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Inhalation Exposure to Surrogate Military Burn Pit Emissions Impairs Systemic Microvascular Function: Linking Pulmonary Insult and Diverse Peripheral Responses

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ABSTRACT

Millions of U.S. troops and supporting personnel have been deployed to military bases in the Middle East. Essentially all personnel on military bases were exposed to the combustion emissions generated by open pit waste burning. Chronic multisymptom illness (CMI) is a term advanced to characterize the complex health effects of inhalation exposures to military burn pits (BPs). Because of the diversity of geography, environmental conditions, and deployment operations, it is very challenging to estimate the number of Veterans affected by CMI, but it has been reported to be in the range of ~40-60%. Despite this overwhelming number of patients, the underlying causes of CMI remain to be identified. The purpose of this study was to replicate BP combustion and deliver these representative emissions to a whole-body inhalation exposure chamber with Sprague Dawley rats. We hypothesized that because the microcirculation is a critical component of health, that normal microvascular function may be disrupted after BP inhalation exposures. A surrogate BP emission generator was used to combust mixtures of wood, rubber, plastic and jet fuel. Resultant emissions were complex mixtures of volatile organic chemicals, polyaromatic hydrocarbons, fine and ultrafine particles. The particle aerodynamic count median diameter was 113 nm with a geometric standard deviation of 2.21. The particle mobility diameter was 78.1 nm with a geometric standard deviation of 1.69. The aerosol mass-size size distribution had a mass median aerodynamic diameter of 288 nm with a geometric standard deviation of 1.72 nm. Rats were exposed for ~4 hr/d at BP emission concentrations of $15.4 \pm 1.6 \text{ mg/m}^3$, for 2, 3, or 6 days. Twenty-four hours later, the spinotrapezius muscle was prepared for intravital microscopy. Tissues were also harvested from different rats in these groups for thorough mechanistic analyses. After 3-6 days of exposure, endothelium-dependent arteriolar dilation was abolished. Adrenergic vasoconstrictor sensitivity was augmented by as much as 50% in the BP exposure groups. Bronchoalveolar lavage revealed robust pulmonary inflammation and cellular infiltration. High-performance liquid chromatography with plasma samples demonstrated significant increases (>50%) in circulating xanthine oxidase, a known

driver of oxidative stress, disruptor of vascular nitric oxide, and thus mediator of endothelial dysfunction. After 3 days of BP exposure, RNA sequencing tissue analyses revealed transcriptional markers of lung inflammation as well as an altered transcriptional immune response in both the lung and spleen. BP inhalation exposure also led to elevated RNA transcripts for the vascular growth factor *Vegfa* and the immune cell trafficking factor *Icam1* in brain hippocampal tissue. These initial microvascular observations demonstrate disruption of typical function and mechanisms that may link pulmonary insult with diverse systemic syndromes characteristic with CMI in Veterans.

INTRODUCTION

Since 2001, millions of U.S. troops and supporting personnel have been deployed to the Middle East and Southwest Asia for various military operations in Afghanistan and Iraq [1]. During these deployments, troops and personnel shared a common health risk among these theaters of conflict; inhalation exposures to military burn pit (BP) emissions. BPs evolved as a means of disposing the significant amounts of waste generated on military bases. It has been estimated that during deployment, an American soldier generates approximately 10 pounds of waste per day, with larger bases generating up to 85,000 pounds per day [2]. Further, activities such as maintaining the base and vehicles, as well as various daily operations all create significant amounts of waste. Collectively, this waste was disposed of by open pit burning in the ground and accelerated with the addition of jet fuel. The result of constant BP operation is the generation of combustion emissions, typically incomplete. It has been estimated that as many as ~200 BPs have been operational, some as large as 10 acres [2, 3]. The amount and scope of inhalable emissions generated by these sources is staggering and likely exposed all personnel in the immediate regions.

Another complex aspect of BP exposure assessments is identifying the number of impacted individuals and the resultant health complications associated with their exposures. The 2022

passage of the “Promise to Address Comprehensive Toxics (PACT) Act into Federal law has enhanced these efforts [4], but more time is necessary to determine the scope and depth of its impact on Veteran Health. A large cross-sectional interview/report from 2024 estimates that greater than 70% of U.S. Veterans deployed to Iraq and Afghanistan reported inhalation exposures to BP emissions, and now approximately 7-16% suffer from pulmonary complications referred to as deployment related respiratory disease (DRRD) [5]. The umbrella diagnosis of DRRD in Veterans Affairs Clinics provides the necessary identification and treatment of affected Veterans [6]. However, given the complexity of BP emissions, it is unlikely that health effects are limited to the lungs [7]. If this is correct, the scope of affected Veterans will be much greater. Originally labelled “Gulf War Illness” in 1991, Veterans exposed to BP emissions present clinically with complex medical conditions. These symptoms include: pulmonary morbidity, cardiovascular morbidity, cognitive impairments, behavioral disorders, fatigue/diminished energetics, compromised immune function, and pain [8]. Currently, Veterans presenting with at least two of these conditions are diagnosed with “Chronic Multisymptom Illness” (CMI) [9]. It has been estimated that ~28% of Gulf War Veterans suffer from CMI [10]. Despite the complexity of these adverse health effects, a common underlying biologic mechanism remains to be determined.

It is interesting that the extrapulmonary events that follow ambient air pollution inhalation exposures have considerable overlap with CMI characteristics such as alterations in autonomic function, cardiovascular function, immune function, bioenergetics, and sleep disorders [11]. We have previously identified systemic microvascular dysfunction after pulmonary exposures to oil fly ash [12], nanomaterials [13, 14, 15], 3-dimensional printer emissions [16] and mountain top mining emissions [17]. Despite its role in every morbidity associated with CMI or their consequences, to date, the microcirculation has not been properly tested as the common denominator of these complex, developing processes. Therefore, the greater purpose of this study was to make initial systemic microvascular function assessments in experimental rats

after being exposed to surrogate military BP emissions. We also sought to assess the initial pulmonary and neural impacts and identify potential mechanistic links of dysfunction.

No standardized model to recapitulate BP inhalation exposures exists. Therefore, the ability to identify causes and mechanisms of CMI is very challenging. Tremendous variability exists with BPs based on geography, base size, scope of base operations, and duration/intensity of combustion [2]. Therefore, it is not possible to precisely replicate an individual BP. Rather, it is feasible to consider some of the most common elements across all BPs. Some of the most universal waste disposed of in all BPs included wood, rubber, and plastic. The use of jet fuel or diesel fuel as an accelerant was also common. Because the process was dependent on feeding waste materials, the process of burning and smoldering produced differential emissions based on temperature. We have created a surrogate combustion emission generator from a re-tasked pellet stove. Custom pellets were fabricated to capture the above-mentioned common characteristics of BPs. The resultant mixed emissions were delivered to an inhalation exposure chamber that contained experimental rats. We hypothesized that BP emission inhalation exposures are associated with systemic microvascular dysfunction in a dose-dependent manner. We also predicted that other physiologic systems would be differentially impacted.

METHODS

Animal Model:

Male, Sprague-Dawley rats (7-8 weeks of age) were purchased from Hilltop Laboratories (Scottsdale, PA) and housed in an AAALAC approved animal facility at the West Virginia University Health Sciences Center. All animals were maintained on a 12-hour light/dark cycle, provided food and water *ad libitum*, and acclimated for at least 72 hours prior to training and/or use. Animals were then randomly assigned to either the Sham-Control (HEPA filtered air) or BP emission inhalation exposure groups (2, 3 or 6 exposure days).

Surrogate BP Emission Generation, Aerosol Characterization, and Inhalation Exposures:

We have re-tasked a pellet stove to combust diverse materials under tightly controlled conditions. This combustion emission generator, aerosol characterizations, and whole-body inhalation exposure system have been previously described [18]. Pellets were fabricated to represent some of the most common materials destroyed in BPs, including (by mass): wood (pine, 80%), plastic (polymethyl methacrylate, 10%) and rubber (10%). Jet fuel with additives (JAA) was used as an accelerant during combustion. Pellets were fed from the hopper to the burn chamber/crucible via an auger at ~6 g/minute. This feedback-controlled system was designed to generate combustion emissions in the temperature range of 220-330 °C. JAA was dripped onto the burning pellets at a rate of 0.25 ml/minute. Combustion emissions were diluted to keep carbon monoxide (CO) and carbon dioxide (CO₂) concentrations tolerable. This resulted in steady-state particle concentrations in the exposure chamber of ~15 mg/m³.

Conditions of combustion and emission generation were measured on-line and off-line in the exposure chamber. Particle concentration was measured with a real-time condensation particle counter (CPC 7075, TSI Inc., Shoreview, MN). Particle size distribution (PSD, nm to µm range) were determined with an electrical low-pressure impactor (ELPI+, Dekati, Tampere, Finland), a scanning mobility particle sizer; and an aerodynamic particle sizing instrument (SMPS 3381 and APS 3321, respectively, TSI Inc., Shoreview, MN). Count Median Diameter (CMD) and geometric standard deviation (GSD) were estimated based on log-normal fits of the PSD data. The SMPS and APS data were used together to determine CMD. A micro-orifice uniform deposit impactor (Nano MOUDI-115, MSP Corp., Shoreview, MN) was used to collect aerodynamic size-classified samples (18 µm - 10 nm) from the exposure chamber. Gravimetric analysis of samples from stages 1-14 were used to estimate the mass mode aerodynamic diameter (MMAD) and GSD for aerosolized combustion emissions. The total gravimetric mass of aerosols was determined for particles collected on a pre-weighed 37 mm diameter, 0.45 µm

pore size polytetrafluorethylene filter (SKC Inc., PA). Air was sampled from the exposure chamber at 1 LPM. Particle morphology was evaluated with transmission electron microscopy (TEM, S-4800, Hitachi, Tokyo, Japan). Particles were collected onto 25 mm diameter, 0.2 μm pore size track-etched polycarbonate filters (Sterlitech Corporation, VA) by drawing air from the exposure chamber at 1 LPM using a pre-calibrated sampling pump with a modified NIOSH 7300 method [19]. After particle collection, filters were prepared and analyzed with TEM. Primary combustion gases such as CO and carbon dioxide CO_2 along with water generation occur in all combustion processes. Therefore, our sampling strategy included real-time measurements of RH and temperature (HMP60, Vaisala, Finland), and CO/ CO_2 (Five Gas Analyzer, AO2000, AAB, Zurich, Switzerland). The chemical constituents in combustion emissions were majorly segregated into: volatile organic compounds (VOCs), volatile carbonyl compounds (VCCs), and polyaromatic hydrocarbons (PAHs). An aggregate total level of organic vapors (e.g., VOCs, VCCs, and PAHs) were measured in real-time using a TVOC2 (Photoionization Detector: PID TV2SXBMX-X, ION Science Ltd, TX). Additional speciation was achieved by collecting emissions in a water trap [18]. The combustion emissions were drawn at 1 LPM from the exposure chamber and sampled in a flask containing 50 mL of ultra-pure water ($\geq 17 \text{ m}\Omega$) and evaluated separately for specific VOCs, VCCs, and PAHs of interest. This offline analysis was performed off-line with a headspace sampler coupled to a gas chromatography/mass spectrometer (GC-MS, Model# 7697A, Model #8890, Model# 7000, respectively, Agilent Technologies Inc., Santa Clara, CA) as described in our previously published study [18]. Chemical speciation for these compounds was identified using the National Institute of Standards and Technology library databases (NIST, Gaithersburg, MD).

Four experimental groups were studied: Sham-Control (HEPA-filtered air) or BP emission inhalation exposure groups (2, 3 or 6 exposure days) at 4 hr/exposure. Temperature, relative humidity, particle concentration, mass concentration, total VOCs and primary combustion gases (CO and CO_2). Total alveolar deposited dose (average $\pm$ standard error) was estimated using

the Multiple Path Particle Dosimetry model (MPPD v3.04) [20]. Clearance was not accounted for in this model. Twenty-four hours after the last exposure, animals were prepared for intravital microscopy or tissue harvesting.

Intravital Microscopy:

At 24 hr. post-exposure, rats were anesthetized with thiobutabarbital sodium (Inactin, 100 mg/kg, i.p.), placed on a heating plate, connected to a thermocouple probe to maintain a 37 °C rectal temperature with an Animal Temperature Controller (World Precision Instruments, Sarasota, Florida). The trachea was intubated to ensure a patent airway, and the right carotid artery was cannulated to measure arterial pressure and heart rate. The right spinotrapezius muscle was then surgically exteriorized for microscopic observation, leaving its innervation and all feed vessels intact. After exteriorization, the muscle was gently secured over an optical pedestal at its *in-situ* length. The muscle was next enclosed in a tissue bath for transillumination and observation. Throughout the surgery and all experimental periods, the muscle was continuously superfused with an electrolyte solution (119 mM NaCl, 25 mM NaHCO₃, 6 mM KCl and 3.6 mM CaCl₂), warmed to 35 °C, and equilibrated with 95% N₂ - 5% CO₂ (pH=7.35-7.40). Superfusate flow rate was maintained at 3-6 mL/min to minimize equilibration with atmospheric oxygen [21].

The animal preparation was then transferred to the stage of an intravital microscope, coupled to a CCD color video camera (BX51WI and DP71, respectively, Olympus, Tokyo, Japan). Observations were made with a 20X water immersion objective (final video image magnification = 1460X). The spinotrapezius microcirculation is an arcading network supplied by feed arteries that deliver blood flow rostrally from the thoracodorsal arteries and caudally from the 11th intercostal artery [22]. Arcade bridge arterioles (first order, 1A) and arcade arterioles (second order, 2A) differ anatomically in length, diameter, and wall thickness, and account for >50% of the pressure dissipation from the feed arteries to the terminal arterioles [23]. It is

therefore possible to study anatomically and physiologically distinct arterioles in the same field of view. One to three arteriolar fields were studied per rat. Endothelium-dependent arteriolar dilation was measured with increasing doses of acetylcholine (ACh, 10^{-6} – 10^{-4} M). Adrenergic sensitivity (vasoconstriction) was assessed with increasing doses of phenylephrine (PE, 10^{-5} – 10^{-3} M). Passive arteriolar diameters were determined at the end of all experiments in the presence of adenosine (ADO, 10^{-4} M). Real-time images were displayed on a high-definition computer monitor and digitally captured for off-line analysis [14, 24]. Arteriolar inner diameters were measured with Image-J software (National Institutes of Health, Bethesda, MD) calibrated with a stage micrometer.

Tissue Harvesting:

Rats were anesthetized in an induction chamber with isoflurane gas (5% induction, 2-3% maintenance). After induction, animals were transferred to a dissecting station. The right carotid artery was cannulated with tapered polyethylene tubing (PE 90). Animals were exsanguinated via this cannula and blood was directly delivered into multiple 3.0 ml vacuum tubes with ethylenediaminetetraacetic acid (EDTA). Vials were centrifuged at 1500 g for 10 minutes. Separated plasma was isolated into 1 ml cryotubes. After exsanguination, a thoracotomy was performed, the heart was collected and the trachea was intubated with PE 240. A ligature was placed around the left bronchus and the airway was closed. Bronchoalveolar lavage (BAL) was performed on the right lung and the left lung was subsequently fixed for histology (see below). Large arteries were then harvested. The liver was harvested next and separated into the caudate, left and right lobes. The spleen was harvested and dissected into two halves. The kidneys, white and brown fat were then harvested. Finally, the brain and thymus were harvested. Unless otherwise noted, all tissues were placed into cryotubes, and flash frozen in liquid nitrogen. All samples were stored in a -80 °C freezer until subsequent assays.

Bronchoalveolar Lavage and Macrophages:

Lung lavage and subsequent assays were performed following our peer reviewed and published methodology [25, 26, 27]. Immediately following euthanasia, right lung lobes were subjected to bronchoalveolar lavage (BAL) using sterile cold phosphate buffer saline. Bronchoalveolar lavage was performed to collect a total of 6 mL of BAL fluid (BALF) by instilling 2 ml ice cold sterile phosphate buffered saline (PBS) through the trachea into the lungs three times. BALF was pooled and processed for cellular and biochemical analyses. BALF was centrifuged at 600 RPM for 5 min at 4 °C and supernatant was collected. To remove the red blood cells, RBC lysis buffer (ACK lysis buffer, Gibco) was added to the cells pellet for 4 min and centrifuged 600 RPM for 5 min at 4 °C. Total protein contents were quantified as a marker for air-blood barrier integrity by Pierce with using BCA kit (Thermofisher Scientific, Waltham, MA). Cytotoxicity was quantified by lactate dehydrogenase (LDH) Assay Kit (Promega, Madison, Wisconsin) as per the manufacturer's instructions. Total Cells were counted in BALF using Countess®, (Thermofisher Scientific, Waltham, MA) automated cell counter. The pelleted cells were resuspended in PBS, and were spin down using Cytospin (Thermo Fisher Scientific, Waltham, Massachusetts) for differential counts. Slides were stained and differential cell count and particle uptake in macrophages were quantified as described by us previously [25, 26, 27]. The BAL supernatant was frozen (-80 °C) until further use in biochemical assays.

Plasma Xanthine Oxidase (XO) Activity Analysis:

The enzymatic activity of XO was determined as we have previously reported [28]. Plasma was snap frozen in liquid nitrogen immediately following harvest. XO activity was measured via electrochemical detection (Dionex UltiMate 3000 RS Electrochemical Detector) of xanthine-driven uric acid production which was separated by reverse-phase HPLC. One unit of XO activity = 1 μ mole of uric acid produced per minute (1U = 1 μ mole/min).

Gene Expression and Analysis in the Lung and Spleen:

Ribonucleic acid (RNA) was isolated from snap frozen lung and spleen as previously described [29]. Total RNA was extracted from snap frozen tissue homogenized in TRIzol

followed by RNA clean up using RNeasy Mini Kit (QIAGEN) per the manufacturer instructions. Quality control and mRNA library preparation of isolated RNA were performed by Novogene, followed by paired-end RNA sequencing using an Illumina NovaSeq platform. FASTQ files provided by Novogene were assessed with FastQC, trimmed, aligned to a reference rat genome using hisat2, and gene-level counts were determined with featureCounts. Differential expression analysis was performed using DESeq2 in R. Gene set enrichment analysis was performed in R after rat-human ortholog mapping using Ensembl annotations.

Gene Expression and Analysis in the Brain:

From a subset of the 3 Day BP exposed rats, brains were rapidly removed from the skull and submerged in RNAlater (Thermo Fisher) to preserve RNA integrity. After RNA preservation, the hippocampus was dissected out of the brain and RNA was extracted using a RNeasy Mini kit (Qiagen) following the manufacturer's protocol. RNA quality and quantity were verified via spectrophotometer (NanoDrop 2000). 2000 ng of RNA from each sample was then reverse transcribed into cDNA using SuperScript IV VILO (Thermo Fisher) following the manufacturer's instructions. qRT-PCR was performed on an ABI 7500 Fast system as previously described (PMID: 22521589) to assess relative expression of vascular and endothelial growth factor A (Vegfa, Rn01511602_m1, Life Technologies,) and the intracellular adhesion molecule-1 (Icam1, Rn00564227_m1). Gene expression values are reported as relative to the mammalian 18s (4318839) housekeeping gene.

STATISTICS and FORMULAS

Systolic and diastolic pressures were measured continuously throughout experiments. Blood pressure and heart rate data was sampled at 10-second intervals during all control and superfusion periods. Mean arterial pressure (MAP) was calculated as: $MAP = \text{diastolic pressure} + (\text{systolic pressure} - \text{diastolic pressure}) / 3$. Resting vascular tone was calculated for each vessel as follows: $Tone = [(D_{pass} - D_c) / D_{pass}] \times 100$, where D_{pass} is passive diameter under

ADO and D_c is the diameter measured during the control period (resting diameter). A tone of 100% represents complete vessel closure, and 0% represents the passive state. To further evaluate arteriolar responsiveness between individual groups with differences in resting diameter, arteriolar diameter was normalized. In this case, arteriolar diameter was expressed as a percent change from control and was calculated for each vessel as follows: Diameter (% change from control) = $[(DSS - D_c) - 1 \times 100]$, where DSS is the steady state diameter achieved during superfusion. All data are reported as means $\pm$ SE, where “N” represents the number of rats studied and “n” represents the number of arterioles evaluated. One to three microvessel segments were studied per rat. Statistical analysis was performed by commercially available software (Sigmaplot 16, Grafitti LLC, Palo Alto, CA). One-way repeated measures ANOVA was used to determine the effect of a treatment within a group, or differences among groups. Two-way repeated measures ANOVA was used to determine the effects of group, treatment and group-treatment interactions on measured variables. For all ANOVA procedures, the Student-Newman-Keuls method for post-hoc analysis was used to isolate pairwise differences among specific groups. Significance was assessed at the 95% confidence level ($P < 0.05$) for all tests.

RESULTS

The conditions of BP combustion emissions in the exposure chamber were extensively characterized during inhalation exposures. Particle size distributions are presented in **FIGURE 1**. The particle aerodynamic count median diameter was 113 nm with a geometric standard deviation of 2.21 (**FIGURE 1A**). The particle mobility diameter was 78.1 nm with a geometric standard deviation of 1.69 (**FIGURE 1B**). The aerosol mass-size size distribution indicated a mass median aerodynamic diameter of 288 nm with a geometric standard deviation of 1.72 nm (**FIGURE 1C**). TEM of captured aerosol samples revealed largely combustion aggregates in the ultrafine range with diverse morphological features. This included complex, heterogeneous chains and fibrous clumps (**FIGURES 2A and 2B**). Real-time measurements of representative

total particles, total chemicals, and combustion gases are presented in **FIGURE 3**. Aerosol concentration was as intended, high and stable (**3A**). A side effect of delivering combustion emissions to the exposure chamber was elevated, but stable CO and CO₂ concentrations (**3B** and **3C**). Due to their inherent instability, Total VOCs were somewhat less stable during combustion emission exposures (**3D**). Offline GC-MS analysis of aerosols collected in the water trap identified speciated chemical (mean $\pm$ standard deviation): formaldehyde ($94.6 \pm 2.93 \mu\text{g}/\text{m}^3$), acetaldehyde ($29.9 \pm 0.75 \mu\text{g}/\text{m}^3$), ethyl benzene ($68.5 \pm 0.82 \mu\text{g}/\text{m}^3$), acrolein ($53.7 \pm 1.45 \mu\text{g}/\text{m}^3$), methyl isobutyl ketones ($31.2 \pm 1.19 \mu\text{g}/\text{m}^3$), methyl methacrylate ($18.6 \pm 0.56 \mu\text{g}/\text{m}^3$), and naphthalene ($5.3 \pm 0.25 \mu\text{g}/\text{m}^3$).

The basal characteristics of the rats in each experimental group are presented in **TABLE 1**. There were no statistical differences in age, mass, MAP or heart rate among the four groups.

The physical characteristics of microvessels at rest for each experimental group are present in **TABLE 2** for arcade bridge arterioles and in **TABLE 3** for arcade arterioles. There were no differences among the groups in resting or passive (maximum) arteriolar diameter for either vessel order. Arteriolar tone was not different among the four groups for either vessel order. Similarly, arteriolar tone was not different within a given group, between larger arcade bridge arterioles and their smaller downstream counterparts, arcade arterioles. This indicates that BP exposures do not alter microvascular anatomy or the distribution of systemic resistance at rest.

A significant increase in BALF total cells was observed after 3 days of exposure to BP combustion emissions (**FIGURE 4A**). This increase was mainly due to an increase in macrophage and neutrophils (**FIGURES 4B, C**). 3 Day BP exposures also significantly increased LDH released in the BALF indicating enhanced cytotoxicity (**FIGURE 4D**). Further evidence of toxicity was obtained from observation of increased number of epithelial cells in the BAL fluid at all time points (**FIGURE 4F**). Moreover, a significant increase in BALF protein levels was observed after three exposures to BP aerosol (**FIGURE 4G**). A greater and persistent increase in lung lavage total protein contents by 3 Days was observed and to a lesser

extent by 2- and 6-Days BP inhalation further confirmed the deleterious impacts in terms of an increase in air-blood barrier permeability (**FIGURE 4G**). Particle uptake was assessed by analyzing the BALF slide images of Sham Control and BP exposed rats (**FIGURES 5A, B**). A significant increase in particle containing macrophages was observed after all exposure days (**FIGURE 5C**).

In the Sham Control group, endothelium-dependent arteriolar dilation was normal. This was demonstrated by a dose-dependent dilation in response to increasing concentrations of ACh in arcade bridge arterioles (**FIGURE 6A**) and arcade arterioles (**FIGURE 7A**). The maximal response to ACh was 78% of the passive diameter observed in the presence of ADO. In the 2 Day BP Exposure group, this microvascular reactivity was intact for both arcade bridge and arcade arterioles. However, in the 3 Day BP Exposure group, arcade bridge endothelium-dependent arteriolar dilation was significantly impaired at the 10^{-5} and 10^{-4} M ACh doses. Regression analysis revealed that the line slope of the collective data points was not different from zero. The individual dose and group differences are more clearly identified in **FIGURES 6B** and **7B**. A similar impairment was observed in the 6 Day BP Exposure group. This observation was consistent for the arcade arterioles in only the 6 Day BP Exposure group. Collectively, this indicates a loss of normal endothelial reactivity after BP exposures in the latter two groups.

In the Sham Control group, adrenergic arteriolar reactivity was normal. This was demonstrated by a dose-dependent vasoconstriction in response to increasing concentrations of phenylephrine in arcade bridge arterioles (**FIGURE 8A**) and arcade arterioles (**FIGURE 9A**). The maximal response to phenylephrine was up to 58% of the total range of reactivity in both orders of arterioles. Vasoconstriction line slopes were not different among the four groups in arcade bridge arterioles only. In the 6 Day BP Exposure group, arcade arteriolar vasoconstriction was significantly augmented as demonstrated by regression analysis. However, in this group, the line slope of the collective data points was significantly less negative

than the other three groups. The individual dose and group differences are more clearly identified in **FIGURES 8B** and **9B**. This observation was consistent for the arcade arterioles in only the 6 Day BP Exposure group. Collectively, this indicates that arcade arteriole vasoconstriction may be attenuated after 6 Day BP exposures. This physiologic relevance of this alteration is unclear, but it does indicate a disruption in vascular reactivity gradients.

To assess potential contributors to circulating oxidant load, we examined the abundance of XO in the plasma of rats exposed to BP emissions for 2, 3 and 6 days. After 2 days of BP inhalation exposures, a significant elevation (~38%) in circulating XO is present as compared to the Sham Control group (**FIGURE 10**). This BP exposure-mediated increase in circulating XO is further amplified (2-fold) by 3 and 6 days of inhalation exposures. This amplification of circulating XO is important as XO is an abundant source of oxidants (H_2O_2 and $O_2^{\cdot-}$) in the vascular compartment that can contribute to diminished vascular function via diminution of nitric oxide (NO) production and/or direct quenching of NO [30].

In select rats from the 3 Day BP exposure group, we performed bulk RNA sequencing of lung and spleen tissue to assess relevant transcriptional responses. We note a marked increase in transcript levels of numerous genes in burnpit exposed rats as demonstrated by a right-shifted volcano plot (**FIGURE 11A**). Select genes with significantly increased transcript counts include genes associated with neutrophils (*Cxcl1*, *Lcn2*, *Cd177*), which is consistent with neutrophil influx seen on cell counts obtained by BAL (**FIGURE 4C**). We also identified increased transcript counts from key genes of the complement system (*C3*, *Cfi*, *C4bpa*) and cellular lysosomal/oxidative pathways (*Gpnmb*, *Gsta2*). In contrast to the lung, we found a marked decrease in transcript levels of several genes in the spleens of burnpit exposed rats as demonstrated by a left-shifted volcano plot (**FIGURE 11B**). Select genes with significantly decreased transcript counts include immune cell recruitment (*Cx3cr1*), stress response (*Creb3l1*), and tissue structure (*Col18a1*). We next performed gene set enrichment analysis of human orthologs of rat genes using hallmark gene sets. We identified a marked positive

enrichment of several Hallmark pathways in the lung including cell cycle associated pathways as well as inflammatory and immune pathways (**FIGURE 11C**). Notably, oxidative phosphorylation was negatively enriched, highlighting a transcriptional shift toward glycolysis that may result from the cellular stress of burnpit emissions. In contrast to the lung findings, we found negative enrichment of several pathways in the spleen including epithelial-mesenchymal transition as well as Wnt/beta-catenin and TGF-beta (**FIGURE 11D**). The sole positively enriched pathway in the spleen was interferon-alpha response.

In the brain, BP inhalation exposure significantly altered gene expression for both *Vegfa* ($F(2,17) = 1.022$, $p = 0.001$) and *Icam1* ($F(2,17) = 1.881$, $p = 0.020$). Post-hoc pairwise comparisons revealed that 3 days of BP exposure elevated *Vegfa* compared to both Sham-Control and 6 Days BP exposure ($p < 0.05$, **FIGURE 12A**). *Icam1* expression showed a similar pattern of elevation after 3 days of BP exposure compared to 6 Days BP exposure ($p < 0.05$, **FIGURE 12B**).

DISCUSSION

This study provides our first observations of the systemic microvascular consequences of inhalation exposures to surrogate military burn pit emissions. The most notable novel observation is the impairment of endothelium-dependent arteriolar dilation. This impairment was evident after three days of BP emission exposures and was maximal by 3 days. Further, novel mechanisms that potentially underlie microvascular dysfunction have been discovered. Most notably, circulating XO activity is similarly altered in that is increased in a dose-dependent manner that is maximal after 3 days of BP emission exposures.

The Military Exposure Guidelines (MEG) for particulate matter (PM) exposures cover multiple deployment considerations and range from 250-600 $\mu\text{g}/\text{m}^3$ for PM_{10} [31]. In 2009 cumulative air quality measurements were made at Joint Base Balad (JBB), near Baghdad, Iraq. While a variety of PM concentrations existed due to base activity and prevailing environmental

conditions, PM concentration ranges were identified. The maximum PM₁₀ concentration was ~10 mg/m³, and the maximum PM_{2.5} concentration was ~3 mg/m³ [2]. The term “burn pit” is somewhat of a misnomer as the practice of their operation entails burning and smoldering, which have been shown to produce PM concentrations of ~40 and ~4 mg/cm³ respectively, at the source [32]. Considering the range of PM concentrations referenced, and range of uncertainties in desert deployment, we selected a target concentration of 15 mg/m³ in our surrogate BP emission inhalation exposures. We too experienced variations in our combustion particle concentrations, distributions, and gases (**FIGURES 1 and 3**). This is a characteristic of combustion. The microvascular impairments reported herein (**FIGURES 6 and 7**) were maximal by three days of BP emission inhalation exposures. In this group of rats, the calculated pulmonary particle deposition was 99 µg (MPPD model results). If we estimate that deployed military personnel were exposed to BP emissions constantly (24 hrs/day), at a ventilation rate of 7.5 LPM, with a lung surface area of 75 m², and a deposition fraction of 0.0985 (MPPD model results), a comparable exposure would be achieved in humans in ~1 day. We acknowledge that our laboratory exposure model/dose may be considered high. However, if we consider the high MEG of 600 µg/m³ and measurements made at JBB of ~10 mg/m³, deployed personnel would achieve the 3D BP dose used herein in ~2-30 days. Given that typical deployments to the Middle East ranged from 9-15 months, we consider our exposure model very relevant.

As the oxidant load in the vascular compartment is tightly associated with microvascular dysfunction, we assessed the abundance of a critical source of oxidant load in the circulation, xanthine oxidase (XO). **FIGURE 10** demonstrates a significant 2-day BP emission exposure-mediated elevation in circulating XO activity versus Sham-Control animals. This increase in circulating XO was further amplified when the rats were exposed for 3 and 6 days. These results are similar to those observed with another inhalation exposure model (nano-titanium dioxide) where circulating XO was increased and was found to contribute extensively to diminished

vascular function [33]. Although contributory roles for XO in microvascular dysfunction were not directly tested herein, it is reasonable to assume our results revealing increased XO activity in the circulation may indicate that this source of oxidants may be mechanistically operative in diminishing microvascular vascular reactivity in our BP emission inhalation exposure model. Future studies will explore this possibility with pre-exposure protective treatments such as nitrate supplementation.

Transcriptional analysis from lung and spleen revealed a marked alteration in immune and inflammatory profiles after 3 days of BP exposure that suggests both direct lung effects of BP emissions as well as systemic extra-pulmonary effects in the spleen. Future research should be directed toward further investigation of the cellular transcriptional response such as single-cell RNA sequencing.

The hippocampus is highly vulnerable to insults due to fragile blood-brain barrier (BBB) and a dense vasculature [34]. Air pollution PM exposure has been associated with hippocampal inflammation and disruptions to BBB [35, 36]. Transient increase in hippocampal *Vegfa* and *Icam1* expression after 3 days of BP emission inhalation exposure suggests an early inflammatory or hypoxia response with alterations in microvascular function. Consistent with this observation, we have previously reported that pulmonary exposure to diverse agents such as residual oil fly ash [12] and nano-titanium dioxide [37] increase venular leukocyte adhesion and rolling. *Vegfa* expression is induced following air pollution exposure via the hypoxia inducible factor (*HIF*)-1 α dependent mechanisms, and it contributes to both angiogenesis and increased vascular permeability [38, 39]. Similarly, previous studies have demonstrated that air pollution exposure is directly related to increased expression of *Icam1*, a key adhesion molecule mediating inflammatory cell trafficking and leukocyte recruitment [40, 41]. Notably, increasing the exposure duration to 6 days resulted in a downregulation of *Vegfa* and *Icam1* relative to the 3D BP exposure group. These results suggest a time-dependent microvascular response to burn pit toxins characterized by endothelial activation and angiogenic responses followed by a

subsequent decline with continuous exposure. Hippocampal *Vegfa* and *Icam1* expression is also consistent with the results of BAL fluid assays (**FIGURE 4**) that show heightened inflammatory responses after 3 days of burn pit exposure but not after 6 days. Some earlier studies have also reported this pattern where prolonged or repeated exposure may attenuate or dysregulate the initial responses. Short term exposure to ozone in rats induced marked permeability and inflammatory responses in lower airways, as demonstrated by enhanced proteins, fibronectin, IL-6, and inflammatory cells in BAL fluid. While a complete disappearance of these responses was observed following 5 days of exposure [42]. In another study, exposure to coarse PM (PM₁₀) in rabbits caused lung and systemic inflammation marked by increased macrophages and elevated serum IL-6 levels, which is related to impaired endothelial function. However, IL-6 returned to baseline in weeks 3 and 4 despite the continued exposure [43]. The observed reduction in inflammatory and microvascular responses may be attributed to adaptive responses or activation of feedback inhibitory mechanisms, which needs further mechanistic studies.

While our exposure paradigm has been demonstrated to produce biologic effects, it certainly does not capture all the materials combusted in burn pits, environmental conditions, or background toxicants such as silica and metals [44, 45]. Therefore, future studies should continue to explore the spectrum of exposures and effects. We have demonstrated the onset of diverse biologic effects that follow BP emission inhalation exposures. A better characterization of the full timeline could help distinguish between the acute phase response and a chronic adaptation. Because the microcirculation underlies most every aspect of health, development and disease [46], a natural evolution of these studies would be to link the microvascular dysfunction reported herein with pathologies specific to chronic multisymptom illness. Finally, as with all diseases, identification of the underlying biologic mechanisms will provide targets for treatment and prevention.

SUMMARY/CONCLUSION

A graphical abstract that integrates our exposure paradigm, biologic effects and proposed mechanisms is presented in **FIGURE 13**. Rats were subjected to whole-body inhalation exposures to emissions resulting from combustion of the most common materials disposed of in military burn pits. This resulted in pulmonary insult, characterized by macrophage particulate uptake, pulmonary inflammation, and cellular infiltration. As with other pulmonary insults, this spilled into the systemic circulation and indiscriminately altered microvascular function at multiple levels. This also produced broad changes in cellular sequencing in the brain, lung, and spleen. Some of the notable impacts of these changes are altered adhesion signaling, inflammation, and XO imbalance. While each of these impacts is capable of impairing systemic microvascular function, the likelihood that a single factor is at play is low. Future studies must continue to identify the full scope of biologic impacts of military burn pit emission inhalation exposures, their interactions, and contributions to the development of CMI.

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ABBREVIATIONS

AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care International

ACh: acetylcholine

ADO: adenosine

ANOVA: analysis of variance

APS: aerodynamic particle sizer

BAL: bronchoalveolar lavage

BALF: bronchoalveolar lavage fluid

BBB: blood-brain barrier

BP: burnpit

CMD: count median diameter

CMI: chronic multisymptom illness

CO: carbon monoxide

CO₂: carbon dioxide

CPC: condensation particle counter

Dcon: control diameter

Dpass: passive diameter

Dss: steady state diameter

DRRD: deployment related respiratory disease

EDTA: ethylenediaminetetraacetic acid

GC-MS: gas chromatography/mass spectrometer

GSD: geometric standard deviation

Icam1: intercellular adhesion molecule 1

JAA: jet fuel with additives

JBB: Joint Base Balad

MMAD: mass median aerodynamic diameter

XO: xanthine oxidase

MAP: mean arterial pressure

MEG: military exposure guidelines

MOUDI: micro-orifice uniform deposit impactor

MPPD: Multiple Path Particle Dosimetry model

NIOSH: National Institute for Occupational Safety and Health

NO: nitric oxide

PACT Act: Promise to Address Comprehensive Toxics Act

PAH: polyaromatic hydrocarbon

PBS: phosphate buffered saline

PE: phenylephrine

PM: particulate matter

PSD: particle size distribution

RH: relative humidity

RNA: ribonucleic acid

SE: standard error

SMPS: scanning mobility particle sizer

TEM: transmission electron microscopy

TVOC: total level of organic vapor

VCC volatile carbonyl compound

Vegfa: vascular endothelial growth factor a

VOC: volatile organic compound

TABLE 1: Animal characteristics in the experimental groups. N, number of animals. Values are means $\pm$ SE.

Experimental Group	N	Age (days)	Mass (g)	Mean Arterial Pressure (mm Hg)	Heart Rate (bpm)
Sham-Control	5	58 $\pm$ 6	269 $\pm$ 21	117 $\pm$ 2	386 $\pm$ 21
2 Day BP Exposure	8	54 $\pm$ 3	273 $\pm$ 14	99 $\pm$ 7	327 $\pm$ 34
3 Day BP Exposure	5	50 $\pm$ 3	258 $\pm$ 18	104 $\pm$ 5	411 $\pm$ 25
6 Day BP Exposure	4	57 $\pm$ 4	310 $\pm$ 27	118 $\pm$ 5	370 $\pm$ 12

TABLE 2: Arcade bridge (first order) arteriolar characteristics. n = number of arterioles. Values are means $\pm$ SE.

Experimental Group	n	D _{rest} (μ m)	D _{pass} (μ m)	Tone (%)
Sham-Control	16	44 $\pm$ 2	106 $\pm$ 4	58 $\pm$ 2
2 Day BP Exposure	14	47 $\pm$ 4	112 $\pm$ 10	57 $\pm$ 5
3 Day BP Exposure	14	54 $\pm$ 5	107 $\pm$ 9	48 $\pm$ 4
6 Day BP Exposure	13	50 $\pm$ 5	108 $\pm$ 9	53 $\pm$ 5

TABLE 3: Arcade (second order) arteriolar characteristics. n = number of arterioles. Values are means $\pm$ SE.

Experimental Group	n	D _{rest} (μ m)	D _{pass} (μ m)	Tone (%)
Sham-Control	10	21 $\pm$ 3	49 $\pm$ 5	54 $\pm$ 6
2 Day BP Exposure	12	25 $\pm$ 4	57 $\pm$ 7	56 $\pm$ 6
3 Day BP Exposure	10	23 $\pm$ 5	50 $\pm$ 6	57 $\pm$ 6
6 Day BP Exposure	6	23 $\pm$ 5	42 $\pm$ 7	45 $\pm$ 6

FIGURE

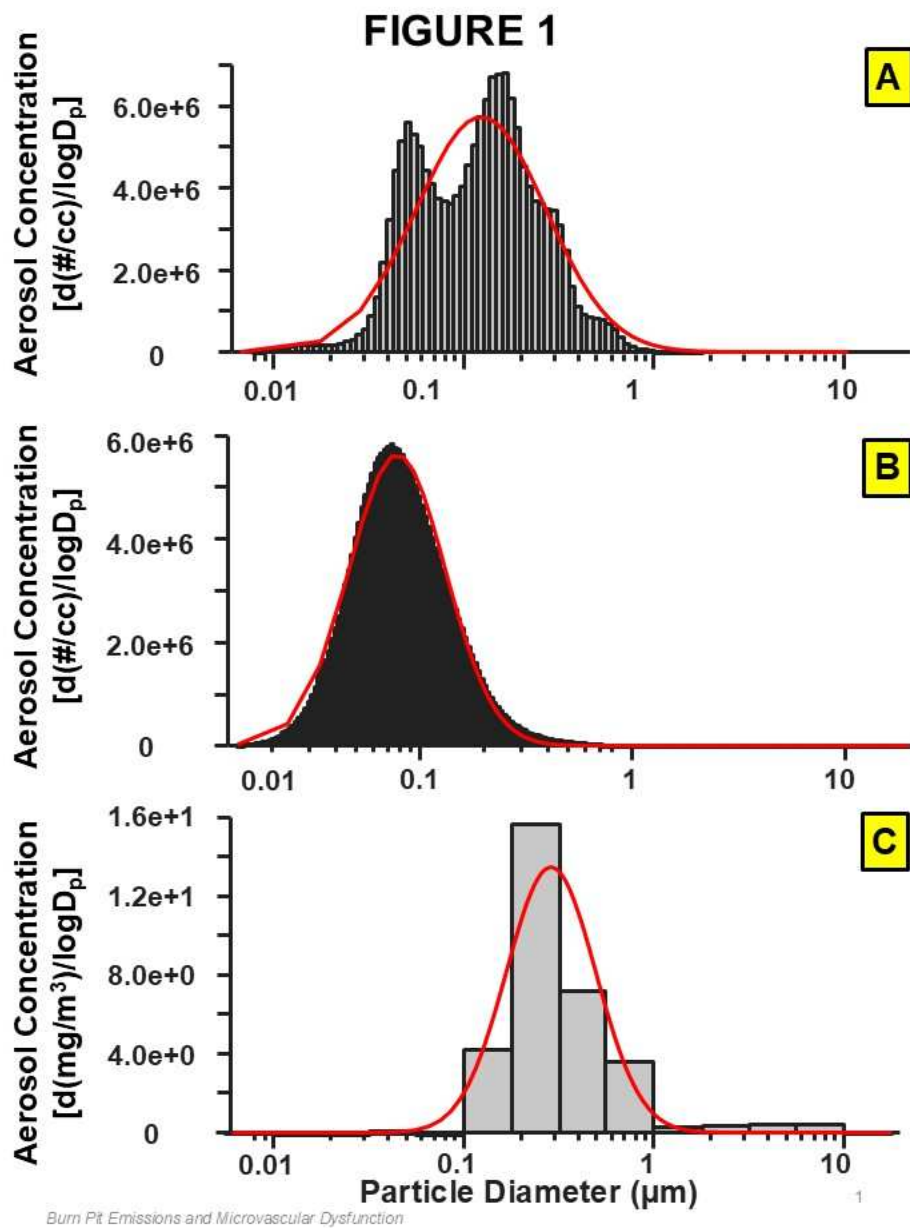
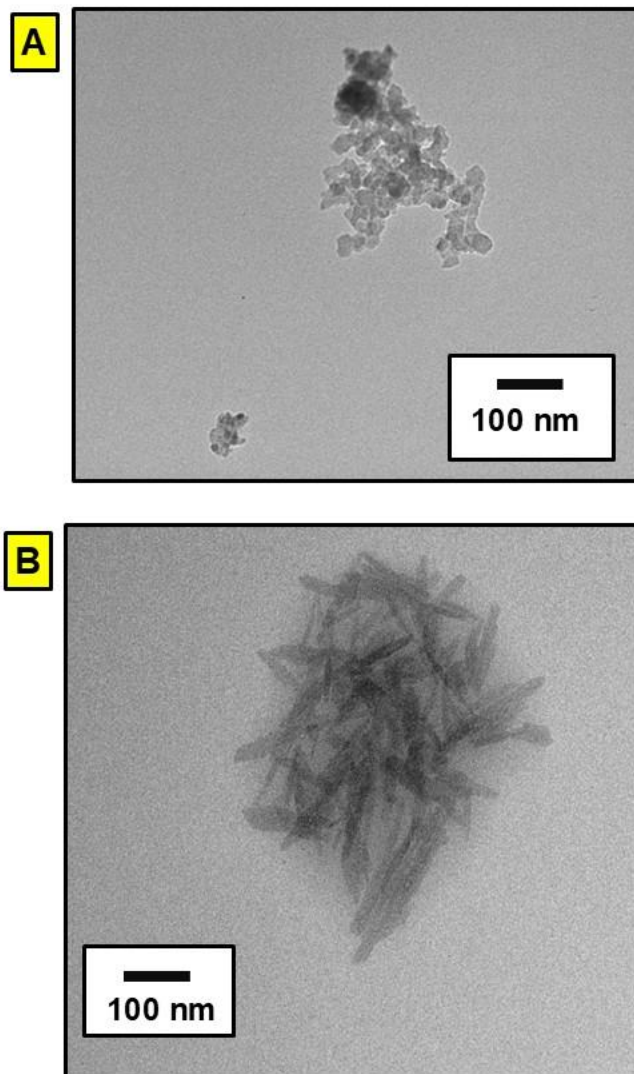


FIGURE 1: BP combustion emissions particle size-distributions. **A)** particle aerodynamic diameter size distribution measured with ELPI+. **B)** particle mobility diameter size distribution measured with SMPS and APS. **C)** mass-based particle size distribution measured with MOUDI. Red lines designate log-normal fits of the data. All measurements were sampled directly from the exposure chamber.

FIGURE 2



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FIGURE 2: Representative transmission electron microscopy images of BP emission particles sampled from the exposure chamber. **A)** Heterogeneous aggregate. **B)** Small fibrous homogeneous aggregate.

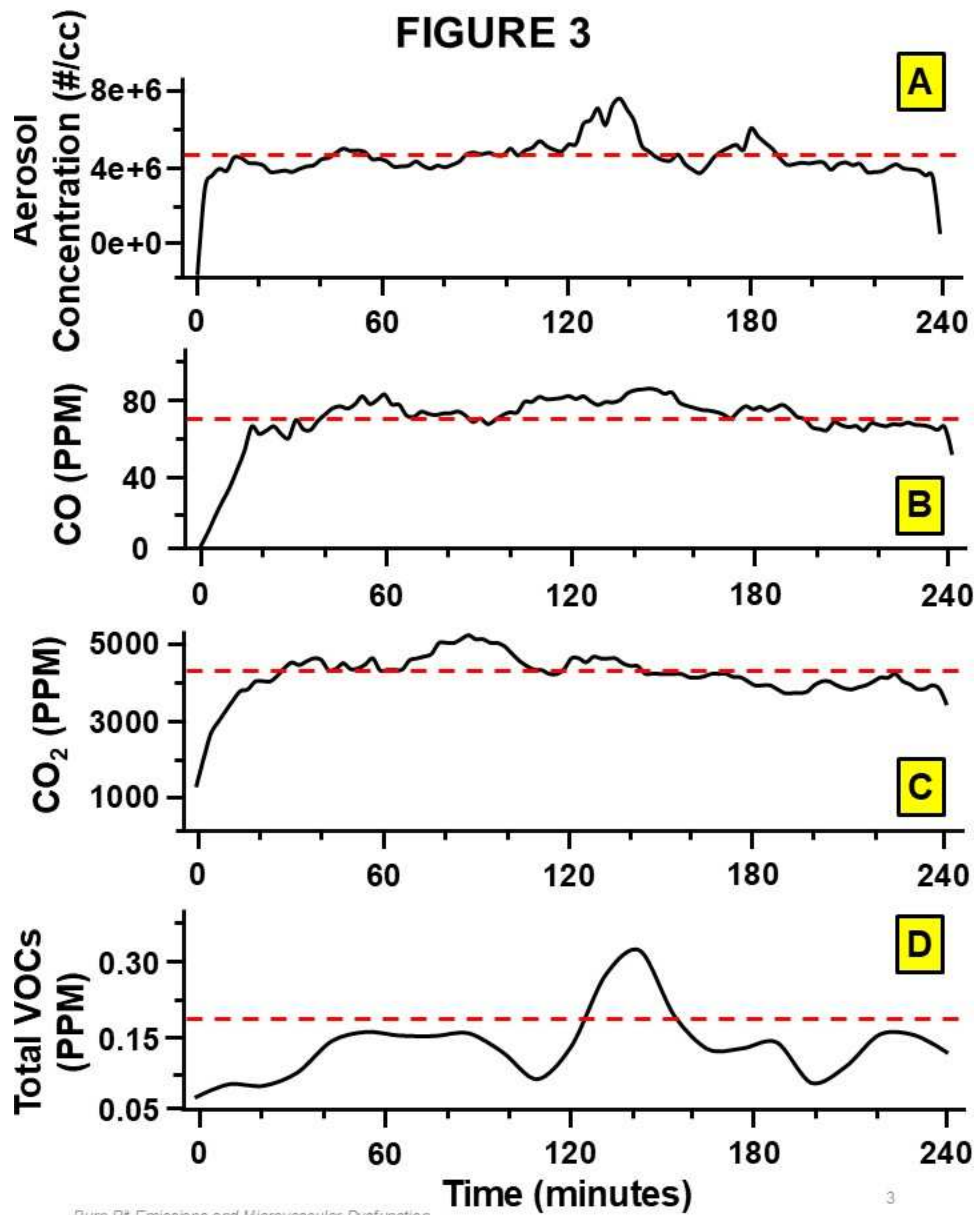
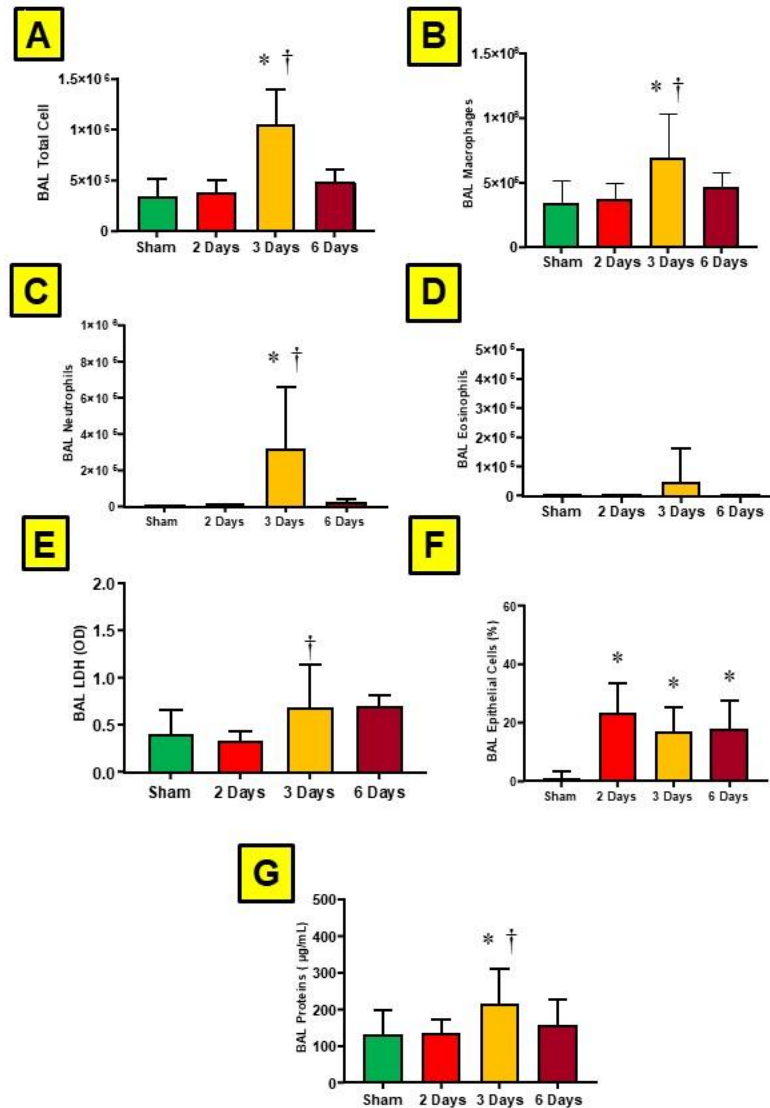


FIGURE 3: Real-time BP emission aerosol characterizations. **A)** Total particle concentration using condensation particle counter (in number #/cc) from exposure chamber. Mean concentration (dashed red line) was $4.8 \times 10^6 \pm 9.5$ #/cc. **B)** Carbon monoxide (CO) concentration measured with Five Gas Analyzer. Mean concentration (dashed red line) was 70 ± 0.2 PPM. **C)** Carbon dioxide (CO₂) concentration measured with Five Gas Analyzer. Mean concentration (dashed red line) was 4400 ± 6.3 PPM. **D)** Total volatile organic compounds (VOCs)

concentration measured with photoionization detector, TVOC2. Mean concentration (dashed red line) was 0.185 ± 0.001 . PPM.

FIGURE 4

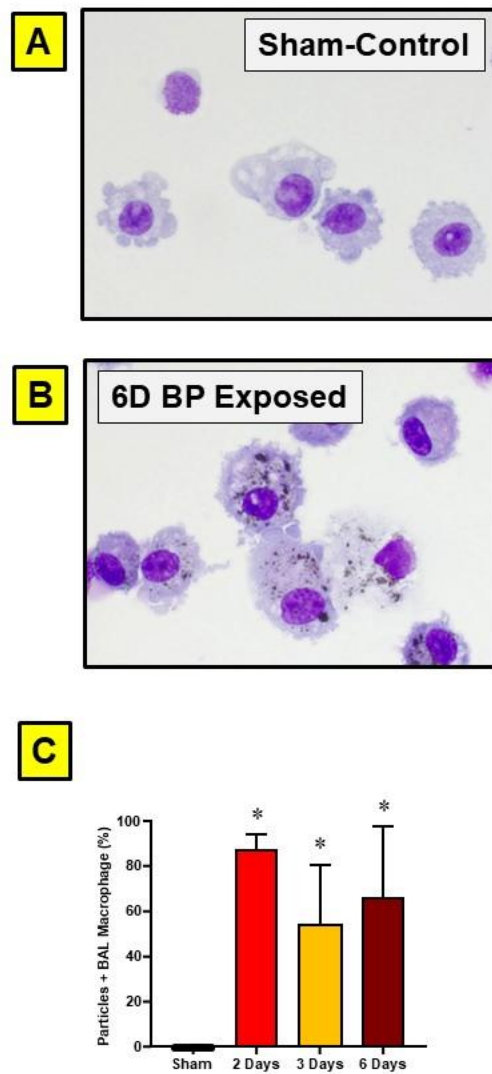


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FIGURE 4: Pulmonary impacts of BP emission inhalation exposures. A) BAL total cell counts. B) BAL Macrophages C) BAL Neutrophils. D) BAL Eosinophils. E) level of released LDH in BALF. F) BAL epithelial cells and G) BAL total proteins. *, $P < 0.05$ vs Sham-Control Group. †, $P < 0.05$ vs 2 Day BP Exposure Group.

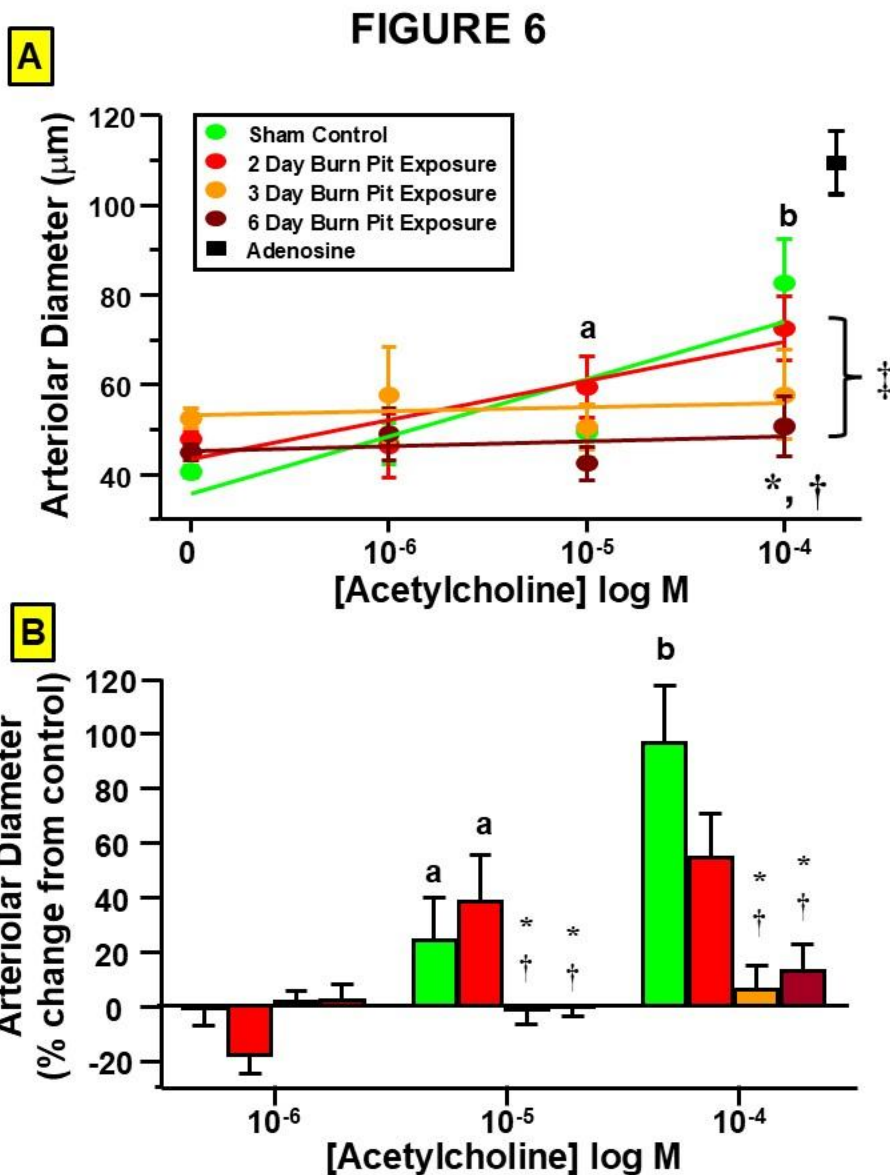
FIGURE 5



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FIGURE 5: Representative pulmonary macrophage morphology after BP emission inhalation exposures in **A)** Sham-Control, and **B)** 6D Day BP Exposure Group. Magnification in both panels is 100X. **C)** Normalized particle and macrophage counts.



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FIGURE 6: Burn pit emission inhalation exposures impair endothelium-dependent dilation in arcade bridge arterioles (1A). **A)** arteriolar reactivity is presented as raw data (micrometers). a, $P < 0.05$ vs control (0 M ACh). b, $p < 0.05$ vs 10^{-6} M ACh. *, $P < 0.05$ vs Sham Control group at same dose. †, $P < 0.05$ BP group at same dose. ‡, $P < 0.05$ for indicated line slopes. Sham Control group line slope equation: $y = 1.3X + 35.7$; $r = 0.88$. Two Day BP Exposure group line slope equation: $y = 0.9X + 43.5$; $r = 0.93$. Three Day BP Exposure group line slope equation: $y =$

0.1X + 53.4; $r = 0.29$. Six Day BP Exposure group line slope equation: $y = 0.1X + 45.2$; $r = 0.37$.

B) arteriolar reactivity is presented as normalized data as percent change from control. a, $P < 0.05$ vs 10^{-6} M ACh. b, $P < 0.05$ vs 10^{-5} M ACh. *, $P < 0.05$ vs Sham Control group at same dose. †, $P < 0.05$ BP group at same dose.

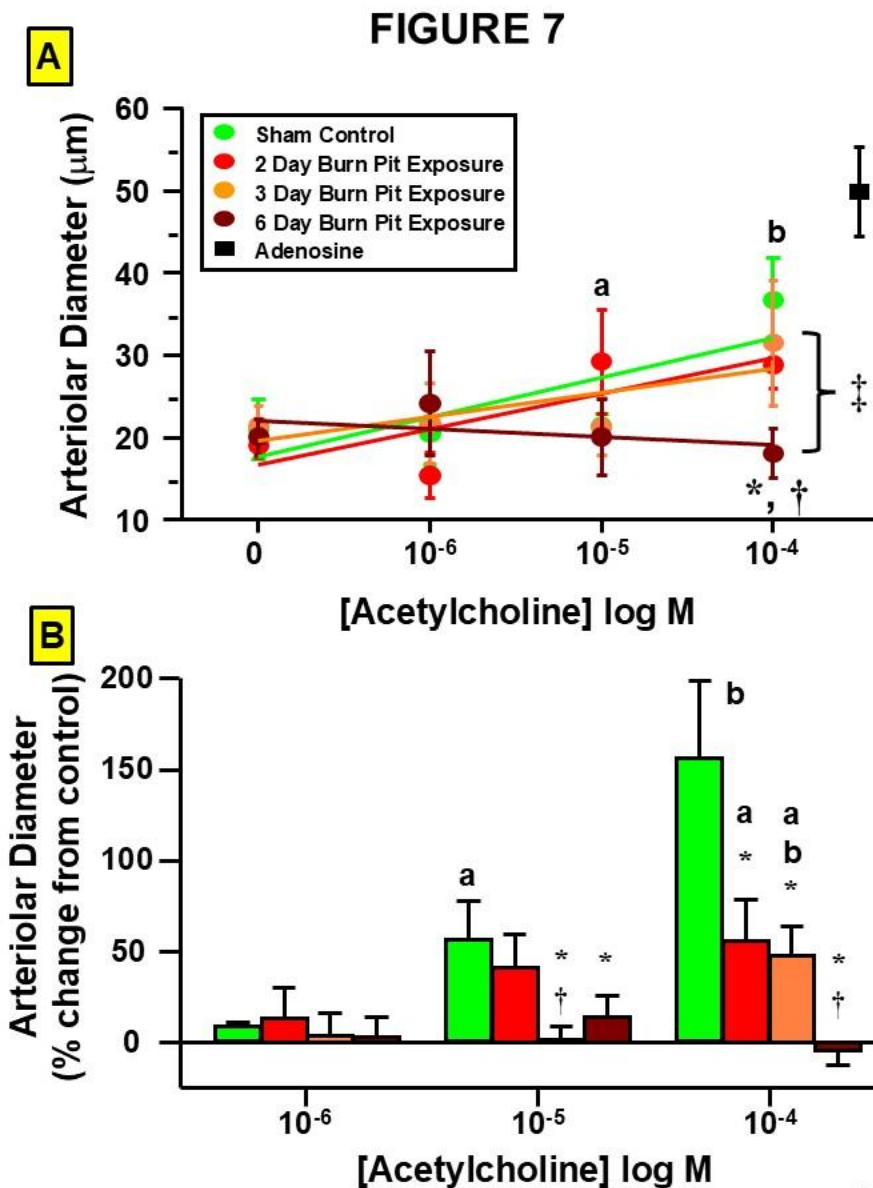
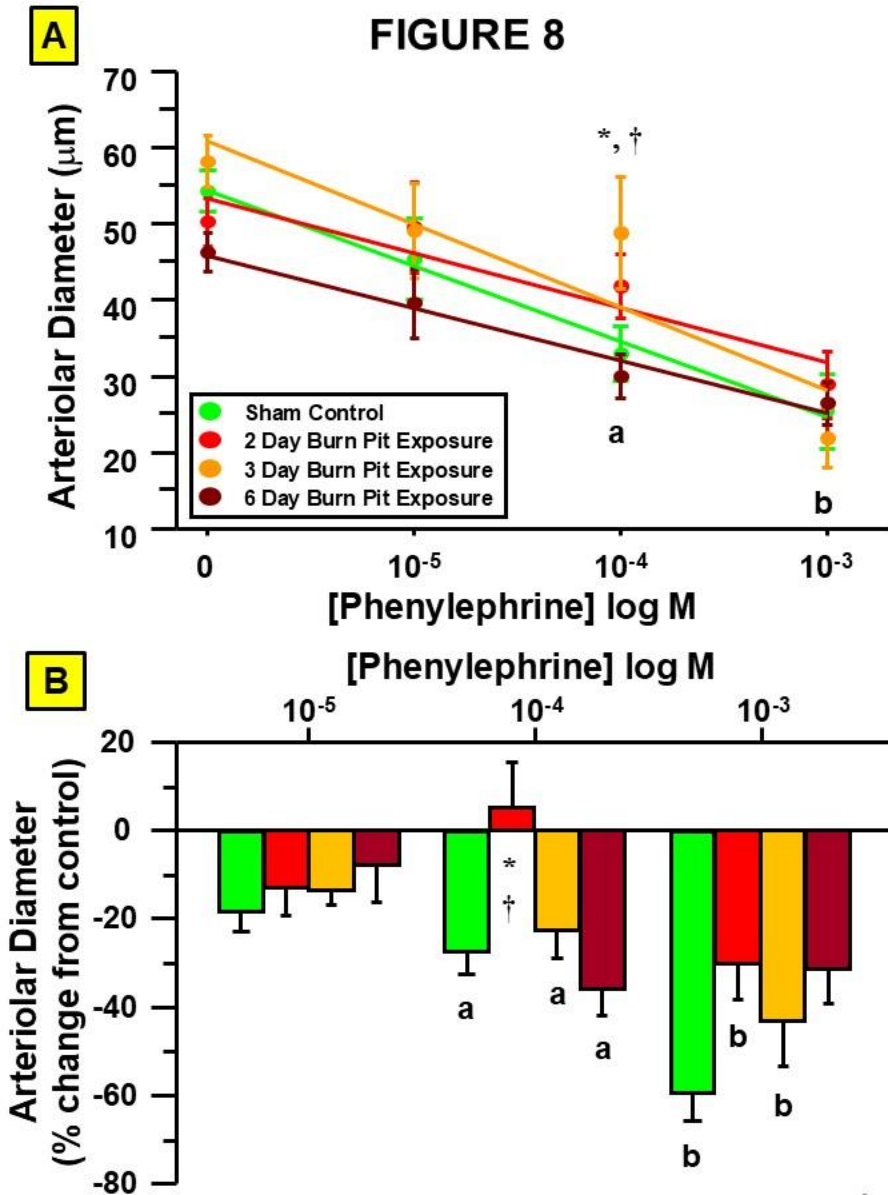


FIGURE 7: Burn pit emission inhalation exposures impair endothelium-dependent dilation in arcade arterioles (2A). **A)** arteriolar reactivity is presented as raw data (micrometers). a, $P < 0.05$

vs control (0 M ACh). b, $p < 0.05$ vs 10^{-6} M ACh. *, $P < 0.05$ vs Sham Control group at same dose. †, $P < 0.05$ BP group at same dose. ‡, $P < 0.05$ for indicated line slopes. Sham Control group line slope equation: $y = 0.5X + 17.7$; $r = 0.79$. Two Day BP Exposure group line slope equation: $y = 0.4X + 16.7$; $r = 0.80$. Three Day BP Exposure group line slope equation: $y = 0.3X + 19.6$; $r = 0.77$. Six Day BP Exposure group line slope equation: $y = -0.1X + 22.1$; $r = 0.51$. **B)** arteriolar reactivity is presented as normalized data as percent change from control. a, $P < 0.05$ vs 10^{-6} M ACh. b, $p < 0.05$ vs 10^{-5} M ACh. *, $P < 0.05$ vs Sham Control group at same dose. †, $P < 0.05$ BP group at same dose.



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FIGURE 8: Burn pit emission inhalation exposures do not alter arcade bridge arteriole (1A) adrenergic sensitivity. **A)** arteriolar reactivity is presented as raw data (micrometers). a, $P < 0.05$ vs control (0 M phenylephrine). b, $p < 0.05$ vs 10^{-6} M phenylephrine. *, $P < 0.05$ vs Sham Control group at same dose. †, $P < 0.05$ BP group at same dose. Sham Control group line slope equation: $y = -1.0X + 54.4$; $r = 1.00$. Two Day BP Exposure group line slope equation: $y = -0.7X + 53.4$; $r = 0.93$. Three Day BP Exposure group line slope equation: $y = -1.1X + 60.9$; $r = 0.90$.

Six Day BP Exposure group line slope equation: $y = -0.7X + 45.9$; $r = 0.99$. **B)** arteriolar reactivity is presented as normalized data as percent change from control. a, $P < 0.05$ vs 10^{-5} M phenylephrine. b, $p < 0.05$ vs 10^{-4} M phenylephrine. *, $P < 0.05$ vs Sham Control group at same dose. †, $P < 0.05$ BP group at same dose.

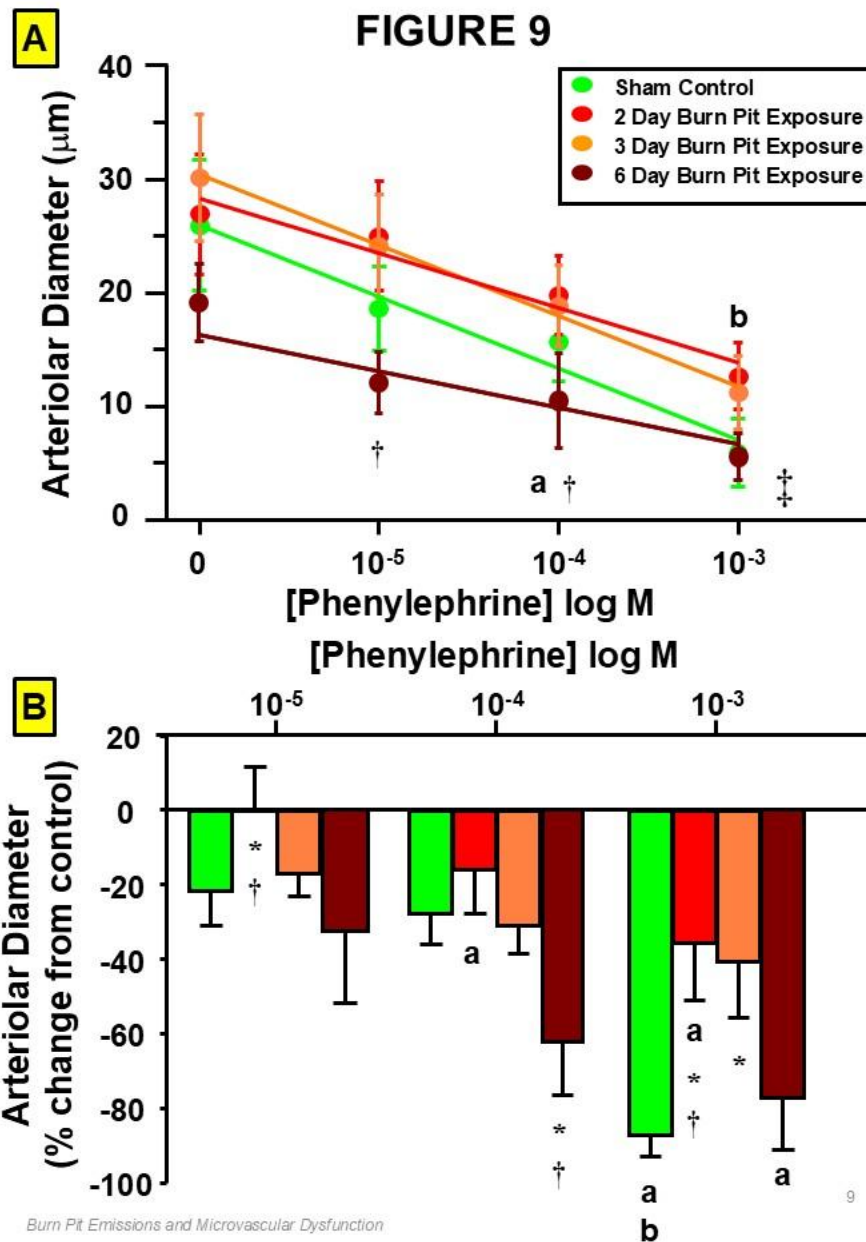
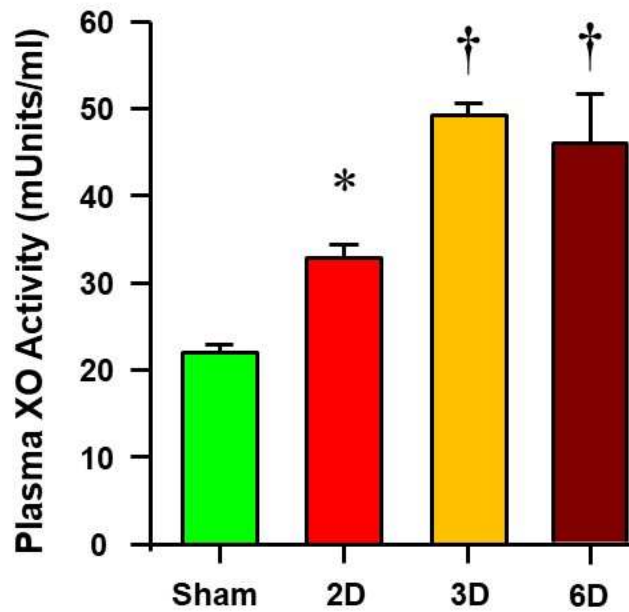


FIGURE 9: Burn pit emission inhalation exposures do not alter arcade arteriole (2A) adrenergic sensitivity. **A)** arteriolar reactivity is presented as raw data (micrometers). a, $P < 0.05$ vs control

(0 M phenylephrine). b, $p < 0.05$ vs 10^{-6} M phenylephrine. *, $P < 0.05$ vs Sham Control group at same dose. †, $P < 0.05$ BP group at same dose. Sham Control group line slope equation: $y = -0.6X + 26.0$; $r = 0.98$. Two Day BP Exposure group line slope equation: $y = -0.6X + 30.4$; $r = 1.00$. Three Day BP Exposure group line slope equation: $y = -0.5X + 28.3$; $r = 0.97$. Six Day BP Exposure group line slope equation: $y = -0.3X + 14.6$; $r = 1.00$. **B)** arteriolar reactivity is presented as normalized data as percent change from control. a, $P < 0.05$ vs 10^{-5} M phenylephrine. b, $p < 0.05$ vs 10^{-4} M phenylephrine. *, $P < 0.05$ vs Sham Control group at same dose. †, $P < 0.05$ BP group at same dose.

FIGURE 10



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FIGURE 10: BP inhalation exposures elevate circulating XO. Shown are the enzymatic activity levels of XO in plasma at days 2, 3 and 6 (2D, 3D & 6D) BP exposure versus Sham Controls. All measures were made 24 hr after last exposure. *, $P < 0.05$ vs Sham Control group. †, $P < 0.05$ vs 2D BP group.

FIGURE 11

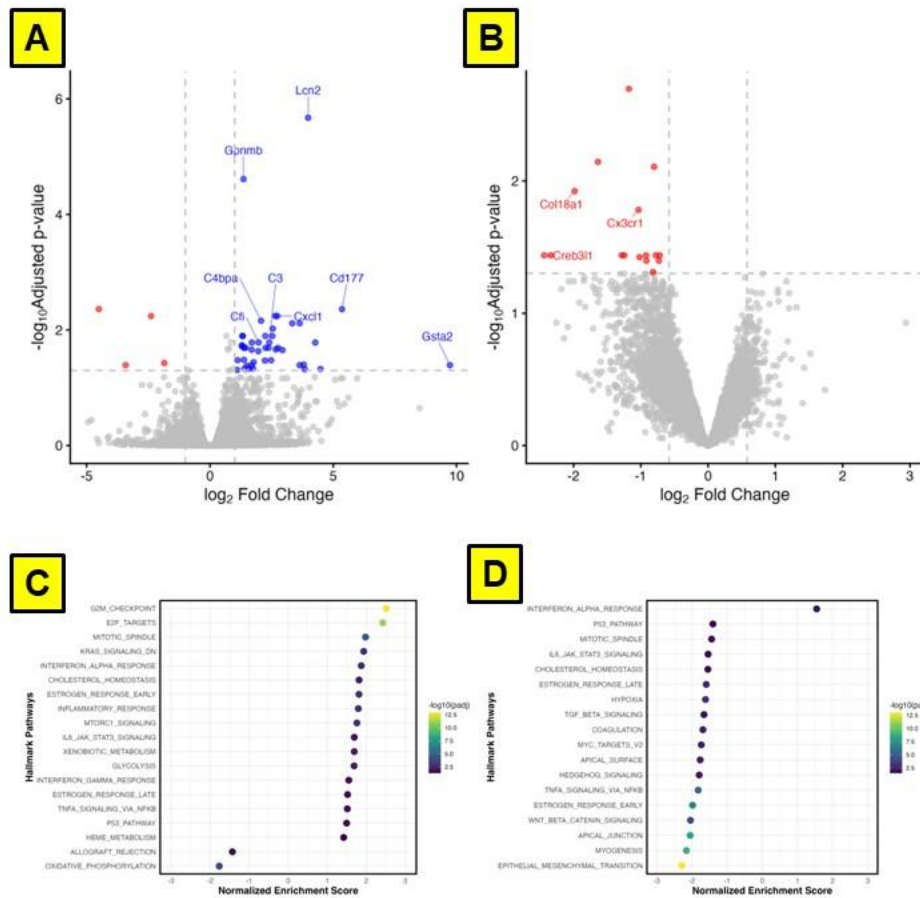


FIGURE 11: BP inhalation exposures inflammatory and immune transcriptional profiles in lung and spleen. **A)** Volcano plots of normalized RNA counts in lung (N=3 sham air controls and N=5 burn pit exposed rats); and **B)** spleen with annotations showing select differentially expressed genes with adjusted P value <0.05. Gene set enrichment analysis of all transcripts using hallmark gene sets in **C)** lung; and **D)** spleen with adjusted P value <0.05. In all panels, results

from Sham Control animals serve as the reference for differential expression and gene set enrichment.

FIGURE 12

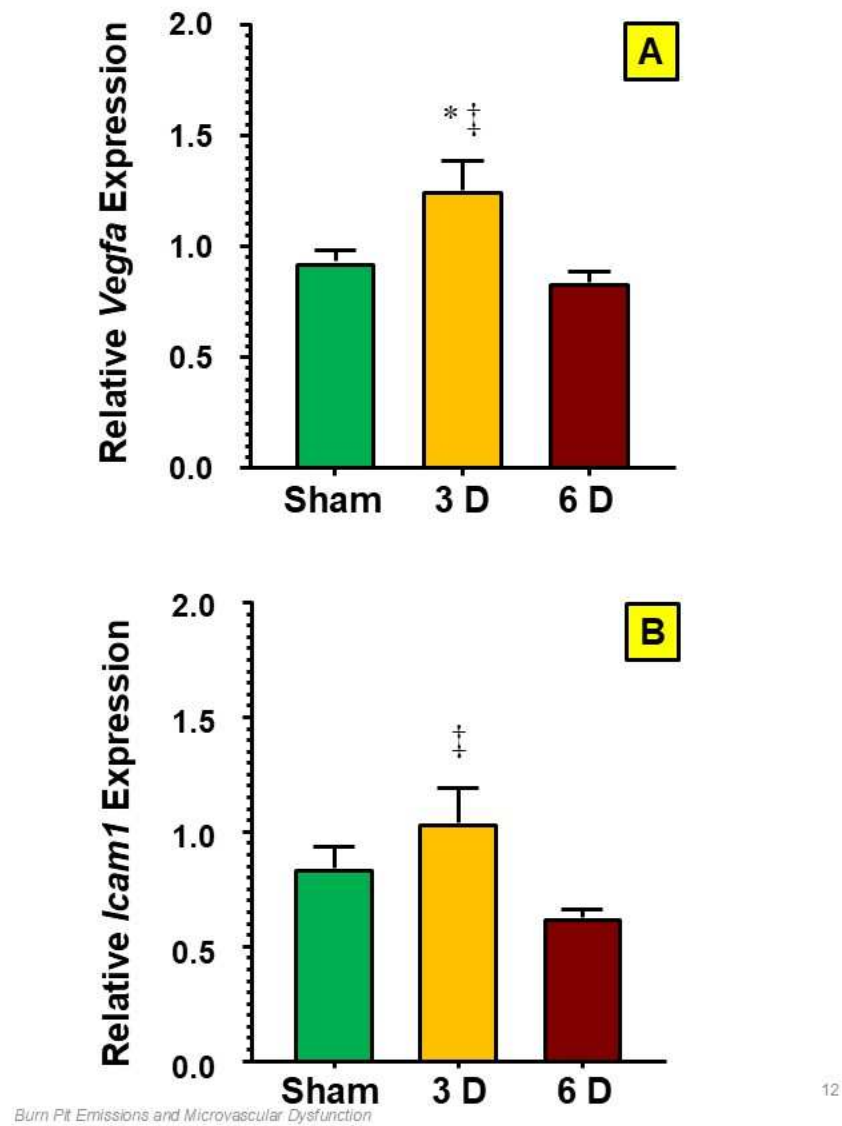
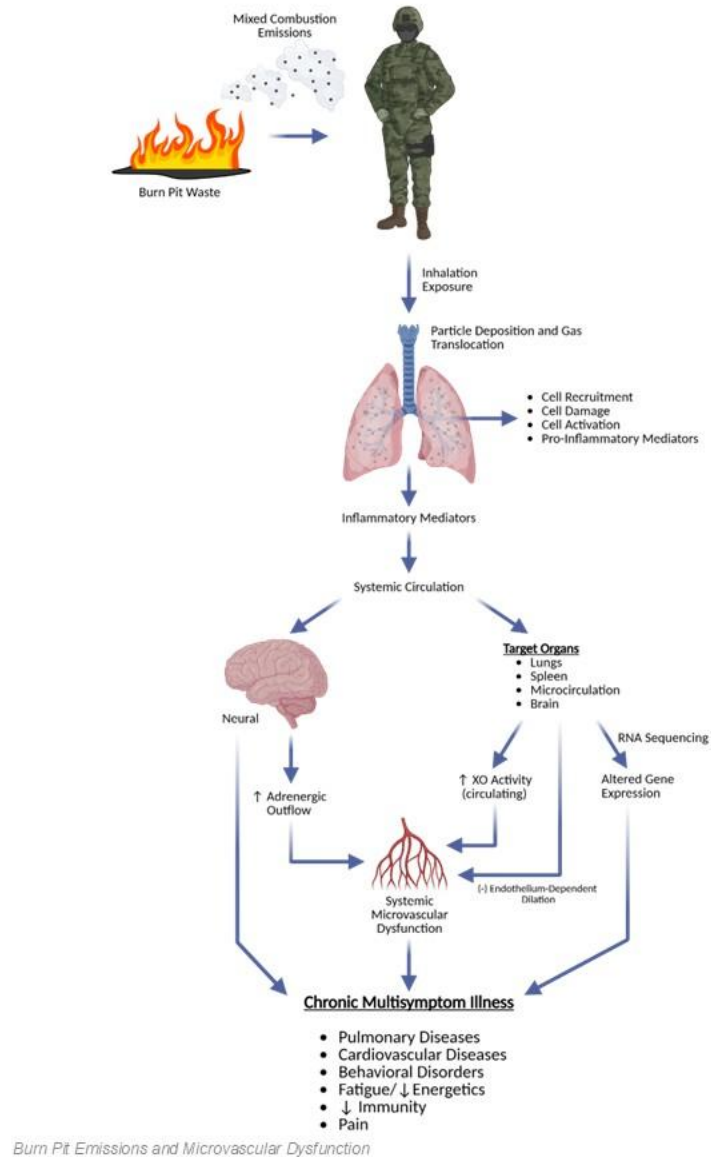


FIGURE 12: BP inhalation exposures alter brain gene expression. **A)** Hippocampal *Vegfa* expression was significantly increased after 3 days of BP inhalation exposure compared to Sham-Control rats or 6 Days BP exposure. **B)** Expression of *Icam1* displayed a similar pattern

of elevation after 3 Days of exposure compared to 6 Days BP exposure. *, $P < 0.05$ vs Sham-Control Group. ‡, $P < 0.05$ vs 6 Day BP Exposure Group.

FIGURE 13



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FIGURE 13: Graphical abstract of exposure paradigm and proposed CMI mechanisms.

DECLARATIONS

Ethical Approval

All procedures and experiments in this study conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (8th Edition) and were approved by the West Virginia University Animal Care and Use Committee (protocol number 1602000621).

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Data Availability

Data available upon reasonable request.

Competing Interests

The authors declare no competing interests.

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