of a custom cyclone-type top (Teague Enterprises) that distributed air within 10% of the mean concentration at six locations in the cage. Gas concentrations were continuously monitored using an in-line photoionization detector (multiRAE: Rae Industries) upstream of the exposure cage. Air exposed mice were exposed to charcoal and HEPA filtered air (7 L/min).



Schematic 3: **Schematic of Flow Cytometry Analysis of Microparticles**. Endothelial microparticles were stained with the antibody cocktail containing Annexin V-Pacific Blue, Flk-APC, Sca-PECy7, CD62E-PE and CD143-FITC for 30 min. Platelet microparticles were stained in a separate tube with Annexin V-Pacific Blue and platelet CD41-FITC antibody. Identical samples with no antibodies were utilized as controls for the gating. Counting beads, added to individual samples were used for data normalization. Samples were analyzed on BD LSR II flow cytometer for 5 min at low speed. Microparticle numbers were quantified in gated populations <1μm in size and positive for Annexin V staining using the FlowJo software. Microparticle subpopulations were further identified based on expression of various surface markers.

DISCUSSION

Multiple lines of evidence suggest that exposure to VOCs, especially benzene and xylene promotes endothelial toxicity. Benzene exposure induced endothelial injury and platelet activation. This was supported by an increase in platelet microparticles and platelet aggregates, as well as, the suppression of hematopoietic progenitor cells in the bone marrow. The second major finding was that exposure to xylene similarly induced endothelial injury and platelet activation, shown by an increase in circulating endothelial microparticles, a decrease in endothelial progenitor cells, augmented platelet-leukocyte adduct formation, and increased circulating platelet microparticles. We also observed an increase in adhesion molecule expression and augmented cholesterol and lipid levels suggestive of dyslipidemia. Finally, the other finding of these experiments is that trichloroethylene exposure decreased circulating endothelial microparticles in male mice and increased platelet-leukocyte aggregates. Following TCE exposure in female mice we only saw modest changes in some of the plasma parameters that suggested a potential alteration to hepatic function. BTEX VOCs are a group of VOCs that consists of benzene, toluene, ethylbenzene, and xylene. Overall, our results suggest that BTEX VOCs, specifically benzene and xylene, may play a more deleterious role than the chlorinated compounds in the context of endothelial activation or injury, and cardiovascular disease. TCE exposure only exhibited modest changes and may be exhibiting toxicities outside

of the context of endothelial injury. Although not much is known about the direct effect of volatile organic compounds on vascular injury and cardiovascular disease, the endothelium has been shown to be susceptible to the effects of toxicants like tobacco smoke, which contains high levels of benzene and various other VOCs (47). Endothelial dysfunction is seen as the earliest sign of injury and precedes other changes to the morphology of the vessel wall. As the endothelium plays a vital role in vascular homeostasis, blood pressure regulation, thrombosis, atherogenesis, plaque stability, and many cardiac functions, dysfunction within the endothelium can alter these processes (10,24,36, 50).

To test the impact that VOCs have on endothelial injury and cardiovascular disease we chose to assess exposure to three different compounds in mice. Benzene is an aromatic compound and is one of the top 20 chemicals produced in the United States. An estimated 6.7 million pounds of benzene is released into the air through manufacturing processes, including rubber and shoe manufacturing (12). Other environmental sources of benzene include tobacco smoke, the processing and use of gasoline, motor vehicle exhaust, and household products like glues, paints, furniture wax, and detergents (12). From the environment, while people are usually exposed to levels of benzene in the low parts per billion (ppb) range, exposure to cigarette smoke and motor vehicle exhaust can lead to levels greater than 50 parts per million (ppm), and occupational exposures can result in levels that exceed 100ppm (35,12). The known toxic effects of benzene include cancer, hematotoxicity, immunotoxicity, and endocrine disruption (20). Additionally, epidemiological and

cohort studies have linked exposure to benzene to an increased risk of cardiovascular disease, but how benzene contributes to cardiovascular disease is not well understood (30, 31, 60). Similarly, xylene is a VOC comprised of a benzene ring with two methyl groups. It is largely released into the atmosphere by sources including combustion smoke, petroleum, auto exhaust, and household products containing solvents (43). Most people are exposed to levels of xylene around 1ppb in the environment, but occupational exposure can average more than 100 ppm per day (43). Acute exposure to xylene has been associated with GI and neurological effects, but chronic exposure in humans is primarily associated with central nervous system and respiratory effects (43). Trichloroethylene (TCE) is a chlorinated solvent and was formerly extensively used as a metal degreaser and component of several consumer products. Although TCE is a VOC, in the environment and at Superfund hazardous sites, it is largely found in the ground water (13). Specifically, it was identified at more than 700 of the 1,300 Superfund hazardous waste sites in soil and ground water (39). This leads to most of human exposure to TCE occurring through contaminated drinking water (13). According to the World Health Organization, the levels of exposure can vary based on the source of the TCE in the drinking water, but in the United States, levels range from 0.1-1 microgram per liter. TCE was reclassified in 2012 as an IARC group 1 human carcinogen and remains on the EPAs priority list of chemicals (13). In addition to cancer, chronic TCE exposure has been associated with immunotoxicity, developmental toxicity, and central nervous system toxicity (13). In an animal model, subacute TCE

inhalation exposure was associated with lipotoxicity and altered glucose handling (69).

In assessing these different VOCs impact on endothelial changes, we measured levels of microparticles that are released from activated or apoptotic endothelial cells and have been established as a sensitive marker of vascular injury (34,44,45,66). Increased levels of circulating endothelial microparticles have been correlated with endothelial dysfunction in patients with coronary artery disease (63), end stage renal failure (4), obesity (19), and diabetes mellitus (29). Additionally, increased levels of activated endothelial cell microparticles are associated with cardiovascular events (45,57). Our data demonstrating that benzene and xylene exposure the circulating levels of endothelial microparticles suggest that these microparticles are sensitive markers of benzene- and xylene-induced endothelial injury. We saw no changes in circulating endothelial microparticles in the TCE-exposed mice. Previous studies have shown that VOC metabolites can induce apoptosis through production of reactive oxygen species and activation of heme oxygenase 1 (HMOX1) (56) and through the activation of caspases 3 and 9, effectors of apoptosis (33). This is supported by our *in vitro* experiments, where we showed that exposure to toxic metabolites of benzene increased activation of caspase-7, caspase-3, and caspase-9. However, to elucidate the specific cellular and mechanistic processes involved in the VOC-induced endothelial injury, further *in vitro* studies are needed.

Our results also show that exposure to VOCs can increase endothelial activation in mice and *in vitro*. This was shown through the use of endothelial

microparticles. The endothelial microparticles contain glycoproteins, Von Willebrand Factor and Factor VIII, which promote the activation of platelets (26,27). Consequently, our data showing the increase in platelet-leukocyte adduct formation in the benzene- and xylene-exposed mice could be secondary to the induced endothelial injury and microparticle formation. We saw modest increases of platelet-leukocyte aggregation in the TCE-exposed male mice and a decrease in the circulating platelet microparticles. A previous study has shown hypercholesteremia following benzene exposure in mice (1). Similarly, our results here show an increase in cholesterol and HDL following xylene exposure. The VOC-induced hypercholesteremia following exposure could be another factor in potentially augmenting platelet activation.

Another aspect of endothelial function is the formation and repair processes of the endothelium. Specifically, we looked at the levels of multiple bone marrow stem cells; common myeloid progenitor (CMPC) and multipotent progenitor cells (MPPC) and hematopoietic progenitor cells (HPC). In the benzene-exposed mice we saw a significant decrease in the levels of HPCs. These progenitor cells give rise to other important components to the endothelium system like endothelial progenitor cells. The depletion of HPCs has been shown to alter endothelial repair processes and vasculogenesis (51). In the xylene-exposed mice, we saw no change in the levels of bone marrow stem cells compared with the air-control, but we did see a decrease in the circulating endothelial progenitor cells, again suggesting a potential impact to vascular repair.

One important element of benzene toxicity is that its chemical structure and properties create a relatively stable hydrocarbon, so most of benzene-associated *in vivo* toxicity is attributed to its metabolites. Benzene undergoes extensive metabolism *in vivo*, mostly in the liver mediated by cytochrome P-450 enzymes. There are several toxic metabolites of interest including t,t-mucondialdehyde, phenol, catechol, hydroquinone, and 1,4-benzoquinone (35). The toxic metabolites of VOCs have been attributed to the hematopoietic toxicity associated with exposure to benzene in both epidemiological and laboratory settings (11,71). Likewise, xylene and TCE are both extensively metabolized *in vivo*. In future studies it will be beneficial to address the toxicity of these metabolites and to determine if any of these play a significant role in VOC-induced endothelial injury and cardiovascular disease. The relative toxicity, or lack of toxicity, of the metabolites may also play a role in the variation in toxic response seen in our studies.

Another relevant consideration in understanding the toxicity of these VOC compounds is the exposure levels. As previously mentioned, the environmental levels of VOCs which most people are exposed to are closer to the ≤1ppm range (35). In our studies we have used higher levels that resemble occupational exposures. Further studies to understand the effects of environmental levels of VOCs would be important to understand the impact of VOC-induced endothelial injury and atherosclerosis on human health.

CONCLUSION

Together our studies suggest that exposure of mice to VOCs, specifically benzene and xylene, is sufficient to induce endothelial injury and modulate platelet activation. Because VOCs are ubiquitous air pollutants, decreasing exposure could significantly reduce air pollution-associated cardiovascular diseases.

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Presentations

1. Poster presentation, S.A. McFall, M. Malovichko, R.J. Keith, D. Riggs, A. Bhatnagar, and S. Srivastava, “EXPOSURE TO VOLATILE ORGANIC COMPOUNDS INCREASES CIRCULATING ENDOTHELIAL CELLS, PLATELET, AND LUNG MICROPARTICLES”, Superfund Research Program, 2019
2. Poster presentation, S.A. McFall, N.S. Wickramasinghe, W. Abplanalp, M.V. Malovichko, I.D. Sithu, D.J. Conklin, T.E. O’Toole, and S. Srivastava, “Mechanisms of Benzene-Induced Endothelial Injury: Role of Heat Shock Proteins”, Superfund Research Program, 2020

Publications

1. Publication. Malovichko MV, Abplanalp WT, McFall SA, Taylor BS, Wickramasinghe NS, Sithu ID, Zelko IN, Uchida S, Hill BG, Sutaria SR, Nantz MH, Bhatnagar A, Conklin DJ, O'Toole TE, Srivastava S. Subclinical markers of cardiovascular toxicity of benzene inhalation in mice. Toxicol Appl Pharmacol. 2021 Nov 15; 431:115742.