Experimental woodsmoke exposure during exercise and an evaluation of blood oxidative stress

by

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Abstract

Introduction: Woodsmoke exposure and exercise elicit oxidative stress in blood. The purpose of this investigation was to understand the effects of woodsmoke exposure on blood oxidative stress measures. **Methods:** Ten males 18-40 years were exposed to woodsmoke during 3 trials (separated by 1 week) while exercising on a treadmill to simulate the workload intensity and smoke exposure doses of wildland firefighting. Participants were exposed Clean Air (0 $\mu g/m^3$, Filtered Air), 250 $\mu g/m^3$ (Low Exposure) and 500 $\mu g/m^3$ (High Exposure) woodsmoke particles (particulate matter <2.5 μm, $PM_{2.5}$) in a randomized counter-balanced crossover fashion while exercising. Blood samples obtained at baseline (Pre), immediately post (Post) and 1 hour following (1Hr) post exposure were assayed for trolox equivalent antioxidant capacity (TEAC), uric acid (UA), lipid hydroperoxides (LOOH), protein carbonyls (PC), nitrotyrosine (3-NT), 8-isoprostane, and myeloperoxidase (MPO) activity and protein content assays. **Results:** Study participants averaged 14.1 % body fat and had an average aerobic capacity of 53.6 $ml \cdot kg^{-1} \cdot min^{-1}$. There were no self-reported respiratory complications by participant following the exposure trials. UA values were lower only following Low Exposure (p=0.042), while plasma TEAC levels were elevated Post (Clean Air and High exposure, p=0.015 and p=0.001 respectively) and at 1Hr (Clean Air and High exposure, p=0.001) time points. LOOH levels were decreased 1Hr Post (High exposure, p=0.036), while plasma 8-Iso levels were elevated in Post samples from both smoke doses (Low exposure p=0.004, High exposure p=0.009). There were no increases in plasma PC levels for any of the 3 trials, while 3-NT values were elevated over Clean Air when Low/High Exposure values were combined (p=0.012). Neither MPO activity nor protein content

was altered following the 3 trials. **Discussion**: The current laboratory simulation of wildland firefighting elicited some indications of oxidative stress, although increases were independent of $PM_{2.5}$ concentrations. Additionally, circulating antioxidants were not consistently depleted and two redox sensitive inflammation markers were unaltered by smoke exposure. Future lab-based woodsmoke exposure studies should employ longer duration exposure times in order to provide an exposure stimulus more related to firefighting scenarios. Future study should continue to identify oxidative biomarkers and physiologic parameters that are most sensitive to woodsmoke exposure leading up to comprehensive field studies.

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CHAPTER I

Introduction

The dangers of air pollution have been a topic of discussion since the early 19th century (123). The burning of biomass in the US plays a pivotal role in indoor and outdoor woodsmoke particulate matter (PM) and the associated adverse health effects. While air pollution is a worldwide problem, specific regions of the United States experience greater seasonal risks due to acute changes in air quality. During the summer months, the northern regions of the Rocky Mountains experiences sporadic forest fires, which increase wood smoke, derived PM exposure to those in closest proximity to the event (1, 2, 131). Outside of the United States, a substantial number of individuals are similarly exposed to wood smoke (2). In 2006, it was estimated wood combustion accounted for 2.7 % of the global burden of disease (1, 2, 104). Wood smoke during the winter months is most commonly derived from residential home heating methods (wood stoves). In the summer, forest fires are a common source of pollution, which impacts both the ambient and indoor environments. The International Agency for Research on Cancer also reported that indoor exposure to biomass combustion (specifically wood) might also act as a carcinogenic agent in humans (28).

Wood smoke combustion yields a very complex mixture including carbon monoxide (CO), aldehydes, polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs) and particulate matter (PM) (82). Exposure to PM is associated with various pulmonary effects including lung function decline, increases asthma incidence and precipitation of a host of other deleterious respiratory symptoms (2). Among the toxic effects caused by PM inhalation is

oxidative stress (2, 114). Numerous epidemiological studies document the detrimental relationship between exposure and particulate matter (126). An example is a health impact surveillance completed in 1997 during severe forest fires in Southeast Asia. The surveillance provided evidence of a 30% increase in outpatient medical attendance for haze related conditions within the general population of Singapore (35). The general consensus among published research does indicate that the mechanism of air pollution induced health effects involves inflammation and oxidative stress both in lung, vascular, and heart tissue (71).

The acute physiologic outcomes of this exposure include coughs, headaches, and eye and throat irritation in otherwise healthy individuals. Epidemiological studies of women in developing countries show that there are associations between biomass cooking fuel and chronic bronchitis (45). Rothman et al. (1991) assessed 69 Northern California firefighters who were nonsmokers for approximately 6 months (108). There were significant increases in cross-seasonal reports of coughing, phlegm, wheeze, and eye and nasal irritation (108). However, of the limited variables examined, eye irritation was the only deleterious outcome associated with firefighting activity ($r = 0.48$, $p<0.001$) (108). Doclos et al. (1990) examined the effect of wildfire smoke on the general population, in relation to large forest fires on emergency room (ER) visits to 15 hospitals in 6 countries in California (34). The calculated observed-to-expected ratios of ER visit during the 2.5-week period observation revealed that there were increases in ER visits for asthma (+40%, $p<0.001$) and chronic obstructive pulmonary disease (+30%, $p=0.02$) (34). The long term and potentially pathological consequences of exposure to woodsmoke are chronic lung disease (e.g. chronic obstructive pulmonary disease, asthma, lung cancer and/or tuberculosis); increase risk of heart disease and stroke. Cohort studies estimate that the relative risk associated with living in an area of higher PM levels leads long term is between

1.06 and 1.76 per 10μg/m^3 (19). Data released by the American Cancer Society cohort study estimated that for each 10μg/m^3 increase in annual average exposure to $PM_{2.5}$, long-term all-cause cardiopulmonary, and lung cancer mortality were increased by approximately 4%, 6%, and 8 % (5), respectively. Gustavsson et al (2001) found an increased risk of myocardial infarction among approximately 3000 Swedish workers with increases cumulative exposure to products from non-vehicular combustion processes (46). Several physiological measures are indicative of acute exposure to woodsmoke including inflammation and oxidative stress. A repeated measures study on adults living in a densely populated city neighborhood in Boston, Massachusetts reported on indicators of inflammation including C-reactive protein (CRP), fibrinogen, white blood cell and platelet counts as well as urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG), a measure of oxidative stress. Results showed significant effect modifications in the levels for 8-OHdG, CRP, fibrinogen, and WBC count for $PM_{2.5}$ association (67).

Occupational exposures to wood smoke vary with the length of exposure and amount of PM content in the woodsmoke combustion. The adverse health effects among populations who are transiently or chronically exposed at elevated levels to date have not been widely investigated (1, 2). Thus, growing concerns are now focused on individuals who receive significant ongoing woodsmoke exposure, such as those who serve as wildlife support personnel, or wildland firefighters. Wildland firefighters experience greater exposure than the general public due to prolonged and frequency of exposure time (126). The primary responsibilities of a wildland firefighter are to suppress fires and employ government land management policies e.g. prescribed burning (1, 2, 41, 105). Data published by the National Interagency Fire Center (NIFC) in 2010, revealed that annually there are 71,971 fires covering 3.5 million acres of forests, and 16,882 prescribed burns covering almost 2.5 million acres of forests in the U.S.

Despite the vast number of forest fires, the U.S Federal Government only employs approximately 15,000 wild land firefighters annually. Wildland firefighters are also tasked with deliberate application of fires as a land management tool. In both firefighting and controlled burn scenarios, the exposure to ambient woodsmoke for extended durations is significant. The purpose of this process is to improve forage value of the forests and reduce wildfire hazard (1, 2). Annually in the US, prescribed burns are applied by firefighters to millions of acreage (1, 2). Few studies have been performed to investigate the effects of agricultural burning on human health. A Canadian study with 428 middle-aged subjects with slight-to-moderate airway obstruction found that one-hour levels of CO and nitrogen dioxide reached 11 ppm (82). In contrast Reinhardt and Ottmar (2000) assessed the breathing zone levels of acrolein, benzene, CO, formaldehyde, carbon dioxide and $PM_{2.5}$ (common components of woodsmoke combustion) among 21 firefighters in California who were exposed during prescribed burns (105). The data revealed that exposures to gases were below the time-weighted average occupational health standards, however there were high-level peak exposures to heavy smoke (105). The corresponding exposures to CO were 4.0 and 2.8 ppm, with peak exposures of 38.8 ppm and 30.5 ppm (2 hour time weighted average) (105).

The main tasks of a firefighter during prescribed burning are: lighting, holding, and mop-up. The tasks may increase the exposure level of the firefighter because they are in direct contact with the fumes. Wildland firefighters are also at a greater risk of exposure due to the inadequate protection provided by their protective gear. Wearing protective gear is essential to protect firefighters from any threatening risks. In general, the standard protective gear is multilayered, which is considered bulky and heavy designed to provide proper thermal protection (83). Despite protective gear's purpose is to contribute to effective protection against hazardous environment,

studies show that the existence of high cardiorespiratory and thermal strain, which may lend to feelings of discomfort (60). The self-perception of discomfort also contributes to the use of PPE that is not adequate to protect against exposure to particulate matter (60). The respirators that are available do not protect against carbon monoxide (CO) (105). Consequently, firefighters usually rely on cotton bandanas, which are not effective in protecting against PM exposure (1, 2, 82). Reinhardt (2004), noted that the common respirators that are used by the firefighters do not provide protection against CO and if they do they are not practical for most firefighting tasks (105).

Combined with the cumbersome attire adding to the discomfort and exposure levels, firefighters also undertake very physically taxing tasks. The physical activity associated with conducting prescribed burns or fighting fires, increases minute ventilation in proportion to the work performed. Physically taxing activities performed by firefighters include toting a hose, lifting patients, and/or using other auxiliary equipment. While most of the work involved in wildland firefighting involves sustained low to moderate physical activity, sporadic bouts of high intensity activity also occur (88).

The physical activity incurred during firefighting may also lead to oxidative damage. Previous studies report associations between oxidative stress and woodsmoke exposure in conjunction with simulated physical activity (via exercise). Danielsen et al. (2008) investigated the level of systemic oxidative stress with markers of DNA damage; strand breaks and oxidized purines. Levels of human 8-oxoguanine DNA N-glycosylase 1 (hOGG1) were significantly increased 20 hours post woodsmoke exposure as compared with 20-Hr post-clean air exposure ($p<0.05$). Currently, there are gaps in literature regarding the emission, acute and chronic exposures, and health effects. On average firefighting requires low intensity physical activity,

however certain actions may require bouts of moderate to vigorous activity. Specifically, it is important to assess the health of wildland firefighters in controlled laboratory settings, as these populations are typically exposed to elevated woodsmoke over a long period of time.

Independent of PM exposure, participation in acute exercise is documented to result in redox perturbation and transient oxidative stress (44, 52, 103). Quantification of oxidative stress in applied exercise studies usually includes a variety of blood biomarkers of antioxidant status and oxidative damage (87, 91, 94). Recent work by Ballmann et al. (2014), McGinnis et al. (2014), and Peters et al. (2015), indicate that post exercise environment may impact post exercise oxidative stress responses (10, 74, 91). Applying this understanding to firefighting, physically active firefighters may experience a significant oxidative stress. Moreover, engagement in physical activity increases minute ventilation, a fact that would enhance exposure while breathing in a smoky environment. To date the impact of physical activity in the presence of woodsmoke is not well understood and requires further investigation, including well controlled laboratory studies. In order to create a scientifically controlled field based study, the current methodological approach employed an exposure chamber with woodstove to generate woodsmoke and a treadmill for exercise performance. The wood used was cured (15% moisture content) western larch. Woodsmoke was delivered directly from a dilution and mixing chamber to each subject via a modified mask respirator. During the inhalation exposure trials subjects walked on a treadmill at a set rate and incline (3.5 mph and 5.7% grade, <57% of maximum) for 1.5 Hrs, which simulated working on a fire line with short breaks (e.g. 20-30 seconds) every 15 minutes.

Based on this rationale, the purpose of the current investigation was to quantify the blood oxidative stress responses of individuals exposed to an episodic woodsmoke event to simulate a

field experience. A randomized repeated measures study design was employed to expose individuals to clean air (0 μg/m^3), 250 μg/m^3 or 500 μg/m^3 wood smoke $PM_{2.5}$. A panel of oxidative stress biomarkers was used to assess outcomes in blood plasma collected before, immediately post and 1 Hr following after 3 identical exercise bouts. Based on prior findings it was broadly hypothesized that exposure to episodic woodsmoke events during controlled physical activity would result in transient elevation of blood oxidative stress markers in proportion to the particulate load. We hypothesize that for all oxidative damage and antioxidant biomarkers there will be a significant change Pre-Post and a return to baseline values in samples examined 1 Hr post exercise. Specifically, it was hypothesized that for the key dependent oxidative damage variables (Protein Carbonyl PC, Nitrotyrosine 3-NT, Lipid Hydroperoxides LOOH, and 8-isoprostanes 8-ISO) and redox related inflammatory markers Myeloperoxidase (MPO activity and protein) will increase in a dose-dependent manner due to exposures of 250 μg/m^3 or 500 μg/m^3 wood smoke $PM_{2.5}$ compared to that of filtered air. Moreover, it was specifically hypothesized that key dependent antioxidant variables (Ferric reducing ability of plasma FRAP, Trolox equivalent antioxidant capacity TEAC, and Uric Acid UA will be decreased post exercise in a dose–dependent fashion following woodsmoke exposure.

CHAPTER II

Literature Review

Particulate Matter

The dangers of air pollution remain a vigorous topic of discussion since first becoming a topic of controversy in the early 19th century (123). Air pollution is any substance introduced into the atmosphere that can damage living things and the environment. Air pollution significantly increases both morbidity and mortality in the general population (32, 93, 116, 119, 123). There is a rapid growth in the amount of published evidence, a fact that is directly linked to a corresponding increase in health risk (18, 19). In 2004, the American Heart Association (AHA) published its first scientific statement detailing medical concerns regarding air pollution and CVD (19, 89). The comprehensive review concluded that short-term exposure to PM air pollution contributes to acute cardiovascular morbidity and mortality. Long-term exposure is associated with a reduction in life expectancy. PM exposure is also associated with subclinical chronic inflammatory lung injury and atherosclerosis (92). According to the World Health Organization long-term exposure to $PM_{2.5}$ contributes to approximately 800,000 premature deaths annually (19). The size and chemical composition of the particles are determinants of its potential to cause harm.

Air pollution composition and a specific examination of woodsmoke

The harmful substances are defined as ambient particles, which include coarse particles with an aerodynamic diameter (AD) of 2.5 to 10 am, fine particles or smaller than pollen-sized (AD <2.5 am; $PM_{2.5}$) and ultrafine particles (AD <0.1 am; UFPs) (90, 132). **Figure 1,** shows the relative size of airborne particulate matter.

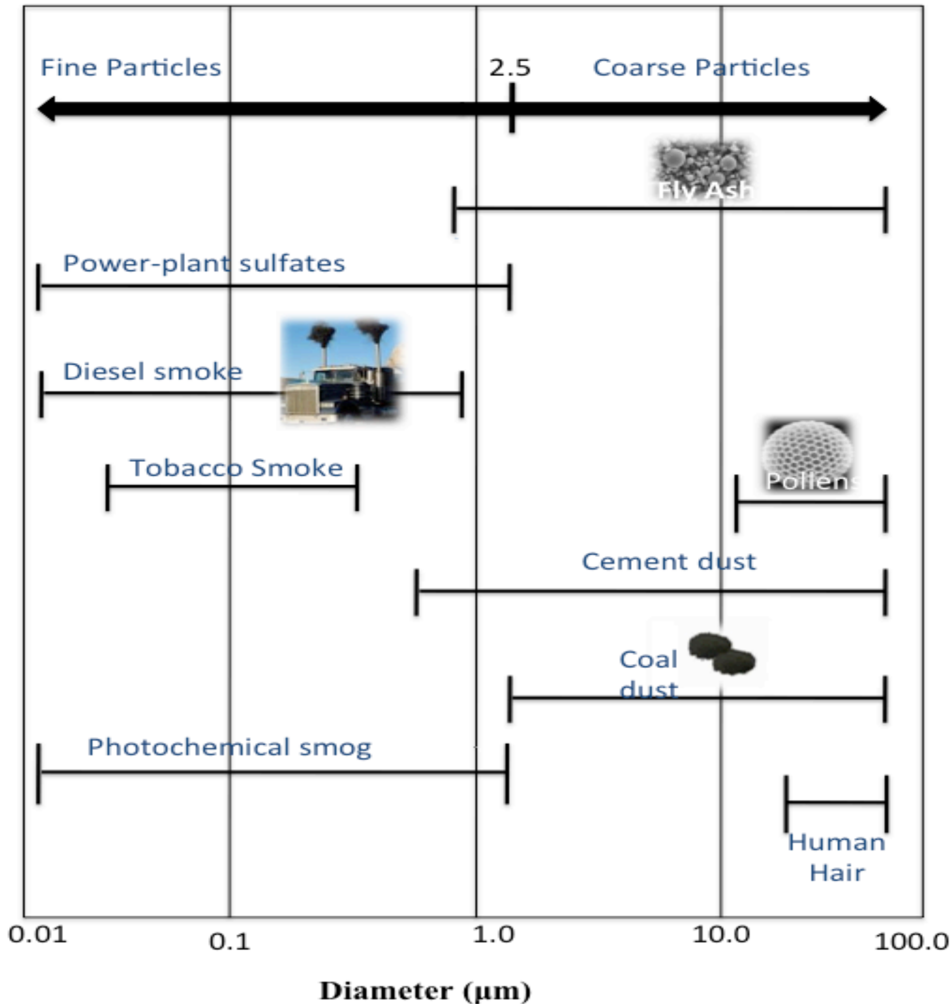

Figure 1. Relative size of particulate matter (PM) and sources of particles. Ambient PM is classified by aerodynamic diameter <10µm (PM10), <2.5µm ($PM_{2.5}$) and <0.1µm (PM0.1). Adapted from Environmental Protection Agency and Heinsohn and Kabel, 1999 (3, 49)).

The smaller the diameter of the particle the more readily they penetrate respiratory passages, and ultimately gain circulatory system access (132-134). The Environmental Protection Agency (EPA) generally compares the relative size of ambient particles to the diameter of a strand of human hair (50-70 µm in diameter) (3, 8). Fine beach sand (90 µm diameter), dust, pollen, and mold are considered PM10 (<10 µm diameter) and of particular interest for our research PM (<2.5 µm in diameter) consists of combustion particles, organic compounds, and metals (3, 8). Airborne particles sometimes travel thousands of miles under favorable weather

conditions of high wind and jet stream currents (84). The chemical composition of the particles depends on their geographical, meteorological, and source-specific variables (123). PM is derived from both human and natural activities (123). PM in the atmosphere and is made up of a complex mixture of both liquids and solids, which include sulfate, nitrate, ammonium and a variety of organic and inorganic compounds (84).

The major sources of PM are power plants, oil refinery, tailpipe and brake emissions from mobile sources, residential fuel combustion, and wildfires (84, 123). The national/international health impact of any pollutant sub-group is difficult to quantify. Nonetheless, the combustion produced by wildfires is estimated to contribute to 2.7 % of the global burden of disease (1, 2). Naeher et al. (2007) (82) notes that biomass in the form of wood and an agricultural waste remains a significant source of energy consumption worldwide, representing approximately 10 % (82). Of that, 90 % is used in its traditional forms as household heating and cooking forms (82). The pollutant composition of woodsmoke observed in occupational exposure studies suggests that the most abundant chemicals include CO, total and respirable particles, silica, aldehydes, benzene, and PAHs (48, 105, 106). A comparison of exposure levels to aldehydes, CO, Carbon dioxide and PM3.5 at the initial attack among fighters in California, Idaho, Montana, and Washington found that there were marked increases associated with longer average work shifts (105). Average time weighted average (TWA) CO exposures at these fires were 2.8 ppm and 4.0 ppm respectively. No CO exposure exceeded the PEL of 50 ppm, some firefighters experienced exposures about the recommended TLV (25 ppm) with maximum exposures of 31 ppm and 39 ppm for their work shift. Findings for average TWA measured 500 $\mu g/m^3$ and 720 $\mu g/m^3$ respectively (105). The study attributed higher exposures to the wind speed and weather conditions during the time of the study (105).

Occupational hazards of woodsmoke

The efforts to reduce woodsmoke exposure largely focus on indoor woodstove interventions designed to provide homes with higher combustion efficiency (1, 2). Perhaps the most common accounts of harmful woodsmoke exposure occur during wildfire season, putting wildland firefighters at significant health risk of pollutant-derived morbidity and mortality. The US Environmental Protection Agency's national ambient air quality standards (NAAQS) are 9 ppm for CO and 35 $\mu g/m^3$ for $PM_{2.5}$ over a 24-Hr period. While, the Occupational Safety and Health Administration (OSHA) uses an overall time weighted average (TWA) and or permissible exposure limits (PEL) to determine exposure over an 8-Hr work period 50 ppm for CO and 15 $\mu g/m^3$ to measure CO and $PM_{2.5}$, respectively (CAFO).

The outlined air quality limits described above are currently exceeded for individuals who are frequently exposed to woodsmoke such as wildland firefighters. Despite recent trends to improve use of respirators and other personal protective equipment, the reality is that these individuals typically conduct their job with little to no protective gear against the hazardous PM (45). Wearing protective gear is essential to protect firefighters from any threatening risks. The protective gear is multilayered, a design that is assumed to provide proper thermal protection (83), however a firefighters' protective clothing should both protect as well as be comfortable to ensure the firefighter's ability to perform optimally (83). However, the current design of the standard protective gear only lowers adherence to PPE use due to the negative perception that they are bulky and heavy (83). Reinhardt 2004, noted that unlike structural firefighters, wildland firefighters do not presently use respiratory protection (105) . Thus, firefighters use alternative methods such as cotton cloth bandanas, which are not effective in protecting against hazardous particles (1, 2, 82). The approximate pore size of a standard cloth bandana used by firefighters in

Yosemite National Park was 100 μm, thus providing no protection against even inhalable particles (PM10) (1).

Firefighters also experience a range of physiological dangers when conducting their daily occupational tasks. Their risks are increased due to their direct contact to smoke and dosage of exposure (82). Epidemiological evidence indicates a significant association exists between elevated PM levels and a number of adverse medical outcomes associated with the wildland firefighting profession (9). Long-term outcomes for life long firefighters include premature mortality, chronic aggravation of respiratory system, lung disease, and cardiovascular disease (IR) (84). Wildfire occurrence is associated with various acute health effects and depending upon the size may affect a substantial number of people exposed to the event. The acute physiologic outcomes of this exposure include coughs, headaches, and eye and throat irritation in otherwise healthy individuals (84). Rothman et al. (1991) assessed 69 Northern California firefighters who were nonsmokers for approximately 6 months (108). There were significant increases in cross-seasonal reports of coughing, phlegm, wheeze, and eye and nasal irritation (108). However, eye irritation was the only activity to be associated with firefighting activity (+ 48 %, $p<0.001$) (108). Additional health concerns for wildlife firefighters such as pro-inflammatory markers. In a study using British Columbia Forest Service, granulocytes and fractions of particulate positive macrophages was increased in the sputum post woodsmoke exposure (126).

Independent of being in close proximity to a fire generating woodsmoke, exposure to more diffuse woodsmoke is detrimental to health. Epidemiological study of women in developing countries indicates an association exists between biomass cooking fuel and chronic bronchitis (45). Similarly, Doclos et al. (1990) examined the effect of wildfire smoke on the general local population. In relation to the occurrences of a large forest fires, emergency room

(ER) visit to 15 nearby hospitals in 6 countries in California were markedly elevated (34). The calculated observed-to-expected ratios of ER visit during the 2.5-week period observation revealed that there were increases in ER visits for asthma and chronic obstructive pulmonary disease by 40 % ($p<0.001$) and 30 % ($p=0.02$), respectively (34). The long term and potentially pathological consequences of exposure to woodsmoke are chronic lung disease (e.g. chronic obstructive pulmonary disease, asthma, lung cancer and/or tuberculosis); increase risk of heart disease and stroke. Related cohort studies provide statistical estimates which indicate that the relative risk associated with living in an area of higher PM levels leads long term is between 1.06 and 1.76 per 10 $\mu g/m^3$ (19). Data released by the American Cancer Society cohort study estimated that for each 10 $\mu g/m^3$ increase in annual average exposure to $PM_{2.5}$, long-term all-cause cardiopulmonary, and lung cancer mortality were increased by approximately 4 %, 6 %, and 8 % (5), respectively. Gustavsson et al. (2001) found that there was an increased risk of myocardial infarction among approximately 3,000 Swedish workers with increases cumulative exposure to products from non-vehicular combustion processes (46).

Both animal and human studies provide well-established associations between cardio-respiratory morbidities and pollutant levels in ambient air with substantial contributions to wood combustion (56, 80, 113, 115). Recent epidemiological analyses indicate that 24-Hr exposure to average levels of ambient particulate air pollution is associated with an increase in all-cause respiratory, and cardiovascular disease mortality (58, 90, 118, 122). Intuitively, a wealth of current research focuses on high respiratory vulnerability (31, 117, 123). In a cross-sectional study the average forced expiratory volume in 1 second, ratio of FEV1 to forced vital capacity, forced expiratory floes, and respiratory volumes of 95 wildland firefighters were lower when compared to 51 police officers working mostly in rural areas within the same area (13). Pro-

inflammatory cytokines, IL-6 and IL-8 were increased post exposure (126). Increases in airway myeloperoxidase (MPO) levels were documented among firefighters serving as part of a hotspot team in National Park Service. Extended periods of exposure may lead to increase levels of inflammatory markers (41). The increased levels of MPO may act as a substantial piece of evidence. Klebanoff (2005) explored the MPO system and its potential involvement in the microbicidal activity of neutrophils and its role in tissue injury (61). The review substantiates the notion that the presence of MPO on the surface of neutrophils may aid in the phagocytosis and destruction of microorganisms, as they are introduced to the cellular environment, hence the increased in levels of MPO following a longer stint of exposure to woodsmoke (61). Further evidence for immune system involvement following woodsmoke exposure, one recent study examined 13 subjects exposed to either experimental wood smoke (240-290 $mg \cdot m^{-3}$) or clean air conditions in an environmental chamber in two 4-Hr sessions one week apart. Findings reveal that serum IL-6 was decreased post exposure; however other markers of inflammation were increased such as serum amyloid A (12). To date, the hazards of woodsmoke exposure are well documented. The idea focuses on the ability of the respirable particles to penetrate the system via blood, which generates ROS and other oxidants leading to the pathogenic effects of woodsmoke (130). Although there is no comprehensive study that provides a direct link between woodsmoke derived PM and lung dysfunction current evidence points to the respiratory system as the initial point of contact, prompting a cascade of systemic events; triggering an inflammatory immune response due to oxidative stress.

Oxidative Stress

Reactive oxygen species (ROS) are oxygen-based free radicals that are highly reactive and contain an unpaired electron in their outer orbital (2, 100). The free radical molecules act as

oxidizing agents in oxidation-reduction reactions (redox) within a biological system (96, 100). Oxidative stress is a scientific term used to describe either ROS generated damage to cellular constituents or an overproduction of oxidants (100). Oxidative stress is defined as an imbalance between the systemic manifestation of ROS and a biological ability to readily detoxify the reactive intermediates and/or repair the resulting damage. Overproduction of oxidants is a relative phenomenon compared to radical quenching capacity of available antioxidants. A more modern understanding of oxidative stress describes it as a pathological imbalance, which results in deleterious outcomes in cellular redox signaling and control systems (57, 100). Thus, oxidative stress is also an essential component to normal cellular signaling and health as well as contributors to disease pathologies (100). While the production of ROS is a necessary cellular process, over production of ROS overwhelm the biological system at the tissue level causing permanent damage (38). A number of physiologic stressors result in the generation of ROS such as exposure to environmental pollutants or physical exercise as described below (38).

Physiological measures that are indicative of acute exposure to woodsmoke include inflammation and oxidative stress. Case in point, a recent repeated measures study conducted in adults living in a densely populated city neighborhood in Boston, Massachusetts measured indicators of inflammation including C-reactive protein, fibrinogen, white blood cell and platelet counts as well as urinary 8-OHdG, a measure of oxidative stress. Results indicate modifications in the levels for 8-OHdG, CRP, fibrinogen, and WBC count were elevated in association with $PM_{2.5}$ exposure (67). The mechanism of toxicity by PM is hypothesized to involve the generation of ROS, oxidative stress, and inflammation (69).

Independent of inflammatory-derived ROS, the combustion produced by woodsmoke contains free radical compounds capable of generating hydroxyl radicals. In-vitro study results

provide evidence that exposure to woodsmoke PM induces oxidative stress, which results in lipid peroxidation and DNA damage in exposed humans (1). Collectively, the few available human-based studies provide consistent understanding that woodsmoke exposure leads to increased oxidative stress, inflammation, and pneumotoxicity (25, 37, 39, 99, 123).

Woodsmoke linked inflammation and oxidative stress

Experimental studies implicate airborne PM (e.g. tobacco smoke) as a direct trigger to activate the mitogen-activated protein kinase (MAPK), and transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) (130) pathways, which are involved in the process of inflammation, apoptosis, proliferation, transformation and cellular differentiation (42, 130). In vitro studies of cultured cells provide further evidence that woodsmoke PM increases expression and production of pro-inflammatory cytokines damaged DNA and oxidative stress. A recent controlled exposure study of woodsmoke in healthy humans investigated outcomes related to oxidative stress and inflammation in exhaled breath condensate from human participants. Differing dosages of woodsmoke derived PM (14, 220, or 354 $\mu g/m^3$) were compared to clean air. There were no differences attributed to PM exposure on the resulting mRNA levels of Interleukin-6 (IL6) ($p=0.12$), Interleukin-8 (IL-8), ($p=0.37$), Tumor necrosis factor alpha (TNF-α) ($p=0.38$), Chemokine ligand 2 (CCL2) ($p=0.96$), Intergrin alpha L chain (ITGAL) ($p=0.69$), Oxoguanine glycosylase (OGG1) ($p=0.58$) or heme oxygenase 1 (HMOX1) ($p=0.61$). Whether corresponding protein concentrations for the aforementioned transcripts and oxidative damage markers were elevated in these samples is unknown and does not appear to have been included in the experimental design (40). Despite a paucity of evidence, some published findings support the notion that woodsmoke increases expression of genes involved in cytokine production. *In vitro* particulates increased the

adherence of THP-1 monocytes to human umbilical vein endothelial cells, potentially due to the increased expression of IL-8 and TNF-α (39).

Synergistic mechanisms of inhalable particulate matter and other components of air pollution (e.g. ozone, NO, soot, heavy metals, and PAHs) provide evidence that porous surfaces of the particles provide a desirable environment for catalyzing the increased generation of ROS (88, 109, 129). The indirect mechanisms of ROS production by woodsmoke are generated by target cells such as lung epithelial cells and pulmonary macrophages upon contact with particulate material (130). Alveolar macrophages and polymorphonuclear neutrophils are phagocytic cells, which are innate to the immune system (33, 72, 130, 135). These cells are endogenous producers of ROS, which enhance microbicidal conditions to eliminate harmful particles (33, 130, 135). Macrophages that also reside within the airways and alveolar spaces release ROS/RNS after the particles are inhaled and the phagocytic process begins (33, 72, 130, 135). The macrophages then release TNF-α, which subsequently generates intracellular ROS (130). Barregard (2006) (12) reported no relationship between subjective symptoms (participants reported mild eye irritation) and woodsmoke exposure. Serum amyloid A levels were increased after woodsmoke exposure compared to that of clean air exposure. C-reactive serum (CRP) levels were elevated by approximately 10% after 3 Hrs of woodsmoke exposure, a finding that did not achieve statistical significance compared to clean air ($p=0.06$). Similarly, numerical increases in urinary 8-iso-prostaglandin$F_{2\alpha}$ did not reach statistical significance as compared to the clean air trial (12). Similar results for inflammatory biomarkers were noted by a Hejl et al. (2013) (50) field that investigated 12 firefighters to investigate the effect of woodsmoke exposure on inflammatory biomarkers. Pre work shift samples were collected at 7:20 AM and 9:47 AM; corresponding post work shift samples were collected 3:05 PM and 7:46 PM. Blood

samples were collected on prescribed burn days which occurred after a 3-to-9 day break between burn days. Study findings were highlighted by a >50 % increases in IL-8, CRP, and ICAM-1 (cellular adhesions molecules involved in recruiting immune cells to the site of an insult) concentrations from pre to post work shift (50). Collectively, these findings indicate that the transient change in combination caused equivocal results.

Given that oxidative stress is dependent upon both an increase in ROS and a corresponding depletion in radical quenching antioxidants, woodsmoke exposure may hold implications for tissue antioxidant concentrations. In support, chronic antioxidant defense system depletion is associated with severe metabolic dysfunction and other deleterious outcomes (130). Chronic antioxidant depletion dysfunction is linked to biological mutations resulting in carcinogenesis. A hallmark of cancer and oxidative stress lies within the chronic inflammation, which is associated with angiogenesis and other disease conditions for which oxidative stress is a contributing factor (129). For instance, the inflammation of a tumor environment is characterized by leukocyte infiltration, neutrophils, macrophages, which produce a host of cytotoxic mediators such as ROS., TNF-α, interleukins, COX-2 (129). Thus, ambient PM generated from combustion of woodsmoke may deplete the endogenous antioxidant capacity, a fact that could have implications for a host of disease conditions in those receiving chronic exposure to air pollution.

Acute exercise and oxidative stress

Acute exercise is one of the most reproducible stimuli for the induction of oxidative stress as quantified in the blood (85, 127). Many prior studies report elevations in blood oxidative stress biomarkers, as an indicator that oxidative is not limited to the cellular compartment. Participation in acute bouts of exercise manifests redox perturbations and transient oxidative stress (44, 52, 102, 103). Paradoxically, the generation of ROS resulting from

exercise also appears to promote the up-regulation of antioxidant defense mechanisms, which subsequently provides protection against intense physical stress (14, 95, 100). Several theories exist to explain sources of oxidative stress via exercise. These theories may apply to various tissues, organs, and cellular location under certain physiological conditions. The mitochondrial theory is based on the premise that exercise increases mitochondrial ROS production due to the elevation oxygen consumption is increased during strenuous exercise. That is, during exercise the respiring mitochondria are believed to release superoxide as a stoichiometric function of total oxygen flux (52, 103). During maximal workloads, the maximal oxygen consumption (VO_2) can increase approximately 20-fold, while muscle fiber levels increase 100-fold (55). Heavy exercise may induce mitochondrial uncoupling as a result of inner membrane damage and hyperthermia (29, 55, 112). Several studies indicate that both muscle and heart mitochondria from animals involved in high intensity chronic exercise demonstrate compromised coupling and disturbance of glutathione (GSH) redox status (55). The mitochondrial theory of ROS production is also supported by the adaptation of mitochondrial antioxidant enzymes (55). In support, observed levels of mitochondrial glutathione peroxidase-1 (GPX) content in rat skeletal muscle are elevated as compared to cytosolic GPX (54, 55). Given that the transcripts for these enzymes are redox sensitive; these data provide strong evidence that mitochondria are a major source of ROS during exercise. In contrast, a study investigating the impact on work intensity on the oxidative stress response to acute resistance training found that both strength and hypertrophy protocols elicited significant elevations in plasma protein carbonyl levels. The finding suggests that there was an undefined threshold for oxidative damage (53, 54). Thus, while mitochondria are clearly a source of ROS during exercise, alternative sources must also be at work.

An alternative source of oxidative stress production has been attributed to the xanthine oxidase pathway. Xanthine oxidase catalyzed reactions is a known source of free radical production in the ischemia and reperfused model (55). Xanthine oxidase and NADPH/NADH oxidases are documented as potent cytosolic ROS producing enzymes. The enzymatic production of ROS is exacerbated by cytosolic calcium elevation, which is an indicator of oxidative stress (100). Alternately, ROS production is also observed in proportion to bioenergetic fatigue. During bioenergetic challenges, ATP is degraded to ADP and AMP. The degraded hypoxanthine is converted to xanthine and uric acid by xanthine oxidase coupled with the electron reduction of O^{2-} (55). Additional sources of ROS include the autoxidation of catecholamines, phagocytic burst by white blood cells, and reactions with disrupted transition metals (e.g. iron and copper) (100). Evidence exists stating that high-intensity exercise mimics the situation of heart IR and may activate the XO pathway (55, 124).

High-intensity workloads may also result in the translocation of neutrophil to the active skeletal muscle, subsequently causing oxidative stress (5, 6, 51, 53, 70, 103, 121). The neutrophil theory is based on the notion that polymorphonuclear neutrophils (PMN) play a critical role in defending from invasion of foreign matter. Blood levels of neutrophil oxidative enzymes such as myeloperoxidase and free radical production may be elevated immediately post exercise (20, 47, 51, 121). Quindry et al., (2003) found neutrophil levels to be highest immediately post maximal treadmill exercise (103). The findings suggest that exercise-induced neutrophilia contributes to oxidative stress (103). Exercise-induced oxidative is a multidimensional paradigm that is mediated by several factors including but not limited to the duration, mode, intensity of exercise, as well as environmental aspects such as altitude and air pollution.

Evidence collected over the last 30 years indicates that the magnitude of oxidative stress during and following exercise is dependent on both exercise intensity and duration. That is, higher intensity and long duration fatiguing exercise bouts are most routinely associated with dramatic increases in biomarkers for oxidative stress (102). During prolonged duration exercise, energy is supplied by the oxidative phosphorylation of ADP via the respiratory chain, providing a steady source of hydrogen peroxide (H_2O_2) via xanthine oxidase which catalyzes two steps in the resulting purine metabolism (55). Alessio and Golfarb (1988) were among the first to provide convincing evidence that exercise duration impacts oxidative stress. They specifically observed that lipid peroxidation measured via Thiobarbituric acid reactive substances (TBARS) were increased in association with extended duration treadmill workloads in rats (4). Several studies support the rationale that exercise intensity generates differing responses (52, 103) in a time dependent fashion. Quindry et al. (2003) found that aerobic and combined aerobic/resistance induces greater oxidative stress (102). Hudson et al. (2008) observed that exercise and oxidative stress is elicited through resistance training and high intensity workload such that the peak rise in oxidative damage markers during the exercise recovery is dependent upon the total work performed (52).

The cellular ROS damage incurred during exercise is often due to modifications to various macromolecule (e.g., proteins, lipids, and nucleic acids) (14). Oxidative damage involves oxidation of amino acid side chains and fragmentation of polypeptides (14). The oxidation of protein is most commonly represented by the formation of carbonyl derivative (14, 68). Lipids experience degradation of polysaturated fatty acids and phospholipids known as lipid peroxidation (14). The quantification of oxidative stress in applied exercise studies typically involves a variety of blood oxidative biomarkers of antioxidant capacity and oxidative damage

(87). Due to the transient time course of oxidative stress markers in response to exercise, many of the discernable observations occur during recovery period (91).

Biomarkers examined within the blood plasma reveal that biomarkers for oxidative modification to lipids and proteins increase following strenuous exercise while antioxidant levels sometimes decline in concentration (102). The increase in circulating oxidative damage markers and decline in antioxidant defenses is typically more discernable immediately following exercise (38). Human studies examining acute exercise and oxidative stress revealed that oxidative stress measures, more specifically TBARS, were increased following both maximal and submaximal and returned to baseline within one Hr of recovery (38). Moreover, Sahlin et al., (1992) investigated the influence of anaerobic exercise on markers of oxidative stress in humans, and found that isometric knee extension at 30% maximal voluntary contraction (MVC) did not influence changes in the lipid oxidative damage marker MDA and the water soluble antioxidant oxidized glutathione (at 20, 40, 60,80 min). However an increased in total glutathione (TGSH) has been observed following acute exercise (14, 110). In another notable example of exercise induced oxidative stress, a 1-Hr isometric handgrip exercise performed at 50% MVC for 45 seconds on and 45 seconds off the contraction phase for 15 minutes (time matched to the subjects' treadmill run time) Alessio et al. (2000) reported increases in LOOH immediately post exercise (5). However, the PC only increased 12 % suggesting that isometric handgrip exercise may increase lipid peroxidation, but only slightly alter protein oxidation (4, 14). The collective results indicate that isometric exercise may contribute to oxidative stress production in high intensity exercise. Eccentric exercise has also been widely examined to investigate oxidative stress. Eccentric muscles actions may produce increase ROS through a variety of biochemical pathways including the inflammatory pathway (14). A study examining an increase in plasma

TGSH through 120 Hrs following a bout of 50 maximal eccentric muscle actions with the elbow flexors, found an increase in TGSH (65). Bloomer et al demonstrated an increase in plasma PC and a non-significant decline in GSH in the days following a bout of 60 elbow flexors (66). An alternative study examining the effects of 70 eccentric actions with knee extensors found that there were no changes in MDA in blood or muscle. However, the findings did demonstrate an increase in two markers of lipid peroxidation, LOOH and 8-ISO during the 4 days following 30 eccentric actions with elbow flexors, which suggest that eccentric exercise, can increase lipid peroxidation during the recovery period (14, 26).

In total, oxidative stress changes can be quantified by a host of oxidative damage markers (lipid, protein, and DNA) in addition to antioxidant markers. The signal changes to exercise (increases versus decreases) are often predictable in an intensity/duration dependent fashion, but time course for these markers may be variable. As such, most oxidative stress researchers choose to examine a panel of biomarkers to better understand the comprehensive nature of redox alterations following acute exercise or other physiologic stressors.

Environmental considerations and exercise induced oxidative stress

Recent work by Ballmann et al. (2014), McGinnis et al. (2014), and Peters et al. (2015), indicate that post exercise environment impacts post exercise oxidative stress responses (10, 74, 91). McAnulty et al. (2005) found that treadmill running elevated core temperatures tremendously (39.5°C) as well as increased plasma F_2-isoprostane level (73). However, exercise in colder temperatures did not elicit a corresponding counter effect (73, 99). Quindry et al. (2012) examined the blood oxidative response post exercise in differing temperatures (99). The analysis of data revealed that biomarkers for antioxidant variables were significantly different between warm and cold trials at both 1 Hr and 3 Hr time points for both FRAP and TEAC. More

specifically FRAP revealed significant elevations during warm trial at POST and 1 Hr time points ($p<0.001$). LOOH and PC measures were used to assess oxidative damage. The results showed a significant time ($p<0.001$) and trial-dependent ($p=0.011$) elevation for LOOH. Post hoc analysis revealed that there were significant elevations only at 1 Hr time point in warm temperature. Post hoc analysis of PC revealed that elevations at 1 Hr ($p=0.039$) and 3 Hr ($p=0.041$) time points. The collective results of the study revealed that blood oxidative responses positively associates oxidative damage increases to environmental temperature changes rather than energy expenditure (99).

The altitude during exercise and exercise recovery appears to impact oxidative stress responses. A recent study by Miller et al. (2013) demonstrated that strenuous multi-day high altitude trekking lead to prolonged oxidative stress response (76). Ballmann et al. (2014), found that altitude induced hypoxia alters exercise adaptions to oxidative stress and metabolism (10). A later study provided more evidentiary support of altitude effects on oxidative stress in a follow-up study that examined altitude threshold at which oxidative stress is blunted. The results showed that there was an time-dependent oxidative stress response for both antioxidant (FRAP, TEAC, UA) measures as well as oxidative stress damage markers (PC and LOOH) at the highest altitudes (3333 m ,5000 m) (91).

In addition to altitude having an effect on oxidative stress, there is reason to believe that exercise may further exacerbate the negative effects caused by exposure to ambient particles. Reinhardt and Ottmar (2000) study looked overall occupational hazards of woodsmoke amongst firefighters (105). The group measured CO, CO_2, benzene, formaldehyde, acrolein, and respirable particulate matter (PM3.5) (105). The key findings demonstrated that work task at the fire sites were contributors to increased exposure levels. Mop-up exposure (the action

smoldering ashes), were lower than direct contact or clearing on the downwind edge of the fire (105). Environmental conditions including air pollution during exercise and physical activity together exacerbates the amount of blood oxidative stress, notably these changes may collectively lead to long-term adverse health outcomes.

Literature review summary relative to the proposed study

Air pollution is a significant problem resulting in acute aggravation of pulmonary tissues. Inflammation and subsequent oxidative stress responses to air pollution exposure are noted. There is reason to believe that the physical activity associated with firefighting in wildland areas may exacerbate air pollution-derived stress responses because of increased pollutant exposure due to elevated ventilator demands. While exercise evokes an oxidative stress response independent of air pollution, the modest physical workloads proposed currently are not likely to elicit dramatic alterations in oxidative damage markers. Nonetheless, physical activity induced oxidative stress is likely to be associated with light physical activity during controlled smoke inhalation. While not within the scope of the current study, repeated exposure to such environmentally induced oxidative stress responses may have important health implications for wildland firefighters. According to this rationale, we undertook the following examination of exercise induced oxidative stress with and without exposure to two doses of woodsmoke. A comprehensive panel of oxidative damage markers and antioxidants was measured in plasma drawn before and after each exercise session in order to quantify the magnitude of the oxidative stress experienced during the 3 conditions.

CHAPTER III

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Graded hypoxia and blood oxidative stress during exercise recovery

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Abstract

10 Altitude exposure and exercise elicit oxidative stress in blood; however, exercise recovery at 5000 m attenuates oxidative stress. The purpose was to determine the altitude threshold at which blood oxidative stress is blunted during exercise recovery. Twelve males 18–28 years performed four-cycle ergometry bouts (60 min, 70% VO_{2max}, at 975 m). In a randomised counterbalanced crossover design, participants recovered 6 h at 0, 1667, 3333 and 5000 m in a normobaric hypoxia chamber (recovery altitudes were simulated by using a computerised system in an environmental chamber by 15 lowering the partial pressure of oxygen to match that of the respective altitude). Oxygen saturation was monitored throughout exercise recovery. Blood samples obtained pre-, post-, 1 h post- and 5 h post-exercise were assayed for ferric-reducing antioxidant plasma, Trolox equivalent antioxidant capacity, uric acid, lipid hydroperoxides and protein carbonyls. Muscle biopsies obtained pre and 6 h were analysed by real-time polymerase chain reaction to quantify expression of hemeoxgenase 1, superoxide dismutase 2 and nuclear factor (euthyroid-derived 2)-like factor. Pulse oximetry data were 20 similar during exercise, but decreased for the three highest recovery elevations (0 m = 0%, 1667 m = −3%; 3333 m = −7%; 5000 m = −17%). A time-dependent oxidative stress occurred following exercise for all variables, but the two highest recovery altitudes partially attenuated the lipid hydroperoxide response (0 m = +135%, 1667 m = +251%, 3333 m = +99%; 5000 m = +108%). Data may indicate an altitude threshold between 1667 and 3333 m, above which the oxidative stress response is blunted during exercise recovery.

25 **Keywords:** *altitude, reactive oxygen species, exercise, oxidative stress*

Introduction

Participation in acute exercise results in redox perturbations and transient oxidative stress (Gomez-Cabrera, Domenech, & Vina, 2008; Hudson et al., 2008; Quindry, Stone, King, & Broeder, 2003). While historically counter-intuitive, oxidative stress due to exercise is now recognised as a stimulus for exercise-induced adaptations (Ristow & Schmeisser, 2011; Ristow & Zarse, 2010). Scientific quantification of oxidative stress in applied exercise studies typically includes various blood biomarkers of antioxidant status and oxidative damage (Pacifici & Davies, 1991; Powers & Jackson, 2008). Given the transient time course for observing oxidative stress responses to exercise, many of these observations occur in recovery from exercise. Adding to the dynamics of redox changes to acute exercise is the compartmental exchange from muscle origin to outcomes in blood (Little, Safdar, Benton, & Wright, 2011; Nikolaidis & Jamurtas, 2009; Nikolaidis et al., 2013; Powers & Jackson, 2008; Powers, Smuder, Kavazis, & Hudson, 2010). Prior evidence clearly indicates that the magnitude of oxidative stress is often proportional to exercise intensity or duration (Alessio, Goldfarb, & Cutler, 1988; Quindry et al., 2003). Recent work by Ballmann et al. (2014) and McGinnis et al. (2014) indicates that the post-exercise environment during recovery also impacts post-exercise oxidative stress responses. Studies from multiple labs reveal that environmental factors, including hypoxia, influence exercise-induced oxidative stress responses (Ballmann et al., 2014; Dosek, Ohno, Acs, Taylor, & Radak, 2007; Miller et al., 2012; Quindry et al., 2013).

In order to provide better scientific control to previously field-based exercise and hypoxia studies,

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in the current methodological approach altitude chambers were used to simulate hypoxia in normobaric environments (Miller et al., 2013; Radak et al., 1997; Sinha, Dutta, Singh, & Ray, 2010; Taylor et al., 2011). Findings from these previous studies indicate that altitude-induced hypoxia during exercise is a direct mediator of oxidative stress (McGinnis et al., 2014; Miller et al., 2012). In a recent and related study, findings demonstrated that exercise performed at 975 m followed by hypoxic recovery at 5000 m attenuated the post-exercise blood oxidative stress responses and blunted post-exercise adaptations in redox-sensitive transcripts in skeletal muscle (Ballmann et al., 2014). Based on this collective understanding, it appears that while oxidative stress is dependent upon the work performed during the exercise bout, recovery environment exerts an independent influence. There is a rationale to suspect that the recovery environment, if experienced at high altitude, may mitigate redox-sensitive exercise adaptations. In application, this understanding may hold implications for recreational hikers or possibly warfighters and others for whom exercise and recovery occur at elevation. Currently, it is unknown what recovery elevation threshold elicits a blunting in the post-exercise oxidative stress response.

Based on this rationale, the purpose of the current investigation was to quantify the blood oxidative stress to normoxic exercise followed by recovery at various post-exercise elevations. A randomised counterbalanced crossover repeated measures study design was employed to examine a panel of oxidative stress biomarkers before and after four identical exercise bouts and the respective recovery environments at simulated altitudes. In addition, muscle biopsies were obtained and redox-sensitive transcript values were quantified from these tissues to give insight into the post-exercise adaptive stimulus. Based on prior findings, it was hypothesised that oxidative stress responses would be attenuated in a threshold-dependent fashion during hypoxic exercise recovery as compared to normoxic exercise recovery.

Materials and methods

Participants

Physically active males (n = 12) between 18 and 28 years of age (48.4 ± 13.1 VO_{2max}; 24.1 ± 3.7; height 185.0 ± 3.5 cm; body mass 84.4 ± 3.8 kg) were recruited from the University of Montana community to take part in the current study. The University of Montana's Institutional Review Board approved the study in accordance with Declaration of Helsinki. Each participant also completed a physical activity readiness questionnaire to determine their physical activity readiness.

Baseline testing

Per cent body fat was determined using hydrodensitometry. Underwater weights were obtained using a digital scale (Exertech, Dresbach, MN). Participants repeated trails until three hydrostatic weight values within 100 g were obtained. Underwater weights were corrected for estimates of residual lung volume (residual lung volume = (0.0115 * age) + (0.019 * height) − 2.24). The relationship between hydrostatic weight and dry land weight was used to calculate body volume and converted to the per cent fat using the Siri equation (BF = (4.95/ρ – 4.50) * 100) (Kravitz & Vivian).

Participants completed a peak maximal aerobic power test on an electronically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA) at the laboratory altitude (975 m) to quantify peak aerobic fitness. The initial workload of 95 W was increased incrementally every 3 min (35 W/stage) until participants achieved volitional fatigue. Gas expiration was collected during exercise and analysed in 15 s intervals using a gas analyser (ParvoMedics, Inc., East Sandy, UT). Subsequent steady-state workloads were determined by the power output associated with VO_{2peak} values (W_{max}).

Steady-state exercise trials

Participants were instructed to abstain from physical exercise 24 h before each trial commenced. Additionally, participants reported to the lab having completed a 12 h overnight fast, where they were instructed to abstain from any alcohol (caffeine was allowed but not on the morning of the trial). Participants were instructed to hydrate *ad libitum* and to be consistently hydrated for all study trials. To ensure compliance, participants completed a 2-day exercise log and a 1-day dietary record, which were replicated prior to all steady-state exercise sessions. For each exercise trial, participants completed four 1 h steady-state exercise sessions at a work rate equivalent to 70% VO_{2max} on a cycle ergometer (Velotron, RacerMate Inc., Seattle, WA). Upon cessation of each exercise bout, participants recovered for 6 h at a randomised simulated altitude chamber of 0, 1667, 3333 or 5000 m (recovery altitudes were simulated by using a computerised system in an environmental chamber by altering the partial pressure of oxygen to match that of the respective altitude). Participants remained in the altitude chamber for the entire duration of the observed recovery period. Independent of hypoxia, the environmental chamber was set at 23°C and 40% relative humidity

for all recovery periods (Tescor, Inc., Warminster, PA). Oxygen saturations were monitored via pulse oximetry (Nonin Onyx Finger Pulse Oximeter, Nonin Medical Inc., Plymouth, USA) by spot check measurements throughout the exercise trial and during the 6 h recovery. The measurements were taken at baseline; 45 min post-exercise, and every hour during the 6 h recovery period. Participants consumed 600 ml of water during the 1 h of exercise and 600 ml during the 5 h post-exercise recovery. The participants were also allowed to consume a Clif Bar. Clif Bar choices were either white chocolate macadamia (the white chocolate macadamia nut bar contained 260 calories per bar and consists of 100% vitamin E, 30% vitamin A, 11% total fat, 16% dietary fibre, 90% vitamin A, 14% total carbohydrate and 18% protein) or chocolate chip (the chocolate chip bar contained 240 calories per bar and consists of 100% vitamin E, 30% vitamin A, 8% total fat, 30%, 15% total carbohydrate and 18% protein) at 0 h of recovery. Participants ate the same flavour bar for all four trials. The study design is illustrated in Figure 1.

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Blood samples

Blood samples were collected pre-, post-, 1 h, 5 h post-exercise from the antecubital vein with sodium heparinised vacutainers (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1000 × *g* for 15 min at 4°C. Plasma was aliquoted and stored immediately at −80°C until subsequent biochemical analysis of oxidative damage and antioxidant biomarkers. Individual aliquots were assayed within a few months of collection and were subject to a single freeze-thaw. In an effort to preserve sample viability upon thawing, plasma aliquots were kept on ice and in the dark to prevent redox alterations.

Muscle biopsies and tissue storage

Genes of interest were measured using quantitative real-time polymerase chain reaction measured pre-exercise and at the 6 h recovery time point. A total of 8 (two samples per trial × four trials) skeletal muscle biopsies (four from each leg) were obtained across the four trials by trained researchers working under

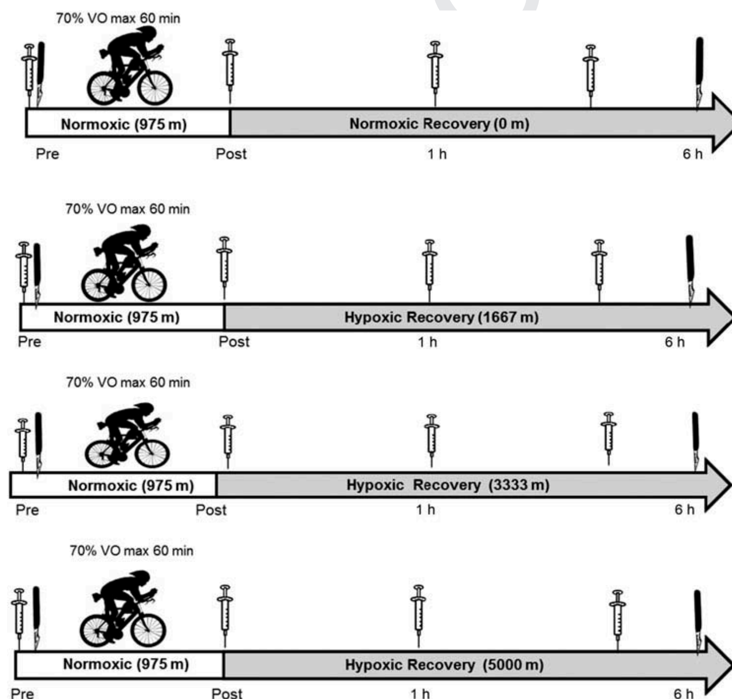

Figure 1. Study design. Participants performed in identical 60 min interval cycle ergometer exercise session at normoxic conditions (975 m altitude) indicated by the *open arrow*. In a randomised counterbalanced crossover design, participants recovered for 5 h at 0, 1667, 3333 and 5000 m (normobaric hypoxia chamber) indicated by a *shaded arrow*. Blood samples were obtained pre-, post-, 1 h post- and 5 h post-exercise. Muscle biopsies were obtained from the vastus lateralis at pre- and 6 h time points.

the supervision of a study physician as approved by the University of Montana's Institutional Review Board. Samples were obtained under a common recovery conditions in terms of fluid and food intake. Based on these study controls leading up and during the trials, the lone change variable was the ambient exercise recovery condition in the environmental chamber. Muscle biopsy samples were taken from the vastus lateralis muscle using a percutaneous needle pre- and 6 h post for each exercise trial. The area was treated with local anaesthesia (1% lidocaine) through subcutaneous and intramuscular injections prior to incision. Following anaesthesia, a small incision (approximately 0.25 in.) was performed and 50–100 mg of tissue was obtained. Incisions were closed with a single suture, supported with Steri-Strip, and covered with sterile adhesive bandage. Muscle tissue samples were immersed in ribonucleic acid later stabilisation solution (Life Technologies, Grand Island, NY) and stored at −80°C until further analysis.

Biochemical assays for oxidative stress

A biochemical assay panel was performed to quantify blood oxidative stress during each exercise recovery trial. To measure total and non-enzymatic antioxidant capacity, ferric-reducing ability of plasma and Trolox equivalent antioxidant capacity assays were performed. The ferric-reducing ability of plasma assay utilises a colorimetric reaction of ferric to ferrous tripyridyltriazine reduction by plasma antioxidants at an acidic pH. The reduction of tripyridyltriazine is proportional to blood plasma antioxidant capacity and was quantified by absorbance spectroscopy at 593 nm (Benzie & Strain, 1996). The Trolox equivalent antioxidant capacity assay measures present antioxidants scavenging of 2,2′ azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) radical anions, thus quenching a quantifiable colorimetric reaction. Calculated Trolox equivalent antioxidant capacity values for each sample were based on standard reactions with calculated values compared to the water-soluble vitamin E analogue Trolox (Erel, 2004). The uric acid assay was used to examine the catalytic activity of peroxidase the generated H_2O_2. Measurements of H_2O_2 were determined by peroxidase catalysed oxidation of chromogenic and fluorigenic substrates or by catalyse-mediated conversion of alcohols to aldehydes, which were measured spectrophotometrically using a reaction mixture containing 3-methyl-benzothiazoline-2-one hydrazone and 3-dimethylaminobenzoic acid. Final plasma uric acid values were determined by comparison with internal standard responses (Kovar, El Bolkiny, Rink, & Hamid, 1990).

To quantify the oxidative damage in blood plasma, protein carbonyls and lipid hydroperoxides were measured. For protein carbonyls, plasma sample protein concentrations were analysed via absorbance spectroscopy according to the methods of Bradford (1976). Plasma samples were diluted to 4 mg · ml^{-1} accordingly and protein carbonyls values determined by a commercially available ELISA (Biocell Corporation Ltd., Papatoetoe, New Zealand) according to the manufacturer's directions (Buss, Chan, Sluis, Domigan, & Winterbourn, 1997). To quantify plasma lipid hydroperoxides, the ferrous oxidation-xylenol orange assay was implemented where oxidised ferrous ions react with the ferrous-sensitive dye contained in xylenol orange forming a complex that is quantified through absorbance spectroscopy at a wavelength of 595 nm (Nourooz-Zadeh, 1999). Calculated adjustments for post-exercise plasma volume shifts were performed for all plasma variables according to established methods (Dill & Costill, 1974).

Transcript analysis from skeletal muscle

In total, 8–20 mg portions of the vastus lateralis skeletal muscle, obtained from post-trial skeletal muscle biopsies, were homogenised in Trizol (Invitrogen, Carlsbad, CA, Cat#15596-018). Samples were homogenised (Tissue Tearor, Biosped Products Inc., Bartlesville, OK) and messenger ribonucleic acid was purified using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol using the additional deoxyribonuclease digestion step (ribonuclease-free deoxyribonuclease set, Qiagen, Valencia, CA). Ribonucleic acid was quantified using a nano-spectrophotometer (nano-drop 2000C, Wilmington, DE). Average ribonucleic acid yield were 274 ± 108 ng · µl^{-1} and the average absorbance ratio at 260:280 was 1.9 ± 0.10, which indicated high-purity ribonucleic acid. The integrity of ribonucleic acid was measured using an Agilent 2100 bioanalyzer using ribonucleic acid nano chips (Agilent Technologies Inc., Santa Clara, CA). The result for the average integrity number was 7.8 ± 0.56, which indicated intact ribonucleic acid. First-strand complementary deoxyribonucleic acid synthesis was achieved using Superscript III-first-strand synthesis system for real-time polymerase chain reaction kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Real-time polymerase chain reaction was performed using 500 nM primers (RPS18:TCC ATC CTT TAC ATC CTT CTG TC; superoxide dismutase 2: CGT CAG CTT CTC CTT AAA CTT g; Hemeoxgenase 1: TCC TTG TTG CGC TCA ATC TC; nuclear factor (euthyroid-derived 2)-like factor: GCA GTC ATC AAA GTA CAA AGC A), 250 nM probe (PrimeTimeqPCR assay, Integrated DNA Technologies), Brillant III Ultra-Fast quantitative real-time polymerase chain reaction master mix

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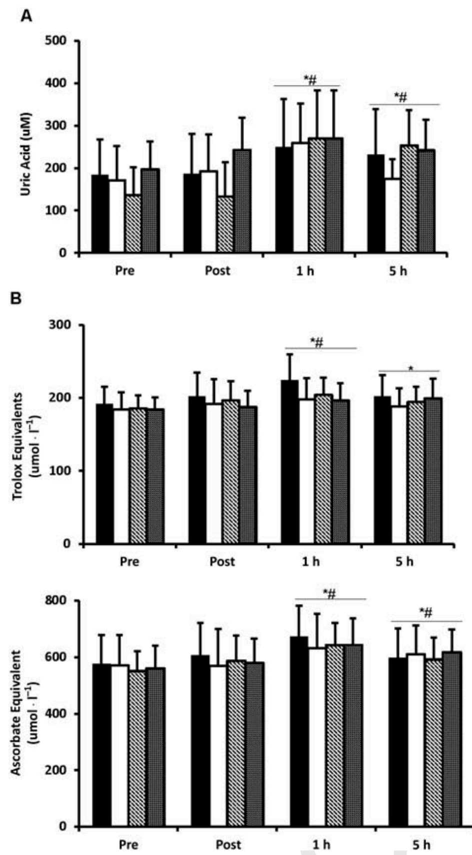


Figure 3. Data are mean ± s. (A) Uric acid values are expressed as uric acid equivalents (μM). (B) Trolox equivalent antioxidant capacity values are expressed as Trolox equivalent antioxidant capacity equivalents (μmol · l^{-1}). (C) Ferric-reducing ability of plasma values is expressed as ascorbate in equivalents (μmol · l^{-1}); *solid black lines* represent 0 m recovery, open *bars* 1667 m, open *stripped bars* are representative of 3333 m and *shaded bars* represent 5000 m above sea level; *significantly different from pre; #significantly different from post.

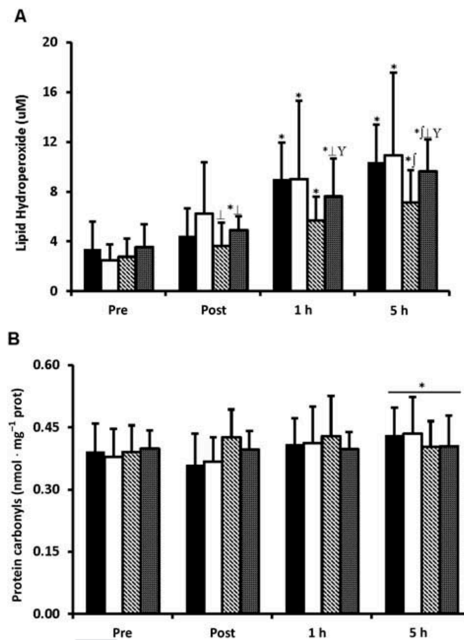
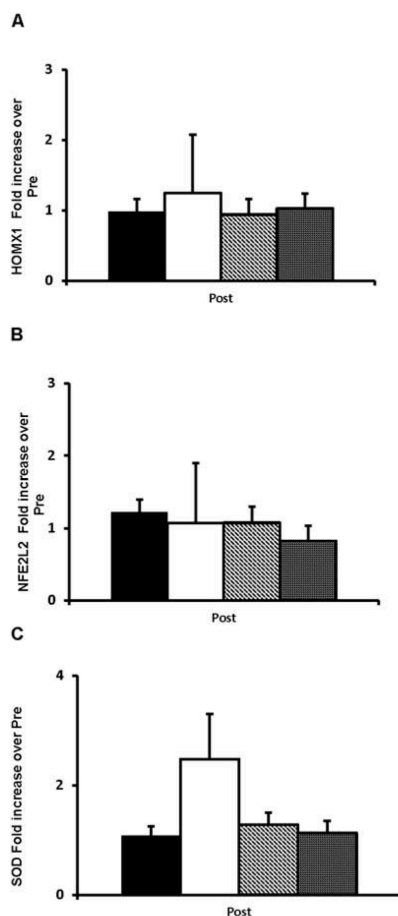


Figure 4. (A) Lipid hydroperoxide values are expressed as lipid hydroperoxide equivalents (μM), *solid black lines* represent 0 m recovery, open *bars* 1667 m, open *stripped bars* are representative of 3333 m and *shaded bars* represent 5000 m above sea level. (B) Protein carbonyl values are expressed in standard comparison to protein carbonyl equivalents (μM), *solid black lines* represent 0 m recovery, *open bars* 1667 m, *open stripped bars* are representative of 3333 m and *shaded bars* represent 5000 m above sea level; *significantly different from respective pre; #significantly different from normoxic recovery; ∫significantly different from 0 m; ⊥significantly different from 1667 m; Ysignificantly different from 3333 m.

coefficient of variation was 7.7% for lipid hydroperoxides and for 5.3% protein carbonyls. Lipid peroxidases exhibited significant time ($P = 0.038$) and trial ($P < 0.001$) main effects with values increasing in all recovery time points. Notably, post hoc analyses revealed that the rise in plasma lipid hydroperoxides was more dramatic in the 0 m recovery climates as compared to 3333 m ($P = 0.011$) and 5000 m ($P = 0.039$). In fact, the mean per cent increase (combined post, 1 h and 5 h) in lipid hydroperoxides was 0 m + 135%, 1667 m + 251% versus 3333 m + 99% and 5000 m + 108%. Analysis of protein carbonyls assay results indicated a significant time ($P = 0.038$) but not trial ($P = 0.909$) effect. Time comparisons revealed a pre-5 h difference only ($P = 0.031$).

Gene expression and QPCR

Mean responses for hemeoxgenase 1, nuclear factor (euthyroid-derived 2)-like factor, and superoxide dismutase 2 are presented in Table II. Neither time ($P = 0.187$) nor trial ($P = 0.211$) effects were observed for hemeoxgenase 1. In similar fashion, neither time ($P = 0.631$) nor trial ($P = 0.565$) main effects were statistically significant for nuclear factor (euthyroid-derived 2)-like factor. Superoxide dismutase 2 transcript levels approached, but did not achieve, significance for



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time (P = 0.070). Trial main effects for superoxide dismutase 2 were not significant (P = 0.146).

Discussion

The key finding from this study is based on plasma lipid hydroperoxide outcomes and suggests an attenuation of the exercise-induced oxidative stress response during recovery occurs at an altitude threshold between 1667 and 3333 m. As presented in Figure 4A, plasma lipid hydroperoxide values at the 3333 and 5000 m were respectively −26% (1 h) and −19% (5 h) as compared to the corresponding 0 and 1667 m recoveries. The results from this study extend prior investigations (Ballmann et al., 2014; McGinnis et al., 2014) to reveal that recovery at a relatively modest altitude will blunt some exercise-induced oxidative stress responses as observed with blood plasma biomarkers. Prior understanding is based on the findings from a series of strategically designed studies, which indicate in aggregate that the exercise-induced increase blood oxidative stress is blunted during recovery in simulated altitude environments. Prior study designs were designed to control for exercise intensity and workload, indicating that ambient environment recovery conditions exert independent effects on post-exercise oxidative stress responses (Ballmann et al., 2014; McGinnis et al., 2014). The general altitude threshold, at which this observance occurs, however, is unknown and serves as the impetus for the current investigation. As a collective body of work, these findings raise important insights regarding exercise, oxidative stress, and adaptations during recovery. Novel findings from the current study may apply to recreational hikers or possibly warfighters who ascend mountains and recover at elevation. Although the presented conclusions are based on speculation, the current understanding is that acute adaptive responses may prove particularly important in environmental extremes where maintenance of fitness and health is of vital importance. The potential implications of these findings are detailed subsequently.

Markers of oxidative damage

Current study findings reveal a time-dependent increase in plasma lipid hydroperoxides following the four exercise challenges throughout the 6 h recovery sampling time window (Figure 4A). However, results also revealed attenuation in lipid hydroperoxides response at the two highest simulated altitudes of 3333 (average −1.7 µm as compared to 0 and 1667 m) and 5000 m (average −1.6 µm as compared to 0 and 1667 m). This key finding can be interpreted to suggest that an altitude threshold occurs between 1667 and 3333 m that

Table II. Gene expression results.

	0 m	1667 m	3333 m	5000 m	Time main effect	Trial main effect
Hemeoxgenase 1	0.98 ± 0.65	1.25 ± 2.8	0.94 ± 0.75	1.03 ± 0.78	P = 0.18	P = 0.21
Nuclear factor (euthyroid-derived 2)-like factor	1.23 ± 1.30	1.07 ± 0.64	1.08 ± 0.50	0.82 ± 0.30	P = 0.63	P = 0.63
Superoxide dismutase 2	1.08 ± 0.60	2.47 ± 2.9	1.28 ± 0.77	1.13 ± 0.74	P = 0.07	P = 0.15

results in a blunting of oxidative stress during post-exercise recovery as determined by plasma lipid hydroperoxides. The results agree with previous findings from an investigation with closely related exercise and environmental study design facets (Ballmann et al., 2014), in addition to prior observations of elevated oxidative stress when exercise is performed at altitude (Dosek et al., 2007). Although the findings suggest that there is a response that occurs at the two highest altitudes, which may infer an altitude threshold, the relationship between altitude and the magnitude of the response remains inconclusive. Historically, the quantitative estimation of protein hydroperoxides has presented some difficulties (Gay & Gebicki, 2003). Over the last decade, improvements have been made in which the ferrous oxidation or lipid hydroperoxide method provided a convenient assay when measuring both lipid and protein hydroperoxide content in samples (Gay & Gebicki, 2003). In lieu of these findings, lipid hydroperoxides were used as an oxidative stress measure opposed to thiobarbituric acid reactive substances because of its relative methodological strength (Gay & Gebicki, 2003).

Significant elevations in protein carbonyls occurred during exercise recovery (+6% at 3333 and 5000 m as compared to 0 m) and confirm that the exercise trial elicited an oxidative stress response (Figure 4B). Given the methodological overlap between the current study and prior studies, this current finding for plasma protein carbonyls agrees with an earlier finding (Ballmann et al., 2014). In this prior study with a similar exercise recovery study design, outcome differences were observed during exercise recovery (Ballmann et al., 2014). There are some obvious applications for this data that can be contextualised during sporting events whereby repeated periods of endurance may be required within a day or over a consecutive number of days, which align with discussion mentioned elsewhere (Cobley, McGlory, Morton, & Close, 2011). Of interest in the current data set is the fact that plasma protein carbonyls were elevated independent of the simulated recovery altitude. We do not currently have a definitive explanation for an altitude-dependent response in lipid hydroperoxides but not protein carbonyls. One possible explanation is that during concentric-dominant exercise similar to the current study, membrane-bound enzymes like xanthine oxidase and nicotinamide adenine dinucleotide phosphate oxidase promote lipid damage that is disproportionate to that of protein (Powers, Nelson, & Hudson, 2011). Previous observation demonstrates that the post-exercise rise in plasma protein carbonyl is dependent upon the total work performed (Hudson et al., 2008). In the Hudson et al. study, strength and hypertrophy squat workouts were normalised for the total amount of work performed. The study demonstrated normalised plasma protein carbonyl responses for differences in recovery time (due to different time requirements to complete the two strength protocols); the magnitude of the rise in plasma protein carbonyls was identical (Hudson et al., 2008). There are obvious methodological differences between the prior strength-based study and the current cardiovascular exercise, but both investigations controlled for workload. As such, it is tempting to speculate that in applied physiology studies like the current investigation lipid biomarkers may be more reflective of the entire exercise recovery period while protein carbonyls were mostly influenced by the more stressful exercise portion (Powers et al., 2011). Further study is needed to resolve these fundamental questions about protein versus lipid oxidative damage markers.

Plasma antioxidant capacity and exercise-induced oxidative stress

To ensure a comprehensive assessment of blood plasma redox status, plasma antioxidant capacity measures were performed to determine total antioxidant capacity and antioxidant potential using Trolox equivalent antioxidant capacity, ferric-reducing ability of plasma and uric acid measurements (Figure 3). These markers indicate that redox-sensitive metabolic activity continues for several hours following exercise cessation (Erel, 2004; Hudson et al., 2008; Quindry et al., 2008). It was previously noted that the post-exercise measurements of Trolox equivalent antioxidant capacity and ferric-reducing ability of plasma values were greatly influenced by plasma concentration of uric acid (Ballmann et al., 2014; Hudson et al., 2008; Quindry et al., 2008). Similar to the previous findings, there was a time-dependent increase in plasma uric acid following exercise. For all groups, there was a significant difference between normoxic recovery and hypoxic recovery. Although there is an intuitive inclination that oxidative stress equates to a lower antioxidant capacity, post-exercise increases in ferric-reducing ability of plasma and Trolox equivalent antioxidant capacity values are typically observed (Ballmann et al., 2014; Hudson et al., 2008; Quindry et al., 2008). Findings probably reflect acute increases in plasma UA values during exercise recovery (Cao & Prior, 1998) due to production of UA in fatiguing muscle that results in a compartmental shift to blood plasma in response (Quindry et al., 2008; 2003). AQ6

Gene expression

Muscle biopsies of the vastus lateralis were obtained with the intent of comparing blood oxidative stress outcomes to redox-sensitive gene transcript changes.

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580 Despite numerical differences in the current investigation, data were variable and did not signify any statistical differences for nuclear factor (euthyroid-derived 2)-like factor (95% CI [−0.27, 0.11]), superoxide dismutase 2 (95% CI[−0.96, −0.02]) or 585 hemeoxgenase 1 (95% CI[−9.45, 50.6]). Current findings are in contrast to a prior study where an elevation of nuclear factor (euthyroid-derived 2)-like factor and superoxide dismutase 2 gene expression was abolished during hypoxic recovery (Ballmann 590 et al., 2014). The expectation that there would be a trial-dependent change is based on the fact that nuclear factor (euthyroid-derived 2)-like factor is a redox-sensitive transcript that has links to over 200 cytoprotective genes that regulate cell growth, cell 595 cycle and help to maintain homeostasis (Lewis, Mele, Hayes, & Buffenstein, 2010). Preceding research findings supported the view that increases in nuclear factor (euthyroid-derived 2)-like factor expression are acutely elevated post-exercise 600 (Ballmann et al., 2014). Transcripts were also measured for two downstream antioxidant enzymes superoxide dismutase 2 and hemeoxgenase 1 for which changes were not statistically significant.

Differences in the gene transcripts from prior find- 605 ings may be the result of the variation among study parameters, where in a previous study participants exhibited greater mean aerobic power (VO_{2max} = 54.4 ± 9.7) as compared to the current study (VO_{2max} = 48.4 ± 13.1). Moreover, the current sam- 610 ple was more heterogenous, with a standard deviation in functional aerobic capacity of 13 $ml \cdot kg^{-1} \cdot min^{-1}$ as compared to the prior study of 9 $ml \cdot kg^{-1} \cdot min^{-1}$. As such, there is reason to believe that similar observations in a more homogenous way may have reached 615 significance. Alternately, there are limitations inherent to application of gene transcript measures to serial biopsies as in the current study. Small-volume, hard-to-obtain samples are subject to day-to-day variability. In the current study, new baseline values were created 620 for each trial and may have masked day-to-day differences. That is, transcript signals are very labile in response to athletic and environmental factors including altitude. While muscle biopsy applied to exercise and performance scenarios can be scientifically 625 powerful, extreme care is needed in order to account for outcome responses due only to the intervention. Further empirical investigation is needed to resolve these methodological considerations in guiding future studies and resulting interpretations.

630 **Study limitations**

The reader should be aware of the following study limitations. First, the oxidative stress biomarkers examined currently in blood plasma are often criticised in whole-body studies due to their labile nature (Powers et al., 2010). In an effort to prevent 635 day-to-day variability concerns, Trolox equivalent antioxidant capacity, ferric-reducing ability of plasma and uric acid were examined from a common "first thaw" plasma aliquot. All assays were performed within a few hours of thaw and exhibited a 640 coefficient of variation less than 2%. Lipid hydroperoxides and protein carbonyls were assayed from dedicated plasma samples on separate days and exhibited coefficients of variation below 5%. Notably, exercise intensity was determined relative 645 to VO_{2max}. This approach does not account for functional differences in lactate threshold, a physiological parameter that could have influenced both oxidative stress and gene transcript outcomes. On a related note, participants from the current study 650 were derived from an academic community located at 975 m in elevation. As such, some elevation-based adaptations may have influenced outcomes in some, particularly if they frequent spent time at higher elevation in the weeks prior to participation 655 in the current study. If correct, there is reason to believe that confounding effects of prior altitude habituation may have had the most influence on gene transcripts. Alternately, the lower partial pressure at 975 m is within the 500–2000 m altitude 660 window considered to be minimally impactful on exercise performance (Bartsch & Saltin, 2008; Gore et al., 2013). Additionally, antioxidant supplementation was not controlled for between participants. The consumption of the Clif Bar, which contains 665 antioxidants, may have added a confounding component to the results. Research has shown that the consumption of antioxidants does quench the reactive oxygen species production (Powers, DeRuisseau, Quindry, & Hamilton, 2004). In 670 future studies, it would be imperative to control for such issues by looking into alternative dietary means during recovery period.

Conclusion

The current study is an important continuation of 675 the investigations of hypoxic exercise-induced oxidative stress by Ballmann et al. (2014), McGinnis et al. (2014) and Quindry et al. (2013). Earlier investigations examined high-altitude exercise, followed by the independent influences of altitude on exercise 680 and recovery. The current study extends upon a linear progression of laboratory investigations where experimental conditions were controlled for by simulating altitude during exercise and recovery in order to observe the effect on oxidative stress 685 response to acute exercise. Current data confirm that exercise recovery at high altitude results in altered redox balance and blood oxidative stress markers and indicates that the altitude threshold for

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this response is above 1666 m. The collective findings of the current study add to the growing body of literature focused on the influence of environmental temperature on exercise-induced oxidative stress (Gomes, Stone, & Florida-James, 2011; McAnulty et al., 2005; Mounier et al., 2009; Pialoux et al., 2010, 2009). With the understanding that exercise and environmental influence on oxidative stress, more reductionistic research approaches with serial muscle biopsies are probably needed to better understand the fundamental mechanisms responsible for redox-sensitive adaptations to exercise and how high-altitude and hypoxic environments influence these responses to acute exercise at the tissue level. Additionally, breath and urinary markers may also prove beneficial in better defining the total body sensitivity to hypoxia/altitude thresholds. Refinements in study design are also needed to better resolve the roll of exercise intensity and altitude exposure on redox changes on blood and muscle measures of oxidative stress. As final consideration, application of more directed research approaches should also work to better resolve the exact altitude threshold (between 1667 and 3333 m as investigated currently) at which redox-sensitive alterations in exercise recovery occur.

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CHAPTER IV

Materials and Methods

Participants

The University of Montana's Institutional Review Board approved the study (Appendix A). Study participants were recruited from the Missoula, Montana community and written informed consent was obtained prior to data collection (Appendix B). To be included in the study participants reported no history of chronic lung disease and were not subject to frequent smoke exposure at home or work (via woodsmoke or wood stoves). Each participant was given a personal information questionnaire and the Physical Activity Readiness Questionnaire (PARQ) prior to the start of the experimental study (Appendix C); to be included in the study the participants had to respond no to all questions. Females were not included in the current study because they make up a small percentage of the target population (wildland firefighters). Prior to arrival in the lab, participants were phoned to verify that they had not developed any respiratory infections and/or other health related changes that would hinder their participation. Participants were fasted 3 Hrs prior at arriving to the laboratory on Day 1.

Study Design

Study participants were recruited by posting flyers on the University of Montana's campus. The target population was University of Montana students, faculty, and staff. Day 1 of the trial was used to determined participants' eligibility for Days 2-4. Participants were asked to fast for three hours prior to coming in on Day 1. Those enrolled in the study received a written and oral informed consent form. Each participant completed the PARQ form and underwent a test to verify that his maximum level of oxygen uptake (VO_2 max) was greater than $40 ml \cdot kg^{-1} \cdot min^{-1}$. The percentage of body fat was determined via underwater weighing technique. On days

2-4, the subjects participated in three exposure trials, each one occurring one week apart. Each subject was exposed to Clean Air (0 μg/m^3), Low Exposure (250 μg/m^3) and High Exposure (500 μg/m^3) wood smoke $PM_{2.5}$, in a randomized cross-over design depicted in **Figure 2.**

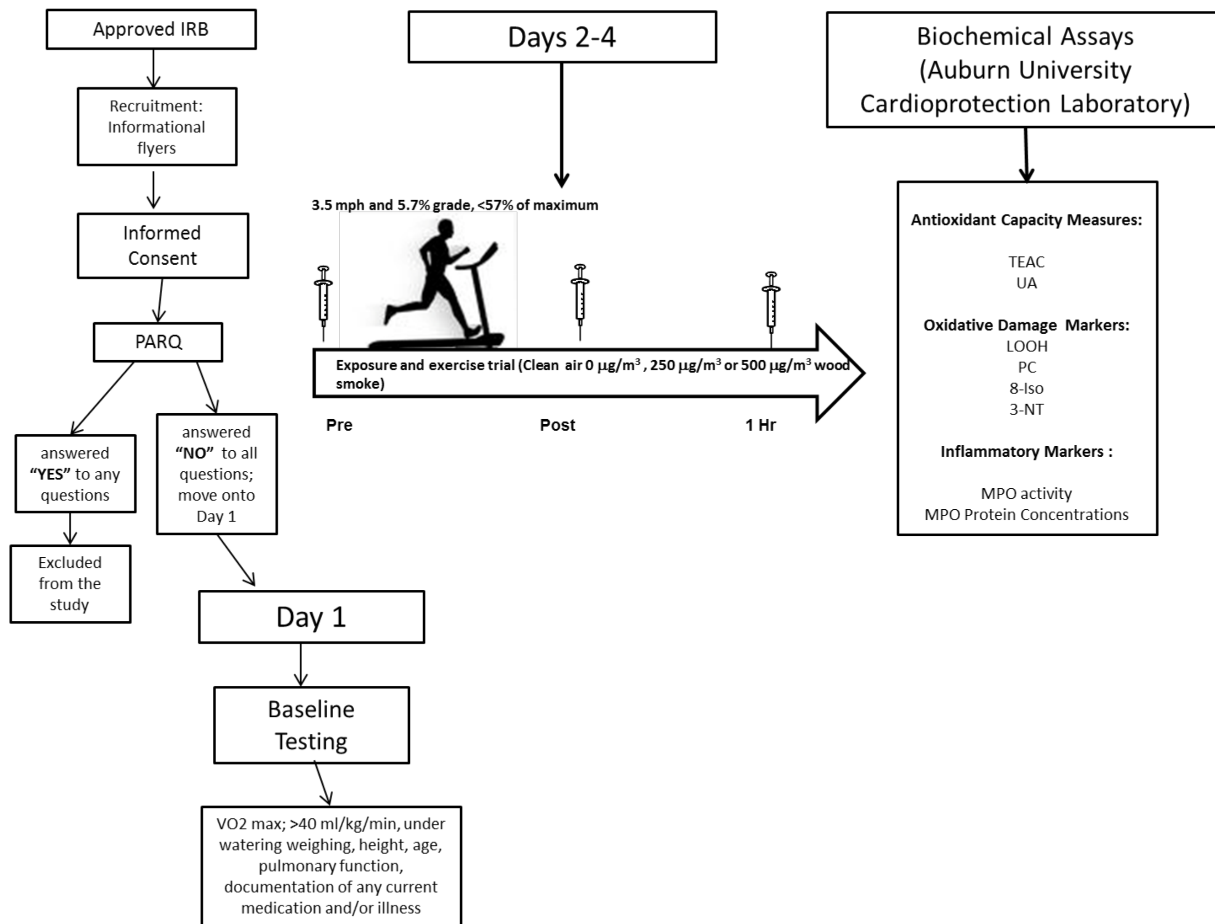


Figure 2. Study design. The study was approved by the University of Montana's IRB. On Day 1 participants underwent baseline testing to record their VO_2 max, weight, age, height, and pulmonary function. Participants were exposed to episodic woodsmoke 1 week apart in a randomized crossover design while walking on a treadmill for 1.5 Hrs indicated by the arrow. Blood samples were taken pre-, post-, and 1Hr post exercise.

Baseline Testing

Personal information collected from participants included age, height, weight, percentage body fat, VO_2 max, respiratory function measurements, and illness and medication history. Percent body fat was determined using hydrodensitometry. The underwater weight of each person was obtained using a digital scale (Exertech, Dresbach, MN). Participants repeated trials

until 3 hydrostatic weight values within 100g were obtained. Prior to obtaining his underwater weights, each participants' height and weight on land were recorded. Underwater weights were corrected for estimates of residual lung volume (residual lung volume = (0.0115*Age) + (0.019*Height)-2.24). The relationship between hydrostatic weight and dry land weight were used to calculate body volume and converted to percent fat using the Siri Equation (BF= (4.95/ρ-4.50)*100) (63). Participants completed a maximal test on an electronically powered treadmill (Model Q5, Quinton Instrument Company, Bothell, WA) to predict aerobic fitness. Maximal oxygen consumption was predicted based on the ACSM walking equation;

VO_2 ($ml \cdot kg^{-1} \cdot min^{-1}$) = [Speed ($m^{-1}$/min) x 0.1] + [(% grade/100) x Speed (m^{-1}/min) x 1.8] + 40 $ml \cdot kg^{-1} \cdot min^{-1}$ (Horizontal and Grade walking (3.7 mph and below) (75). Percent grade was increased incrementally every 3 minutes (+10 % grade) until subjects demonstrated signs of volitional fatigue; the protocol is presented in **Table 1**.

Table 1. Workload, duration, and estimated METs for maximal aerobic test

Stage	Speed	Grade	Time (min)	METs
I	1.7 mph	10%	3:00	4.6
II	2.5 mph	12%	3:00	7
III	3.4 mph	14%	3:00	10.1
IV	4.2 mph	16%	3:00	13.5
V	5.0 mph	18%	3:00	14.9
VI	5.5 mph	20%	3:00	17
VII	6.0	22%	3:00	19.3

Woodsmoke Preparation

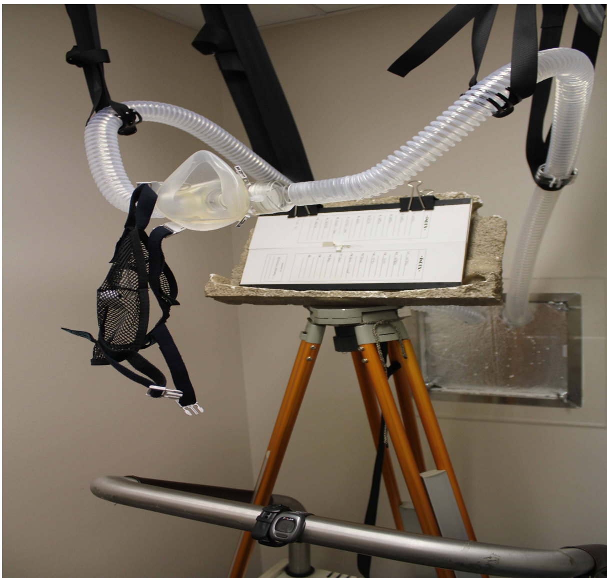
The Pulmonary Physiology Core located within the Center for Environmental Health Sciences at the University of Montana was modified to conduct the human woodsmoke exposure trials. A woodstove (Englander, England Stove Works, Inc., Monroe, VA) was used to generate wood smoke for exposure trials. The wood was western larch (*Larix occidentalis Nutt*) which was cured to approximately 15% moisture content. The building and maintenance of the fire was

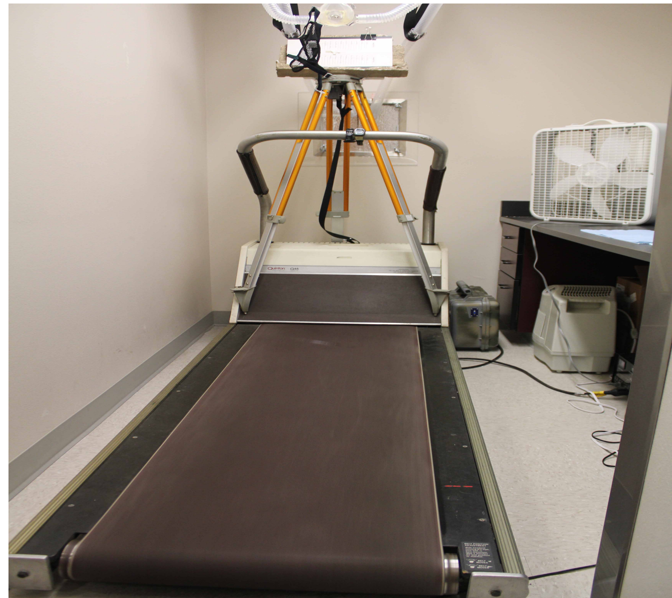
kept consistent throughout the study. The fires were built 25 minutes prior to the start of each exposure trial by using 1kg of wood as well as kindling with 1-2 pages of newspaper. Three hundred grams of wood was then added every 15-20 minutes over the two-hour period. The fire was maintained by removing ash if necessary to ensure a constant layer of ash (approximately 0.5-1 inch deep).

Exposure Trials

Participants were exposed once to clean air 0 $\mu g/m^3$, 250 $\mu g/m^3$ and 500 $\mu g/m^3$ woodsmoke particles ($PM_{2.5}$) in a randomized fashion while exercising for 1.5 Hrs. The lower concentration (250 $\mu g/m^3$) of woodsmoke particulate matter is comparable to concentrations of woodsmoke recorded when biomass is burned for cooking or other heating purposes in homes lacking ventilation (30) and consistent with the concentration used in other European human/biomass exposure studies (11, 12, 27, 40, 43, 107, 111, 120, 125). The higher level of woodsmoke exposure (500 $\mu g/m^3$) used is comparable to human exposure studies conducted by Ghio et al. (2012) (43), where study participants were exposed to an average concentration of 485 $\mu g/m^3$ over a 2=Hr time course. Throughout each trial $PM_{2.5}$ and CO levels were monitored. The concentrations of woodsmoke PM were established and maintained on the basis of information from two $PM_{2.5}$ monitors linked into the system. The clean air was adjusted manually as a function of observed PM levels just upstream from subject exposure. During exposure, woodsmoke was delivered directly from the dilution and mixing chamber to the subject via a modified mask respirator with continuous $PM_{2.5}$ measurements measured by two DustTrak II (DustTrak, TSI, Model 8530, Shoreview, MN). The temperature, humidity, CO, and carbon dioxide in the mask and exercise room were monitored with a Q-Trak (TSI Inc., Shoreview, MN). Images of the treadmill and woodstove are shown in **Figure 3**.

During the smoke exposure, subjects were asked to walk on a treadmill at a set rate and incline of (3.5 mph and 5.7% grade, <57% of mean maximum aerobic power) for 1.5Hrs (with a short break e.g. 20-30 seconds) every 15 minutes to simulate working on a fire line. Each experimental trial took approximately 1.5 Hrs.





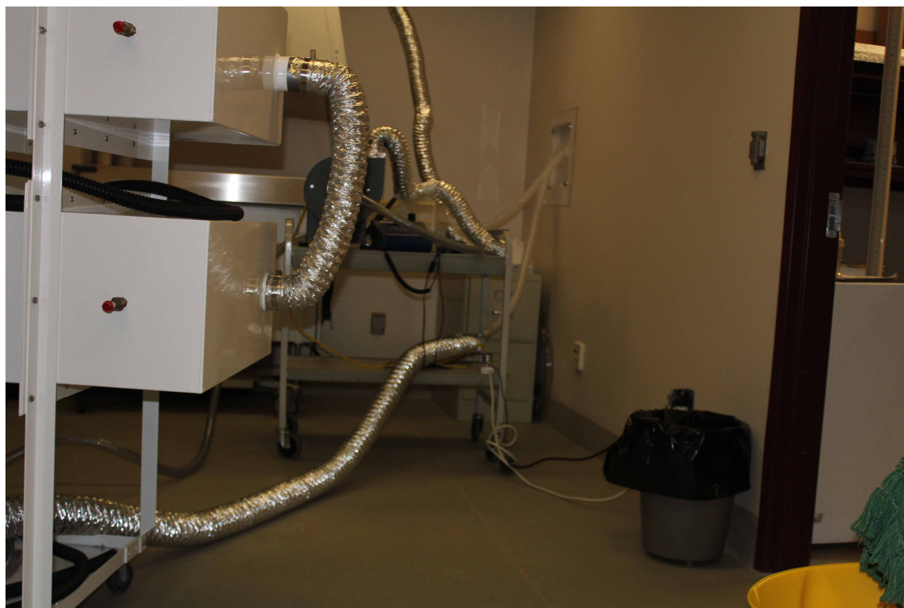


Figure 3. Instrumentation for exercise exposure trials. During exposure, woodsmoke was delivered directly from the dilution and mixing chamber to the subject via a modified mask respirator with continuous $PM_{2.5}$ measurements measured by the DustTrak II (DustTrak, TSI, Model 8530, Shoreview, MN). During the smoke exposure, subjects walked on a treadmill at a

set rate and incline of (3.5 mph and 5.7% grade, <57% of mean maximum aerobic power) for 1.5 Hrs.

Blood Samples

For the three exposure trials, blood samples were collected pre- (Pre), post - (Post), and 1- (1Hr) hour post exercise/exposure from the antecubital vein with sodium heparinized vacutainers (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1000xg for 15 minutes at 4°C during each visit. Plasma was aliquoted and stored immediately at -80°C until subsequent biochemical analysis of oxidative damage and antioxidant biomarkers. Samples were then packaged on dry ice and shipped to Auburn University. Individual where individual aliquots were stored at -80°C. All assays were completed within a few months of sample collection and were subjected to a single thaw. To preserve sample viability, aliquots were kept on ice and in the dark to prevent environmental redox alterations.

Biochemical Panel

To quantify blood oxidative stress a panel of markers was chosen for both oxidative damage and for antioxidant content. The oxidative damage markers were LOOH, PC, 8-ISO, and 3-NT. The antioxidant markers were TEAC, and UA. Additional markers of redox related inflammatory markers were examined, included MPO activity and protein content.

Biochemical Assays for Antioxidant Capacity Measures

The TEAC assay quantifies any antioxidants capable of scavenging 2, 2’ azinobis 3-ethyl-benzothiazoline-6-sulfonic acid) radical anions, thus quenching a quantifiable colorimetric reaction. The assay was performed using a 96-well plate (Sigma-Aldrich, St. Louis, MO). The assay work solution was made by combining 2.5 mM Trolox solution (31.1 mg Trolox + 50 ml 10 mM PBS) with 50 mM Glycine buffer (600 ml deionized water + 2.25 g Glycine with pH adjusted to 4.4). The glycine peroxidase mixture (5.2 mg peroxidase +3 ml 50 mM Glycine

buffer) was added to the 50 mM glycine buffer. Next ABTS solution (490.0 mg ABTS +597 ml of glycine buffer with peroxidase solution) was added. The final component added was 375 μl of 22 mM H_2O_2 [20 μl 30% (8.8 mM) H_2O_2 + 8000 μl deionized water], and then combined with 375 μL of 22mM H_2O_2 to 597 ml Glycine-peroxidase-ABTS solution). The standards were made by serial dilution from the 1.0 mM Trolox standard stock solution (7.5 ml 10 mM PBS + 5 ml 2.5 mM Trolox) (concentrations were measured in μM; 200, 100, 50, 25, 0). Calculated TEAC values for each sample are based on a standard reaction with calculated values compared to the water-soluble vitamin E analogue Trolox (36). See Appendix (D) for stepwise details.

To measure the catalytic activity of peroxidase generated by H_2O_2 in each sample, the uric acid (UA) assay was performed. The assay was performed using 1.5 mL UV/VIS spectroscopy cuvettes (Sigma-Aldrich, St. Louis, MO). Uric acid stock solution was made by adding 8 mL of (3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) (28 mg MBTH + 200 mL PBS; pH 4.5), 8 ml of 3-(Dimethylamino) benzoic acid (DMAB) (750 mg DMAB+150 mL PBS; pH 6.5) and 0.9 ml peroxidase (from Horseradish; 5 mg + 142.86 ml PBS; pH 8). The standards were made and diluted serially using the uric acid stock solution (17.1mg uric acid +150 mL deionized water; concentrations were measured in μM; 600, 300, 150, 75, and 0). 300 mL of test solution (3 ml of uricase + 30 ml of PBS) or blank (PBS pH 8.0) was added to the cuvette. Next 60 μl of standard or sample was added in 30-second intervals to the appropriate cuvette. The mixture was incubated at 37° C for 10 minutes. Work solution (480 μl) was added to each cuvette in 30-second intervals and allowed to incubate at 37° C for 15 minutes. Measurements of H_2O_2 were determined by peroxidase catalysis-oxidation of chromogenic and florigenic substrates or by catalyze mediated conversion of alcohols to aldehydes, which were measured spectrophotometrically using a reaction mixture containing 3-

methyl-benzothiazoline-2-one hydrazone and 3-dimethylaminobenzoic acid. Final plasma UA values were determined by comparison with internal standard responses (62). See Appendix (D) for stepwise details.

Biochemical Assays for Oxidative Stress

Oxidative damage in blood plasma was quantified using protein carbonyls (PC), lipid hydroperoxides (LOOH), 8-Isoprostane (8-ISO) and nitrotyrosine assays (3-NT). For PCs, plasma sample protein concentrations were analyzed via absorbance spectroscopy according to the methods of Bradford (16). Plasma samples were diluted to 4 mg/ml and PC values were measured by a commercially available ELISA kit (Biocell Corporation Ltd, Papatoetoe, NZ) (22). PC standards were oxidized 24 hours prior to the start of the ELISA kit as instructed by the manufacturer (add 25 μl of deionized water to tubes K1-6, and control tube, (incubate overnight at room temperature). On day two, plasma was thawed and the Bradford assay was performed to determine protein content for further analysis. In preparation for the Bradford assay, samples were diluted 1:100 with PBS. Bradford reagent (285 μl) was added to each well followed by 15 μl of standard or sample. The plate was allowed to incubate for 20 minutes at room temperature in the dark. Following the incubation period the plate was read at 595 nm (16). Reagents were made according to the kit's instructions. The samples were diluted based on the standard procedures for samples containing 4-35 mg protein per ml. Following the ELISA procedure the absorbance was read at 450 nm. See Appendix (D) for stepwise details.

Plasma LOOH was quantified by implementing the ferrous oxidation-xylenol orange assay. LOOH standard solution (9 μl of 80 % cumene + 50 ml of MEOH) was used to serially dilute the standard curve concentrations (were measured in mM; 20, 10, 5, 2.5, and 0). The reaction was done within individual 1.5 micro centrifuge tubes (Sigma-Aldrich, St. Louis, MO).

10 μl of TCEP (blank) or MEOH (test) was added to in triplicate to each tube. Next, 60μL of standard or sample was added the TCEP or MEOH tubes were and the tubes incubated for 30 min at room temperature. Work solution was made by adding 19.6 ferrous ammonium sulfate + 20 ml of H_2SO_4, + 158.4 mg butylated hydroxytolene +180 ml MEOH + 15.2 mg xylenol orange. Work solution was added (900 μl) to each tube in 45 second intervals. The tubes were then incubated at room temperature in the dark for 45 minutes. At the completion of the incubation time the tubes were centrifuged at 10X gravity. 830 μl of supernatant was decanted into cuvettes. During the reaction, oxidized ferrous ions react with the ferrous sensitive dye contained in xylenol orange to form a complex that is quantified through absorbance spectroscopy at a wavelength of 595nm (86). See Appendix (D) for stepwise details.

Blood plasma 8-Isoprostane are concentrations were measured using a specific immunoassay enzyme (EIA) kit (Cayman Chemical, Ann Arbor, MI). The isoprostanes are a family of eicosanoids of non-enzymatic origin that are produced by random oxidation of tissue phospholipids by oxygen radicals (59, 97). 8-isoprostanes is a marker of lipid oxidative damage. Prior research revealed elevated levels among heavy smokers. Plasma of healthy volunteers contains modest amounts of 8-isoprostane (40-100 pg/ml). Quantification is based on the competition between 8-isoprostane and an 8-isoprostane-acetylchoinesterase (AChE) conjugate (8-Isoprostane Tracer). Samples were diluted (40μL sample to 200 μL deionized water). EIA buffer was added (100 μL) to NSB wells and 50 μL to B_0 wells. Standards (100 μL 50ng/mL 8-isoprostane) were made and 50μL were added to the standard wells. Samples were added (50 μL) to each well. 8-isoprostane tracer was added (50 μL) to each well except TA and Blank. Then, 50 μL of 8-isoprostane antiserum was added to all wells except TA, NSB, and blank wells. The plate then incubated for 18 hours at 4°C. On day 2 the plate was washed and Ellman's

reagent was added. The enzymatic reaction is read by spectroscopy at 412nm. Tracer bound to the well is inversely proportional to the amount of free 8-isoprostane present (79). See Appendix (D) for stepwise details.

The presence of nitrotyrosine in biological fluids such as plasma and/or urine has been indicated as a marker for oxidative stress in vivo (25). Nitrotyrosine is produced by the modification of protein tyrosine residues by peroxynitrite generation via the reaction of nitric oxide and superoxide (25). All reagents were brought to room temperature. Standards were prepared with 1 mL 1X wash buffer by pipetting. Wash buffer 1X was prepared by adding 25 mL 20X wash buffer to 475 ml deionized water. An 8-point standard curve was then prepared by reconstituting the 3-NT 4000 ng/ml stock standard with 1 ml 1X wash buffer. Then serial dilutions were done by first adding 600 μl to tube 2. Next, 300 μl of tube 1 contents was transferred to tube 2, repeated for tubes 3-7. Samples were diluted with 1X wash buffer then 50 μl standard or samples were added to each well. Horseradish peroxidase (HRP) Detector Antibody 2X (50 μl) was pipetted into each well and the plate was allowed to incubate for 2 hours at room temperature on an orbital plate rocker at 300 rpm. Wells were aspirated and washed four times with 300 μl of 1X wash buffer. Following the wash step, 100 μl HRP Development Solution was added to each well. An immediate color change was recorded at 450 nm. See Appendix (D) for stepwise details.

Biochemical Assays for Redox Sensitive Inflammatory Markers

MPO protein content

Myeloperoxidase (MPO) is a heme-containing enzyme belonging to the XPO subfamily of peroxidase (61, 81). MPO binds albumin on the macrophage mannose receptor, cytokerain 1 on vascular endothelial cells, high molecular weight kininogen, and the integrin CD11b/CD18 on

neutrophils (17, 128). The interactions promote MPO clearance and a reduction of nitric oxide (17, 61, 81). Quantitative measures of MPO were obtained by an immunoassay (EIA) technique (R&D Systems, Minneapolis, MN), which quantified the bound MPO (17). Samples were prepared by diluting 10-fold (20 μl of sample + 180 μl of Calibrator Diluent RD6-58). Standards were serial diluted according the manufacturer's directions. All reagents were made according to the directions outlined by the kit's manufacturer. Assay diluent was added (100 μl) to each well. Standard, control or sample was added (50 μl) per well and the plate was then allowed to incubate for 2 hours at room temperature on an orbital shaker. The plate was aspirated and each well was washed with 400 μl of wash buffer. MPO conjugate (200 μl) was added to each well and the plate was allowed to incubate for 2 hours at room temperature on a shaker. The plate was aspirated and washed at the conclusion of the 2-hour incubation period. Following the wash step, substrate solution (200 μl) was added to each well and the plate incubated for 30 minutes at room temperature, on the benchtop and covered with foil to protect from light. The reaction was stopped within the plate by adding 50 μl of stop solution to each well. Color change was observed. The enzymatic reaction is read by a spectroscopy at 450 nm. See Appendix (D) for stepwise details.

MPO activity measures

MPO activity was measured using a colorimetric activity (EIA) kit (Sigma Aldrich, St. Louis, MO). The kit is designed to detect the formation of hypochlorous acid, which readily reacts with taurine to form taurine chloramine. Taurine chloramine reacts with the chromophore TNB, which results in a colorless product of DTNB. One unit of MPO activity is defined as the amount of enzyme that hydrolyzes the substrate and generates taurine chloramine to consume 1.0 μmol of TNB per minute at 25° C. 50 μL of reaction mixture was added to each positive control,

sample, and sample blank. To optimize the reaction to our samples, preliminary experiments were performed in which assay plates were read at 30 minutes, 60 minutes and 120 minutes to ensure values were in linear range. At each sample time 2 μl of stop mix was added. At 120 minutes the plate was read and 2 μl Stop solution was added to the plate. The plate was allowed to incubate for 10 minutes, and then 50 μl of TNB reagent or standard was added to the sample and control wells. The plate was allowed to incubate for an additional 10 minutes. Absorbance measures were read at 412 nm. See Appendix (D) for more stepwise details.

Statistical Analysis

The purpose of the present study was to examine blood oxidative stress responses of individuals exposed to an episodic woodsmoke event to simulate a field experience. A randomized repeated measures study design was employed to expose individuals to Clean Air (0 $\mu g/m^3$), 250 $\mu g/m^3$ or 500 $\mu g/m^3$ wood smoke $PM_{2.5}$. Given the full repeated measures study design employed, planned comparisons were used to determine differences among the three trials for Pre, Post, and 1Hr time points. Additional time-dependent relationships were examined within each of the three trials. Specifically, paired-sample t-tests were conducted to examine oxidative stress outcomes for the key dependent variables (37). All values are presented as means ± standard error (SEM). Significance was set at $p \leq 0.05$ *a priori*.

CHAPTER V
Manuscript for Journal Submission

Experimental woodsmoke exposure during exercise and an evaluation of blood oxidative stress

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Abstract:

Introduction: Woodsmoke exposure and exercise elicit oxidative stress in blood. The purpose of this investigation was to understand the effects of woodsmoke exposure on blood oxidative stress measures. **Methods:** Ten males 18-40 years were exposed to woodsmoke during 3 trials (separated by 1 week) while exercising on a treadmill to simulate the workload intensity and smoke exposure doses of wildland firefighting. Participants were exposed Clean Air (0 μg/m^3, Filtered Air), 250 μg/m^3 (Low Exposure) and 500 μg/m^3 (High Exposure) woodsmoke particles (particulate matter <2.5μm, $PM_{2.5}$) in a randomized counter-balanced crossover fashion while exercising. Blood samples obtained at baseline (Pre), immediately post (Post) and 1 hour following (1Hr) post exposure were assayed for trolox equivalent antioxidant capacity (TEAC), uric acid (UA), lipid hydroperoxides (LOOH), protein carbonyls (PC), nitrotyrosine (3-NT), 8-isoprostane, and myeloperoxidase (MPO) activity and protein content assays. **Results:** Study participants averaged 14.1 % body fat and had an average aerobic capacity of 53.6 ml·kg^{-1}·min^{-1}. There were no self-reported respiratory complications by participant following the exposure trials. UA values were lower only following Low Exposure (p=0.042), while plasma TEAC levels were elevated Post (Clean Air and High exposure, p=0.015 and p=0.001 respectively) and at 1Hr (Clean Air and High exposure, p=0.001) time points. LOOH levels were decreased 1Hr Post (High exposure, p=0.036), while plasma 8-Iso levels were elevated in Post samples from both smoke doses (Low exposure p=0.004, High exposure p=0.009). There were no increases in plasma PC levels for any of the 3 trials, while 3-NT values were elevated over Clean Air when Low/High Exposure values were combined (p=0.012). Neither MPO activity nor protein content was altered following the 3 trials. **Discussion**: The current laboratory simulation of wildland firefighting elicited some indications of oxidative stress, although increases were independent of

$PM_{2.5}$ concentrations. Additionally, circulating antioxidants were not consistently depleted and two redox sensitive inflammation markers were unaltered by smoke exposure. Future lab-based woodsmoke exposure studies should employ longer duration exposure times in order to provide an exposure stimulus more related to firefighting scenarios. Future study should continue to identify oxidative biomarkers and physiologic parameters that are most sensitive to woodsmoke exposure leading up to comprehensive field studies.

Introduction

The dangers of air pollution remain an important public health topic (123). The burning of biomass in the US is the primary source of indoor and outdoor woodsmoke particulate matter (PM) associated with adverse health effects. In 2006, it was estimated that wood combustion accounted for 2.7 % of the global burden of disease (1, 2, 104). Woodsmoke combustion yields a very complex mixture including carbon monoxide (CO), aldehydes, polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs) and PM (82). The acute physiologic outcomes to PM exposure include coughs, headaches, and eye and throat irritation in otherwise healthy individuals. Epidemiologic studies further establish the association between the incidence of biomass cooking fuel and chronic bronchitis (45). Moreover, cross-seasonal examination of non-smoking firefighters reveals an undeniable link between woodsmoke exposure and symptoms of coughing, phlegm, wheeze, and eye and nasal irritation (108).

Occupational exposures to woodsmoke vary with the length of exposure and amount of PM content in the woodsmoke combustion. Wildland firefighters experience greater woodsmoke exposures than the general public (126). Compounding the problem, wildland firefighter exposure is magnified by inadequate access to high quality protective gear. Combined with the cumbersome attire adding to the discomfort and exposure levels, firefighters also undertake very physically taxing tasks. The physical activity incurred during firefighting may also lead to oxidative damage. Previous studies document the association between oxidative stress and woodsmoke exposure in conjunction with physical activity (via exercise). Danielsen et al. (2008) investigated the level of systemic oxidative stress with markers of DNA damage; strand breaks and oxidized purines (27). The levels of strand breaks were significantly decreased at 3 Hr and 20 Hr post exposure ($p<0.05$) (27). Quantification of mRNA levels of hOGG1 were significantly

increased 20 hours post woodsmoke exposure as compared with 20 hour post clean air exposure ($p<0.05$) (27). Independent of air pollution exposure, participation in acute exercise elicits redox perturbations and transient oxidative stress responses (44, 52, 103). A series of recent studies by our group indicates collectively that the post exercise environment may impact the production of oxidative stress as quantified by a panel of blood biomarkers (10, 74, 91). While a rationale exists to suspect that wildland firefighters experience oxidative stress when they inhale woodsmoke on the job, little research is currently available to quantify the degree to which this outcome may occur.

A number of scientific approaches can be employed to understand the impact of smoke exposure during physical activity. In order to create a laboratory-based study that simulates outdoor woodsmoke exposure, the current methodological approach included woodsmoke exposure during light intensity exercise on a treadmill. The purpose of the current investigation was to quantify the blood oxidative stress responses of individuals exposed to an episodic woodsmoke event designed to simulate a field experience. A randomized repeated measures study design was employed to expose individuals to Clean Air (0 $\mu g/m^3$), Low Exposure (250 $\mu g/m^3$) and High Exposure (500 $\mu g/m^3$) woodsmoke $PM_{2.5}$ while performing an identical exercise bout. A panel of oxidative stress biomarkers was used to assess outcomes in blood plasma collected before, immediately post and 1Hr following the 3 exposure trials. Based on prior findings it was broadly hypothesized that exposure to episodic woodsmoke events during controlled physical activity would result in transient elevation of blood oxidative damage markers and a corresponding decline in blood plasma antioxidant content. Additionally, it was hypothesized that indices of oxidative stress would be altered in proportion to the smoke exposure dose.

Materials and Methods

Participants

The University of Montana's Institutional Review Board approved the study (Appendix A). Study participants were recruited from the Missoula, Montana community and written informed consent was obtained prior to data collection (Appendix B). To be included in the study participants reported that they had no history of chronic lung disease and were not subject to frequent smoke exposure at home or work (via woodsmoke or wood stoves). Each participant was given a personal information questionnaire and the Physical Activity Readiness Questionnaire (PARQ) prior to the start of the experimental study (Appendix C), to be included in the study the participants had to respond "no" to all questions. Females were not included in the current study because they make up a small percentage of the target population (wildland firefighters). Prior to arrival at the lab, participants were phoned to verify that they had not developed any respiratory infections and/or other health related changes that would hinder their participation. Participants were fasted 3 hours prior to arriving at the laboratory on Day 1.

Study Design

Study participants were recruited by posting flyers on the University of Montana's campus. The target population was University of Montana students, faculty, and staff. Day 1 of the trial was used to determined participants' eligibility and baseline testing while Days 2-4 were the exercise/exposure trials. Participants were asked to fast for three hours prior to coming in on Day 1. Those enrolled in the study received a written and oral informed consent form. Each participant completed the PARQ form and underwent a test to verify that their maximum level of oxygen uptake (VO_2 max) was greater than 40 $ml \cdot kg^{-1} \cdot min^{-1}$. The percentage of body fat was determined via underwater weighing technique. On days 2-4, the subjects participated in three exposure trials each one occurring one week apart. Each subject was exposed to Clean Air (0

μg/m³), Low Exposure (250 μg/m³) and High Exposure (500 μg/m³) woodsmoke $PM_{2.5}$, in a randomized cross-over design depicted in **Figure 1.**

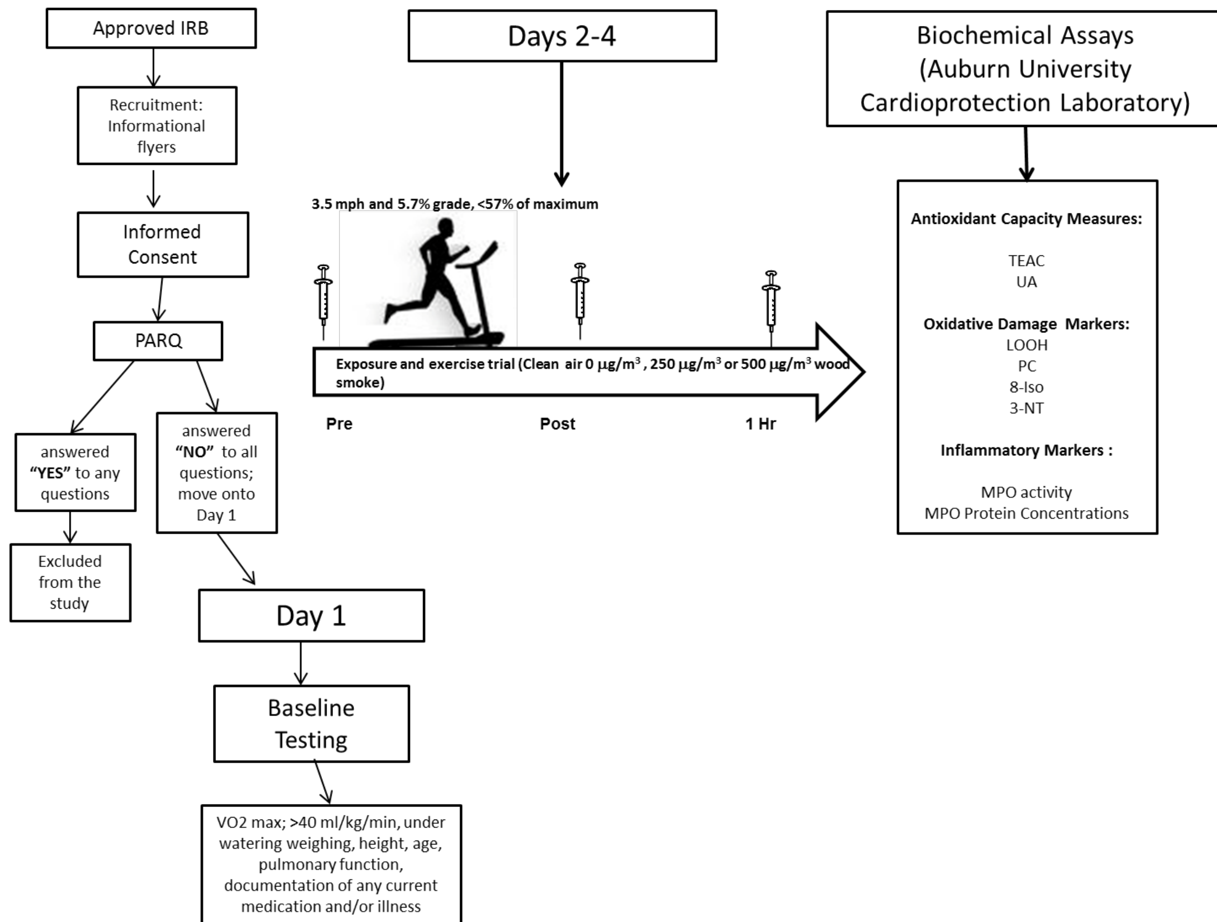

Figure 1. Study design. The study was approved by the University of Montana's IRB. On Day 1 participants underwent baseline testing to record their VO_2 max, weight, age, height, and pulmonary function. Participants were exposed to episodic woodsmoke 1 week apart in a randomized crossover design while walking on a treadmill for 1.5 hours indicated by the arrow. Blood samples were taken pre-, post-, and 1Hr post exercise.

Baseline Testing

Personal information collected from participants included age, height, weight, percentage body fat, VO_2 max, respiratory function measurements, and illness and medication history. Percent body fat was determined using hydrodensitometry. The underwater weight of each person was obtained using a digital scale (Exertech, Dresbach, MN). Participants repeated trials until 3 hydrostatic weight values within 100g were obtained. Prior to obtaining their underwater

weight, participants' height and weight on land were recorded. Underwater weights were corrected for estimates of residual lung volume (residual lung volume = (0.0115*Age) + (0.019*Height)-2.24). The relationship between hydrostatic weight and dry land weight were used to calculate body volume and converted the percent fat using the Siri Equation (BF= (4.95/ρ-4.50)*100) (63). Participants completed a peak maximal aerobic power test on an electronically powered treadmill (Model Q5, Quinton Instrument Company, Bothell, WA) to estimate peak aerobic fitness. Maximal oxygen consumption was predicted based on the ACSM walking equation;

VO_2 (ml/kg min) = [Speed (m/min) x 0.1] + [(% grade/100) x Speed (m/min) x 1.8] + 3.5 ml/kg min (Horizontal and Grade walking (3.7 mph and below) (75). Percent grade and speed were increased incrementally every 3 minutes until subjects demonstrated signs of volitional fatigue; the protocol is presented in **Table 1**.

Table 1. Workload, duration, and estimated METs for maximal aerobic test

Stage	Speed	Grade	Time (min)	METs
I	1.7 mph	10%	3:00	4.6
II	2.5 mph	12%	3:00	7
III	3.4 mph	14%	3:00	10.1
IV	4.2 mph	16%	3:00	13.5
V	5.0 mph	18%	3:00	14.9
VI	5.5 mph	20%	3:00	17
VII	6.0 mph	22%	3:00	19.3

Woodsmoke Preparation

The Pulmonary Physiology Core located within the Center for Environmental Health Sciences at the University of Montana was modified to conduct the human woodsmoke exposure trials. A woodstove (Englander, England Stove Works, Inc., Monroe, VA) was used to generate wood smoke for exposure trials. The wood was western larch (*Larix occidentalis Nutt*) cured to approximately 15% moisture content. The building and maintenance of the fire was kept

consistent throughout the study. The fires were built 25 minutes prior to the start of each exposure trial by using 1kg of wood as well as kindling with 1-2 pages of newspaper. Three hundred grams of wood was then added every 15-20 minutes over the two-hour period. The fire was maintained by removing ash if necessary to ensure that a constant layer of ash (approximately 0.5-1 inch deep) was present.

Exposure Trials

Within the Inhalation and Pulmonary Physiology Core, a wood stove was used to generate wood smoke for the exposure trials. Participants were exposed once to Clean Air (0 $\mu g \cdot m^{-3}$), Low Exposure (250 $\mu g/m^3$) and High Exposure (500 $\mu g/m^3$) woodsmoke particles ($PM_{2.5}$) in a randomized fashion while exercising for 1.5 hours. The Low Exposure concentration (250 $\mu g/m^3$) of woodsmoke particulate matter is comparable to concentrations of woodsmoke recorded when biomass is burned for cooking or other heating purposes in homes lacking ventilation (30) and consistent with the quantity used in other European human/biomass exposure studies (11, 12, 27, 40, 43, 107, 111, 120, 125). The High Exposure level of woodsmoke exposure (500 $\mu g/m^3$) used is comparable to human exposure studies conducted by the EPA's Ghio et al.(2012) (43), in which study participants were exposed to an average concentration of 485 $\mu g/m^3$ over a 2 hour time course. Throughout each trial $PM_{2.5}$ and CO levels were monitored. The concentrations of woodsmoke PM were based on adjustments of two $PM_{2.5}$ monitors linked into the system. The clean air was adjusted manually as a function of observed PM levels just upstream from subject exposure. During exposure, woodsmoke was delivered directly from the dilution and mixing chamber to the subject via a modified mask respirator with continuous $PM_{2.5}$ measurements measured by the DustTrak II (DustTrak, TSI, Model 8530,

Shoreview, MN). The temperature, humidity, carbon monoxide (CO), and carbon dioxide in the mask and exercise room were monitored with a Q-Trak (TSI Inc., Shoreview, MN).

During the smoke exposure, subjects were asked to walk on a treadmill at a set rate and incline of (3.5 mph and 5.7% grade, <57% of estimated VO_2 max) for 1.5 hours (with a short break e.g. 20-30 seconds) every 15 minutes to simulate working on a fire line. Each experimental trial took approximately 1.5 hours.

Blood Samples

For the three exposure trials, blood samples were collected pre- (Pre), post –(Post), 1-(1Hr) hours post exposure from the antecubital vein with sodium heparinized vacutainers (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1000xg for 15 minutes at 4°C during each visit. Plasma was aliquoted and stored immediately at -80°C. Samples were shipped to Auburn University on dry ice and stored at -80°C until subsequent biochemical analysis of oxidative damage and antioxidant biomarkers. Individual aliquots were assayed within a few months of sample collection and were subjected to a single thaw. In an effort to further preserve sample viability, plasma aliquots were kept on ice and in the dark to prevent environmental redox alterations.

Biochemical Panel

To quantify blood oxidative stress a panel of markers was chosen for both oxidative damage and for antioxidant content. The oxidative damage markers were LOOH, PC, 8-ISO, and 3-NT. The antioxidant markers were TEAC, and UA. Additional markers of redox related inflammatory markers included MPO activity and protein content.

Biochemical Assays for Antioxidant Capacity Measures

Trolox equivalent antioxidant capacity (TEAC) was performed to measure the total and non-enzymatic antioxidant capacity. The TEAC assay quantifies any present antioxidants scavenging of 2, 2' azinobis 3-ethyl-benzothiazoline-6-sulfonic acid) radical anions, thus quenching a quantifiable colorimetric reaction. The assay was performed using a 96-well plate (Sigma-Aldrich, St. Louis, MO). The assay work solution was made by combining 2.5 mM Trolox solution (31.1 mg Trolox + 50 ml 10 mM PBS) with 50 mM Glycine buffer (600 ml deionized water + 2.25 g Glycine; pH to 4.4). The glycine peroxidase (122 μl) mixture (5.2 mg peroxidase + 3 mL 50 mM Glycine buffer) was added to the 50 mM glycine buffer. Next ABTS solution (490.0 mg ABTS + 597 ml of glycine buffer with peroxidase solution) was added. The final component added was 375 μl of 22 mM H_2O_2 [20 μl 30% (8.8 mM) H_2O_2 + 8000 μl deionized water, and then combine 375 μL 22mM H_2O_2 to 597 mL Glycine-peroxidase-ABTS solution]. The standards were made by serially diluting the 1.0 mM Trolox standard stock solution (7.5 ml 10 mM PBS + 5 ml 2.5 mM Trolox) (concentrations were measured in μM; 200, 100, 50, 25, 0). See Appendix (D) for stepwise details. Calculated TEAC values for each sample are based on a standard reaction compared to the water-soluble vitamin E analogue Trolox (36).

To measure the catalytic activity of peroxidase generated by H_2O_2 in each sample, the uric acid (UA) assay was performed. The assay was performed using 1.5 ml UV/VIS spectroscopy cuvettes (Sigma-Aldrich, St. Louis, MO). Uric acid stock solution was made by adding 8 ml of (3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) (28 mg MBTH + 200 ml PBS; pH 4.5), 8 ml of 3-(Dimethylamino) benzoic acid (DMAB) (750 mg DMAB + 150 ml PBS; pH 6.5) and 0.9 ml peroxidase (from Horseradish; 5mg + 142.86 ml PBS; pH 8). The standards were made and serially diluted using the uric acid stock solution (17.1 mg uric acid +150 ml deionized water; concentrations were measured in μM: 600, 300, 150, 75,

and 0). A dose (300 ml) of test solution (3 ml of uricase + 30 ml of PBS) or blank (PBS pH 8.0) was added to the cuvette. Next 60 μl of standard or sample was added in 30 second intervals to the appropriate cuvette. The mixture incubated at 37° C for 10 minutes. 480 μl of work solution was added to each cuvette in 30 second intervals and allowed to incubate at 37° C for 15 minutes. Measurements of H_2O_2 were determined by peroxidase catalyzed oxidation of chromogenic and florigenic substrates or by catalysis mediated conversion of alcohols to aldehydes, which were measured spectrophotometrically using a reaction mixture containing 3-methyl-benzothiazoline-2-one hydrazone and 3-dimethylaminobenzoic acid. Final plasma UA values were determined by comparison with internal standard responses (62). See Appendix (D) for stepwise details.

Biochemical Assays for Oxidative Stress

Oxidative damage in blood plasma was quantified using lipid hydroperoxides (LOOH), 8-Isoprostane (8-ISO), protein carbonyls (PC), and nitrotyrosine (3-NT) assays. For PC and 3-NT experiments, plasma sample protein concentrations were analyzed via absorbance spectroscopy according to the methods of Bradford (16). Plasma samples were diluted to 4 $mg \cdot ml^{-1}$ and PC values were measured by a commercially available ELISA kit (Biocell Corporation Ltd, Papatoetoe, NZ) (22). PC standards were oxidized 24 hours prior to the start of the ELISA kit as instructed by the manufacturer (add 25 μl of deionized water to tubes K1-6, and control tube, L incubate overnight at room temperature). On day two, plasma was thawed and Bradford assay was performed to determined protein content for further analysis. In preparation for the Bradford assay, samples were diluted 1:100 with PBS. Bradford reagent (285 μl) was added to each well followed by 15 μl of standard or sample. The plate was allowed to incubate for 20 minutes at room temperature in the dark. Following the incubation period the plate was read at 595 nm (16). Reagents were made according to the kit's instructions and as needed. The

samples were diluted based on the standard procedures for samples containing 4-35 mg protein per ml. Following the ELISA procedure the absorbance was read at 450 nm. See Appendix (D) for more detailed stepwise details.

Plasma LOOH was quantified by implementing the ferrous oxidation-xylenol orange assay. The LOOH standard (9 μl of 80 % cumene + 50 ml of MEOH) was used to serially dilute the standard curve concentrations were measured in mM: 20, 10, 5, 2.5, and 0). The reaction was done within individual 1.5 micro centrifuge tubes (Sigma-Aldrich, St. Louis, MO). 10 μl of TCEP (blank) or MEOH (test) was added in triplicate to each tube. Next, 60 μl of standard or sample was added to the TCEP or MEOH tubes and the tubes incubated for 30 minutes at room temperature. While incubation was ongoing the work solution was made (19.6 ferrous ammonium sulfate + 20 ml of H_2SO_4, + 158.4 mg butylated hydroxytolene +180 ml MEOH + 15.2 mg xylenol orange). Work solution was added (900μL) to each tube in 45-second intervals. The tubes incubated at room temperature in the dark for 45 minutes. At the completion of the incubation time the tubes were centrifuged at 10Xg. The supernatant (830 μl) was decanted into cuvettes. During the reaction oxidized ferrous ions react with the ferrous sensitive dye contained in xylenol orange to form a complex that is quantified through absorbance spectroscopy at a wavelength of 595 nm (86). See Appendix (D) for stepwise details.

Blood plasma 8-Isoprostane concentrations were measured using a specific immunoassay enzyme (EIA) kit (Cayman Chemical, Ann Arbor, MI). The isoprostanes are a family of eicosanoids of non-enzymatic origin that are produced by random oxidation of tissue phospholipids by oxygen radicals (59, 97). 8-isoprostanes have been proposed as a marker of antioxidant deficiency and oxidative stress. Prior research has revealed that elevated levels among heavy smokers. Plasma of healthy volunteers contains modest amounts of 8-isoprostane

(40-100 pg·ml^{-1}). Quantification is based on the competition between 8-isoprostane and an 8-isoprostane-acetylchoinesterase (AChE) conjugate (8-Isoprostane Tracer). Samples were diluted (40 μl sample to 200 μl deionized water). EIA buffer was added (100 μl) to NSB wells and 50 μl to B_0 wells. Standards (100 μl 50 ng·mL^{-1} 8-isoprostane) were made and 50 μl was added to the standard wells. Samples were added (50 μl) to each well. 8-isoprostane tracer was added (50 μl) to each well except TA and Blank. Then, 50 μl of 8-isoprostane antiserum was added to all wells except TA, NSB, and blank wells. The plate then incubated for 18 hours at 4°C. On day 2 the plate was washed and Ellman's reagent was added. The enzymatic reaction is read by spectroscopy at 412 nm. Tracer bound to the well is inversely proportional to the amount of free 8-isoprostane present (79). See Appendix (D) for stepwise details.

The presence of 3-NT in biological fluids such as plasma and/or urine has been indicated as a marker for oxidative stress in vivo (25). Nitrotyrosine is produced by the modification of protein tyrosine residues by peroxynitrite generation via the reaction of nitric oxide and superoxide (25). All reagents were brought to room temperature. Standards were prepared with 1 ml 1X wash buffer by pipetting. Wash buffer (1X) was prepared by adding 25 ml 20X wash buffer to 475 mL deionized water. An 8-point standard curve was then prepared by reconstituting the 3-NT 4000 ng·ml^{-1} stock standard with 1 mL 1X wash buffer. Then serial dilutions were done by first adding 600 μl to tube 2. Next, 300 μl of tube 1 contents were transferred to tube 2; this was repeated for tubes 3-7. Samples were diluted with 1X wash buffer then 50 μl standard or samples were added to each well. Next, each well received 50 μl of 2X HRP Detector Antibody and the plate was allowed to incubate for 2 hours at room temperature on an orbital plate rocker at 300 rpm. Wells were aspirated and washed four times with 300 μl of 1X wash buffer.

Following the wash step, 100 µl HRP Development Solution was added to each well. An immediate color change was recorded at 450 nm. See Appendix (D) for stepwise details.

Biochemical Assays for Redox Sensitive Inflammatory Markers

MPO protein content

Myeloperoxide (MPO) is a heme-containing enzyme belonging to the XPO subfamily of peroxidase (61, 81). MPO binds albumin on the macrophage mannose receptor, cytokerain 1 on vascular endothelial cells, high molecular weight kininogen, and the integrin CD11b/CD18 on neutrophils (17, 128). The interactions promote MPO clearance and a reduction of nitric oxide (17, 61, 81). Quantitative measures of MPO were obtained by an immunoassay (EIA) technique (R&D Systems, Minneapolis, MN), which quantified the bound MPO (17). Samples were prepared by diluting 10-fold (20 µl of sample + 180 µl of Calibrator Diluent RD6-58). Standards were serially diluted according to the manufacturer's directions. All reagents were made according to the directions outlined by the kit's manufacturer. Assay diluent was added (100 µl) to each well. Standard, control or sample was added (50 µl) per well and the plate was then allowed to incubate for 2 hours at room temperature on an orbital shaker. The plate was aspirated and each well was washed with 400 µl of wash buffer. MPO conjugate (200 µl) was added to each well and the plate was allowed to incubate for 2 hours at room temperature on a shaker. The plate was aspirated and washed at the conclusion of the 2-hour incubation period. Following aspiration and wash step, 200 µl substrate solution was then added to each well and the plate incubated for 30 minutes at room temperature, on the benchtop and covered with foil to protect from light. The reaction was stopped within the plate by adding 50 µL of stop solution to each well. Color change was observed. The enzymatic reaction is read by a spectrophotometer at 450 nm. See Appendix (D) for stepwise details.

MPO activity measures

MPO activity was measured using a colormetric activity (EIA) kit (Sigma Aldrich, St. Louis, MO). The kit is designed to detect the formation of hypochlorous acid, which readily reacts with taurine to form taurine chloroamine. Taurine chloramine reacts with the chromophore TNB, which results in a colorless product of DTNB. One unit of MPO activity is defined as the amount of enzyme that hydrolyzes the substrate and generates taurine chloramine to consume 1.0 μmol of TNB per minute at 25° C. Reaction mixture (50 μL) was added to each positive control, sample, and sample blank. Plates were read at 30 minutes, 60 minutes and 120 minutes to ensure values were in linear range. At each sample time 2 μl of stop mix was added. At 120 minutes the plate was read and 2 μl of Stop Solution was added to the plate. The plate was allowed to incubate for 10 minutes, and then 50 μl of TNB reagent or standard was added to the sample and control wells. The plate was allowed to incubate for an additional 10 minutes. Absorbance measures were read at 412 nm. See Appendix (D) for stepwise details.

Statistical Analysis

The purpose of the present study was to examine blood oxidative stress responses of individuals exposed to an episodic woodsmoke event to simulate a field experience. A randomized repeated measures study design was employed to expose individuals to Clean air, 250 $\mu g \cdot m^{-3}$ or 500 $\mu g \cdot m^{-3}$ wood smokes $PM_{2.5}$. Given the full repeated measures study design employed currently, planned comparisons were used to determine differences among the three trials for Pre, Post, and 1HR time points. Additional time-dependent relationships were examined within each of the three trials. Specifically, paired-sample t-tests were conducted to examine oxidative stress outcomes for the key dependent variables (37). All values are presented as means ± standard error (SEM). Significance was set at $p \leq 0.05$ *a priori*.

Results

Participant characteristics and performance data

Study participants' physical characteristics and performance data are presented in **Table 2.** Nine of the ten subjects completed all trials, with one subject completing only the Low Exposure and High Exposure trials. The average body fat for the recruited participants was 14.1% and average aerobic power was of 53.6 $ml^{\cdot -1}kg^{-1}min$. There were no self-reported developments of respiratory related complications by participants following the conclusion of the exposure trials.

Table 2. Participant characteristics and performance data

Characteristics	
Participants (*n*)	10
Age (years)	26.4 ± 3.5
Height (cm)	178.1 ± 3.0
Body mass (kg)	79.0 ± 11.8
Percent fat (%)	14.1 ± 3.4
Exercise performance	
Estimated VO_2 peak ($ml^{\cdot -1}kg^{-1}min$)	53.6 ± 6.9

Plasma antioxidant capacity

Plasma antioxidant capacity assessed by UA and TEAC are presented in **Figures 2-3** respectively. An analysis of combined exposure was also assessed. UA values were largely unaltered by the 3 trials, with the lone difference being a Pre-Post decline during the Low Exposure trial (p=0.042). Given that a dose-response effect was not observed between Low/High Exposure trials, the values were combined and compared to Clean Air using identical analyses described above. Combined Low/High Exposure values were similarly decreased Pre-Post

Exposure (p=0.032). In contrast, plasma TEAC levels were elevated Post (Clean Air and High Exposure, p=0.015 and p=0.001 respectively) and at 1Hr (Clean Air and High Exposure, p=0.001 and p=0.031 respectively). Combined Low/High Exposure analyses were compared to Clean Air using as averaged Low and High Exposures means. There were no observed differences for TEAC between the combined Low/High Exposure trial and Clean Air.

Biomarkers for plasma oxidative damage

Biomarkers for oxidative damage were quantified by the LOOH and 8-ISO, and protein modification biomarkers PC and 3-NT. Mean values are presented in **Figures 4-7**, respectively. Analysis of LOOH findings revealed a decreased 1Hr post (High exposure, p=0.036) compared to baseline values. As described previously, combined exposure analyses were performed and the data reveal a decrease in combined exposure Pre-1Hr post (p=0.011). Analysis of the 8-ISO data revealed a mean Pre-Post increase for both exposure trials (Low Exposure p=0.004, High exposure p=0.009). Combined exposure analysis confirmed the increase from Pre to immediately Post (p=0.002). Plasma PC levels were unaltered in response to any of the 3 exercise/exposure trials, although in combined Low/High Exposure analyses revealed a numerical difference that approached significance as compared to Clean Air (p=0.053). Analysis of the 3-NT assay results indicated Post trial differences existed between Clean Air and High Exposure (p=0.014). The Low Exposure trial yielded a numeric difference between Pre-Post that approached significance (p=0.076), and Clean Air-Low Exposure neared significance for Post (p=0.069) and 1Hr (p=0.088) recovery sample times. More compelling, combined Low/High Exposure analyses elicited a Pre-Post elevation in plasma 3-NT (p=0.049), and Clean Air-Low/High Exposure differences existed Post trial (p=0.012).

Biochemical Assays for Redox Sensitive Inflammatory Markers

Biomarkers for inflammatory response were measured by MPO activity and protein concentration assays presented in **Figures 8-9**, respectively. MPO activity was unaltered by any of the 3-exercise/exposure trials. Analysis of combined Low/High Exposure was also assessed and confirmed the aforementioned negative findings for MPO activity. Analysis of plasma MPO protein concentrations were increased Post Low Exposure and High Exposure ($p=0.035$ and $p=0.019$, respectively) relative to baseline values. Combined exposure analysis confirmed an increase immediately Post ($p=0.005$) as compared to Pre values.

Discussion

Wildland firefighters experience doses of woodsmoke that are potentially detrimental to health, but the extent to which this is true is not well quantified. The current controlled laboratory study was designed to simulate physical activity in wildland firefighters. Participants were exposed to Clean Air and Low/High Exposure woodsmoke concentrations in a randomized crossover fashion. Key findings reveal that modest elevations in oxidative damage markers were observed; 2 of 4 oxidative damage markers (8-ISO and 3-NT) were elevated in a smoke-dependent fashion. Moreover, blood plasma antioxidant values were not consistently depleted following the smoke exposure trials suggesting that the oxidative stress incurred during woodsmoke exposure did not deplete plasma antioxidant capacity appreciably. Finally, redox sensitive markers for inflammation were similarly unimpacted by woodsmoke exposure. The overarching conclusion of the current study is that based on the current observations of elevated plasma 8-ISO and 3-NT following Low and High doses of woodsmoke exposure, we conclude that woodsmoke PM inhalation induces systemic oxidative stress. Our interpretation is that the observed smoke-induced oxidative stress was at best a modest effect, however this finding was probably mitigated by the fact that the simulated exposure trial was as little as 1/10 of the exposure time experienced by firefighters in the field. A detailed discussion of these points follows.

Oxidative damage and controlled woodsmoke exposure

Current results indicate an elevation in the mean value for plasma 8-ISO. Moreover, analyses of 3-NT revealed that combined Low/High exposure results were elevated over the Clean Air trial. Our interpretation that woodsmoke exposure induced oxidative stress is based

collectively on 8-ISO and 3-NT findings, but it should be noted that these effects were independent of smoke exposure dose.

8-ISO is a relatively stable prostaglandin compound produced *in vivo* by free radical-catalyzed peroxidation (79). In contrast to 8-ISO, LOOH values were unaltered by our 3 trials, although given the nature of LOOH reactivity and the dose of woodsmoke given currently, this finding was not altogether surprising. Thus, disparate findings between these two lipid-based oxidative damage markers may be based on the fact that LOOH may be more representative of membrane damage 8-ISO (78, 79). Had our participants been exposed to woodsmoke for durations akin to actual firefighting scenarios, it is likely that quantifiable increases in plasma LOOH would have been observed. In support, Ayciek et al. (2007) found LOOH to be significantly higher in active and passive smokers than in control individuals (7). In the current investigation two indices of protein oxidative damage were also investigated, 3-NT and PC. The key finding in this regard was that plasma 3-NT was elevated following woodsmoke exposure. 3-NT is sensitive to tyrosine modification and is sensitive to oxidative stressors linked to exercise and other stimuli. Xiao et al. (2005), for instance, examined the post-translational modification of protein tyrosine nitration under oxidative stress conditions induced by exposure to diesel exhaust particles. The results revealed 3-NT modifications on ten proteins including GAPDH, MnSOD and components of the proteasome (136). In contrast to 3-NT, plasma PC values were unaltered in the current investigation. The results of this study appear to confirm biochemical understanding of these biomarkers in that tyrosine modifications tend to occur more rapidly to 3-NT in biological systems and may therefore be more sensitive than PC (23). Thus, a longer woodsmoke exposure duration may be imperative to elicit noticeable responses in plasma PC as investigated currently.

Another aspect of the current study design that may have limited the magnitude of oxidative damage pertains to the low intensity of exercise prescribed for the exposure 3 trials. In support, Quindry et al. (2013) previously demonstrated that the magnitude of exercise-induced oxidative stress response is proportional to exercise intensity (102). Previous studies support the notion that increases in oxidative damage markers may be intensity driven. For instance, using two anaerobic exercise protocols it was previously demonstrated that moderate-and-high-intensity exercise resulted in a measurable oxidative stress response (52). More specifically, Bloomer et al. (2004), review concluded that high intensity anaerobic exercise may lead to acute oxidative modifications (14). In contrast, exercise prescribed in the current study did not implement an intense workload. The current methods could be modified to include intermittent bouts of 20-30 seconds of sporadic vigorous activity to simulate the actual in field activity.

Plasma antioxidant content and controlled woodsmoke exposure.

We hypothesized that plasma antioxidants would be decreased in response to woodsmoke exposure. In contrast to our hypothesis for TEAC, we observed a rise in plasma TEAC values that was independent of smoke exposure. Numerous prior findings of elevated antioxidant capacity have been observed previously, but these outcomes are typically associated with long duration (12, 67) or high intensity (52, 77) exercise. However, the low intensity exercise workload was very unlikely to have elicited such a response in these participants. In support, Quindry et al. (2003) found that plasma antioxidant levels were increased only after maximal-intensity exercise (103). The biochemical interpretation of elevated plasma antioxidant capacity is due to acute rises in circulating UA due to accelerated purine metabolism in fatigued skeletal muscle. In support, approximately 20% of the multifaceted TEAC assay value is recognized to be due to UA concentrations (24). However, this explanation is incongruent with

the current data set where modest significant decreases in UA were observed following the Low Exposure trial only. While numeric decreases in UA were observed following the High Exposure trial, they did not achieve statistical significance, a fact that dampens an interpretation of smoke-induced depletion of plasma antioxidant capacity in the current study. As discussed for the oxidative damage markers, there is reason to believe that longer duration smoke exposure may have elicited more consistent alterations in plasma antioxidants. In support, of the current findings Kurmi et al. (2013) found depletions in glutathione when exposed to woodsmoke (64).

Redox dependent inflammation markers

In the current study, we examined the MPO protein content as well as activity to investigate indices of redox related inflammation. Results show that MPO activity was unchanged by the 3 exercise/exposure trials while MPO protein content was only modestly elevated following woodsmoke exposure. While it may be tempting to speculate that elevated MPO protein content is indicative of oxidative stress related to inflammation, the absolute values are still below what is held to be par with "clinically significant" elevations (21, 98). Although we did not find significant alterations in MPO, previous studies implicate MPO measures as an indicator of oxidative stress in acute exercise in humans (103). Naher (2007) attributes short-term exposure to altering the pulmonary immune defense system (82). Quindry et al. (2003) investigated neutrophil-generated superoxide levels and found that they were highest immediately post maximal treadmill exercise (103), a fact that reinforces current recommendations for additional physical activity and longer duration smoke exposure for this type of study design.

Study limitations and recommendations for future research

The current study appears to be limited by smoke exposure time, and perhaps the physical activity duration and intensity. Additionally, smoke dose had no appreciable influence on any biomarker investigated currently. The smoke concentration used in the current study was a low representative dose of wildland firefighting experiences. In support of this, Reinhardt et al. (2004) reported that on average firefighters experience up to 2930 $\mu g \cdot m^{-3}$ of woodsmoke PM, with average levels around 720 $\mu g/m^3$ when fighting wildland fires directly at the fire line (105). The lower concentration of woodsmoke PM in the current study was (250 $\mu g/m^3$), which is comparable to concentrations recorded when biomass is burned for cooking or heating purposes in homes, consistent with European human biomass smoke exposure studies (30). The higher level of exposure (500 $\mu g \cdot m^{-3}$) is comparable to EPA studies, in which subjects were exposed to an average concentration of 485 $\mu g/m^3$ over a two-hour period (43).

In addition to considerations of smoke dose, exposure time was as little as $1/10^{th}$ the duration of common firefighter work shifts (2). Typically, firefighters are exposed to large quantities of woodsmoke combustion over a 12+ hour work shift (1, 2). In 2006, Barregard et al. used a study design similar to the current investigation. However, participants were exposed to clean air or woodsmoke over a 4-hour period. Their findings indicated positive increases in oxidative stress and inflammatory markers (12), which further supports the current recommendation for longer duration exposure. Given that wildland firefighters are known to work extended shifts on consecutive days, the oxidative stress, and workload experienced remains unknown. Thus, within the spirit of imposing a realistic oxidative stress balanced against an ethical exposure dose in research volunteers, similar lab experiments to be conducted should consider an exposure dose of 500 $\mu g \cdot m^{-3}$ and a longer exposure time of approximately 4 hours.

Moreover, we employed an oxidative biomarker panel that included multiple variables for oxidative damage, antioxidant capacity, and redox sensitive inflammation within blood plasma. It is important to note that a ‘positive’ finding of oxidative stress does not necessitate an increase in all of these markers. In support, we have previously identified time-dependent increases in one or multiple biomarkers (10, 52, 74, 91). Moreover, these findings are sensitive to the environment (10, 74, 76, 91, 101) and exercise stimulus (5, 14, 103). However, we are unaware of a related prior study, which uses comprehensive biomarker panel of oxidative stress, where all biomarkers yielded significant alterations along a common time frame. As such, we recommend consideration of additional biomarkers to post-exposure sampling times in order to quantify which biomarkers are most sensitive to the air pollution event. Furthermore, we recommend that in addition to extending the duration of smoke exposure, future investigations of this type should incorporate bursts of higher intensity exercise to simulate vigorous exercise to better simulate the work demands of firefighting.

Conclusions

The average wildland fighter career is estimated to be approximately 8 years (15). While the work is seasonal, the deleterious health implications are clear. The long term goal of this research line is to quantify the impact of woodsmoke exposure through readily identifiable biomarkers, like oxidative stress. Oxidative stress outcomes can then be linked to a host of pathophysiological outcomes. With this better understanding, relationships between smoke-induced oxidative stress and physiologic dysfunction (e.g., respiratory, vascular dysfunction) can be quantified. Further work can then be conducted to understand the extent to which these events occur during actual firefighting. With this collective knowledge, protective equipment and firefighting strategies can be optimized to preserve firefighter health. Future laboratory study is

needed to quantify acute smoke-induced oxidative stress. The biomarker panel employed currently should be extended to observe which biomarkers are most sensitive to the smoke stimulus for oxidative damage, antioxidant capacity and inflammation. As tested under future experimental conditions of longer exposure times and more representative exercise stimuli, these markers should be linked to physiologic outcomes. Once a testing scenario is optimized, extensive field-testing should be performed to quantify the detrimental impact of woodsmoke exposure on oxidative stress.

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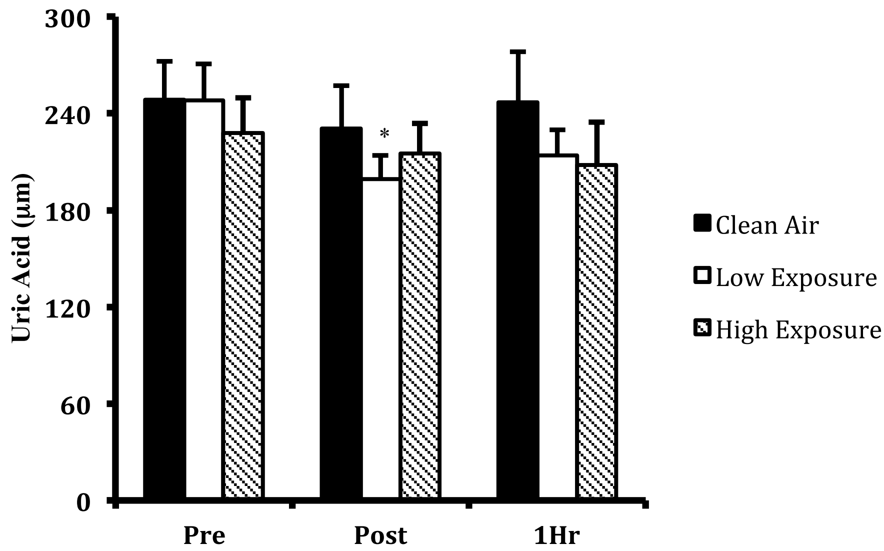
Figure 2 A



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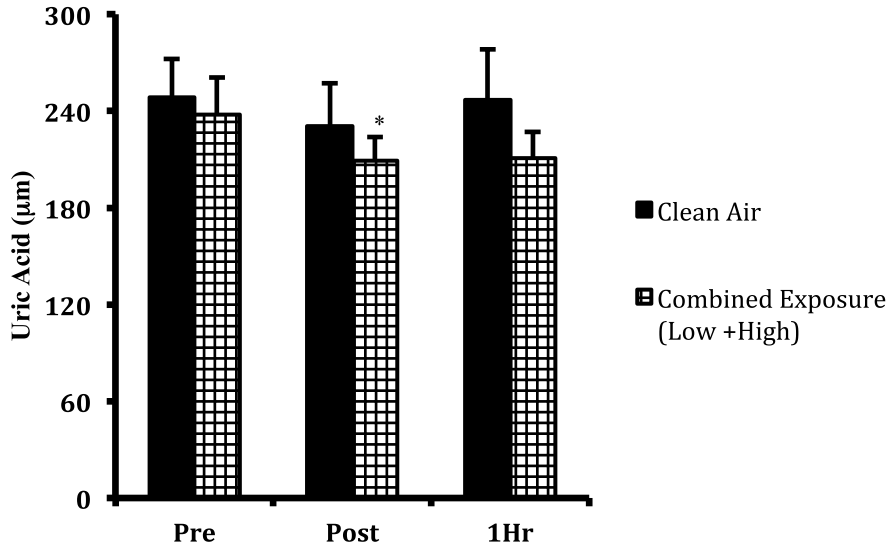

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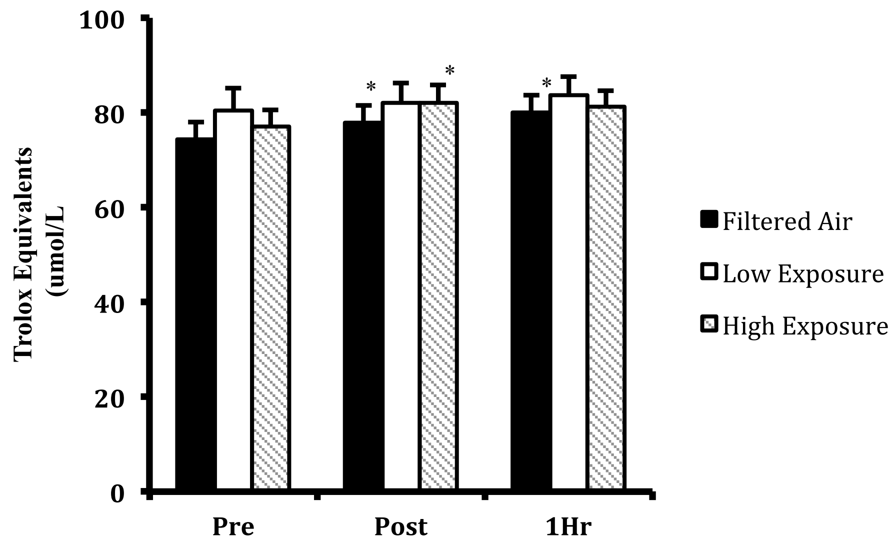

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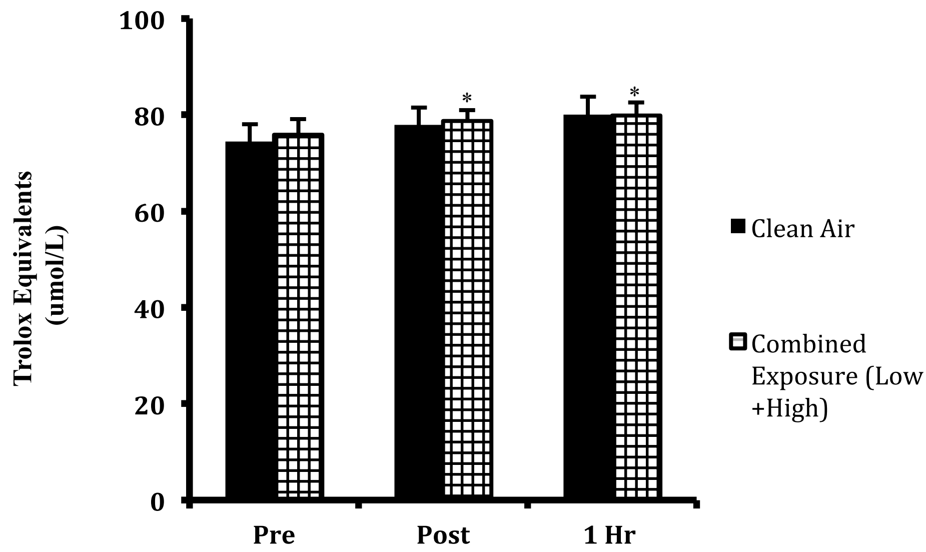

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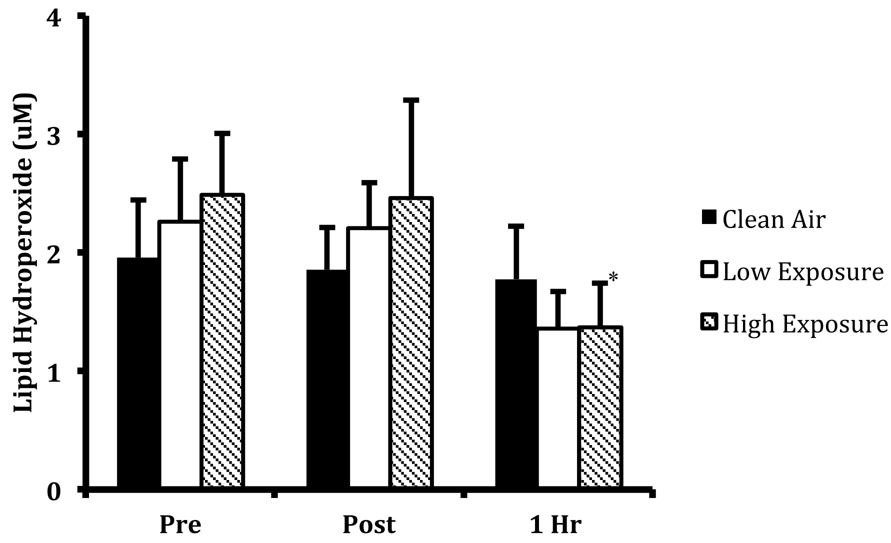

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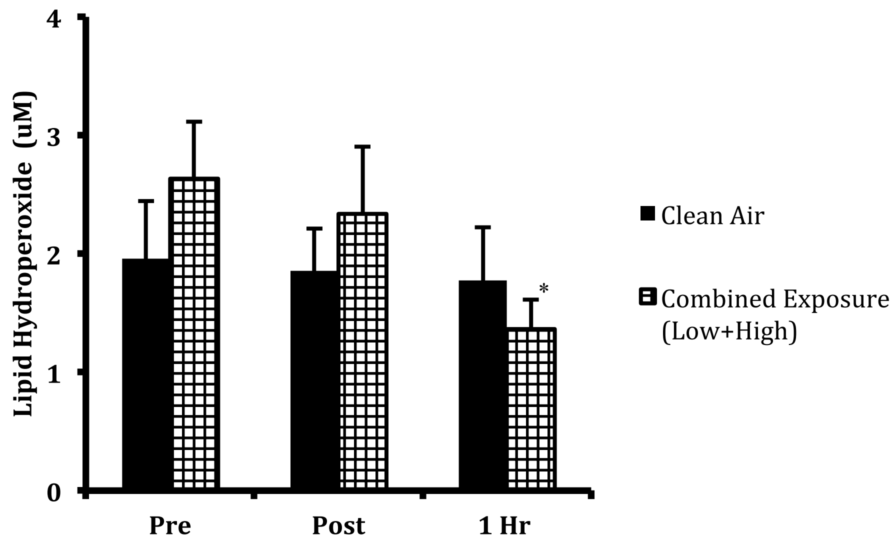

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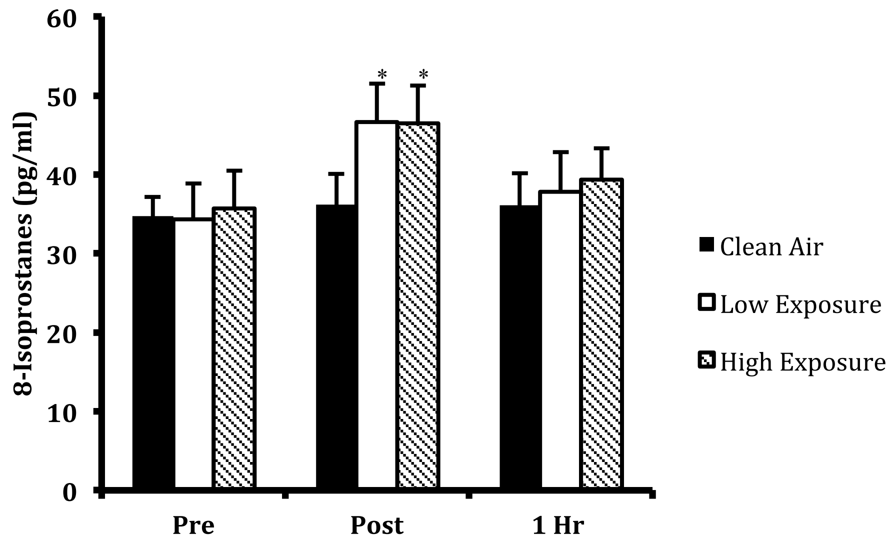

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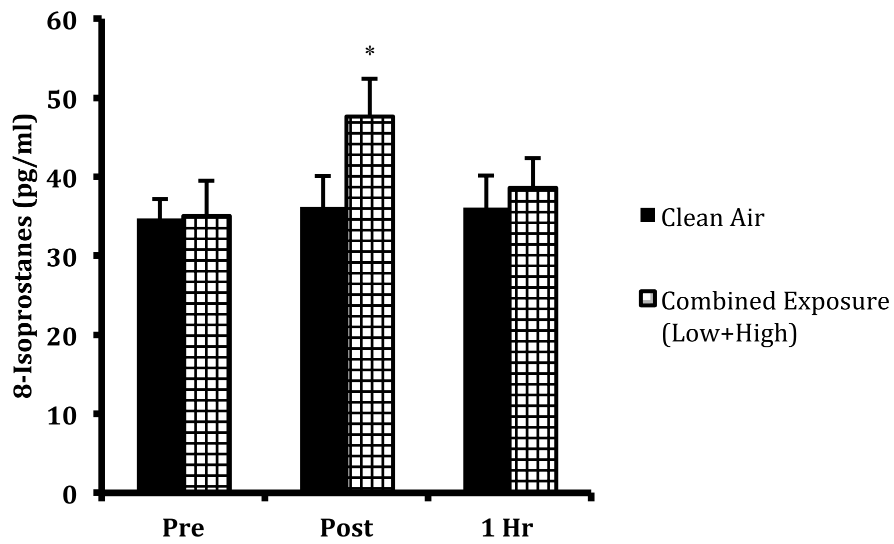

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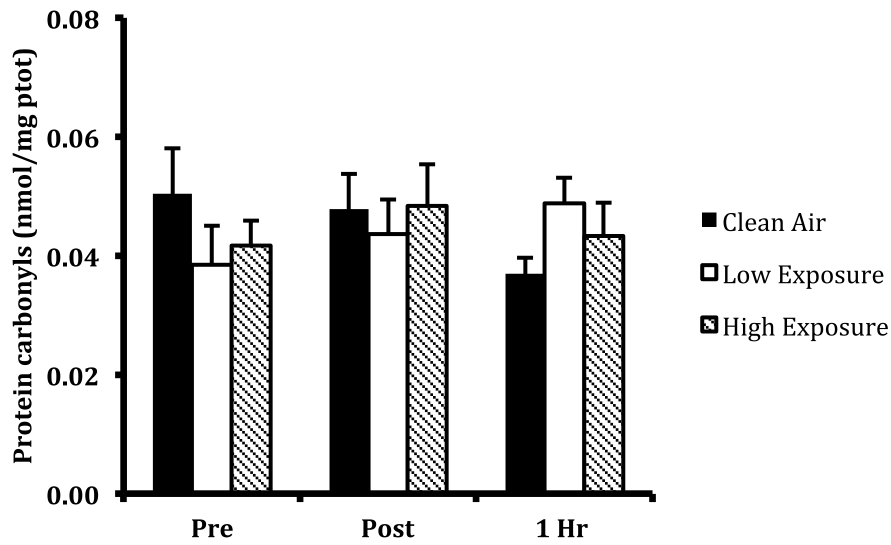

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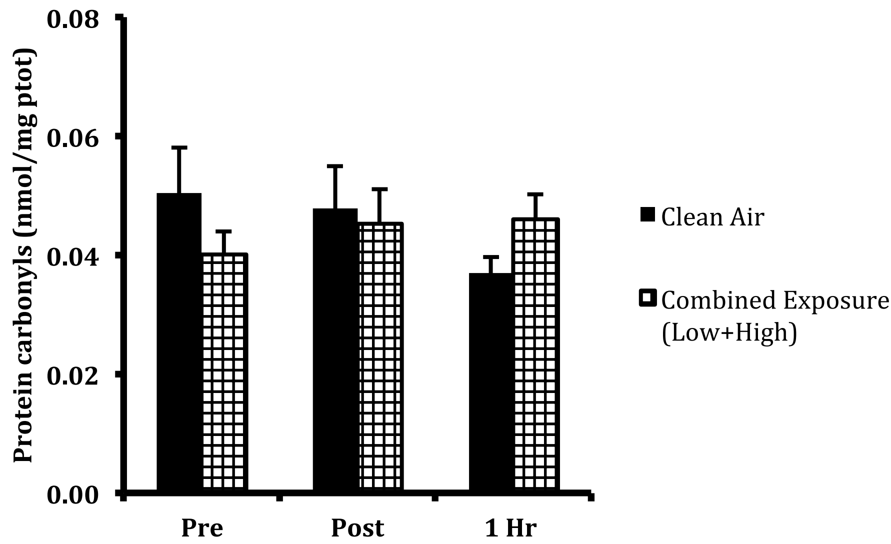

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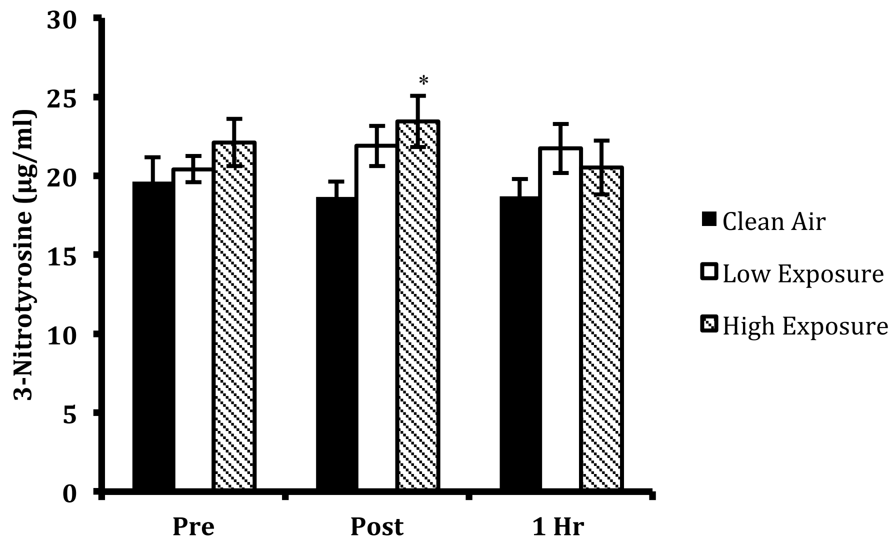

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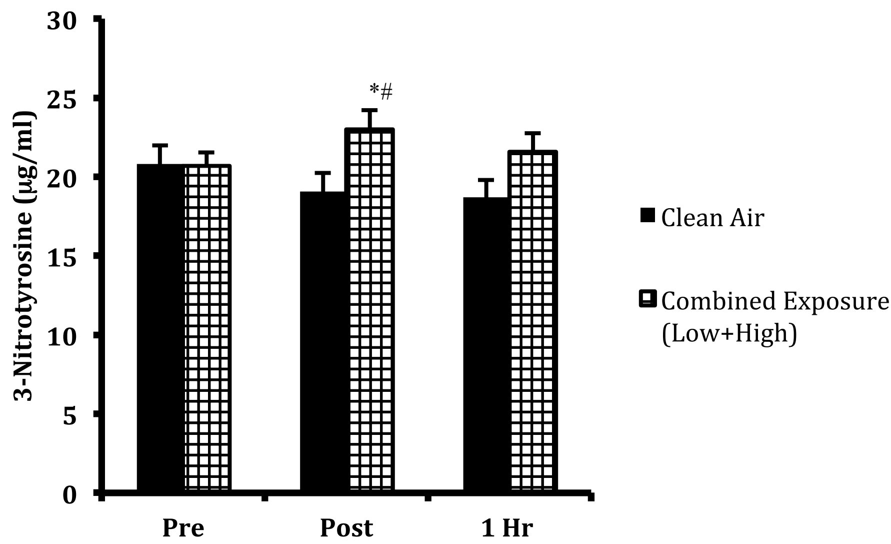

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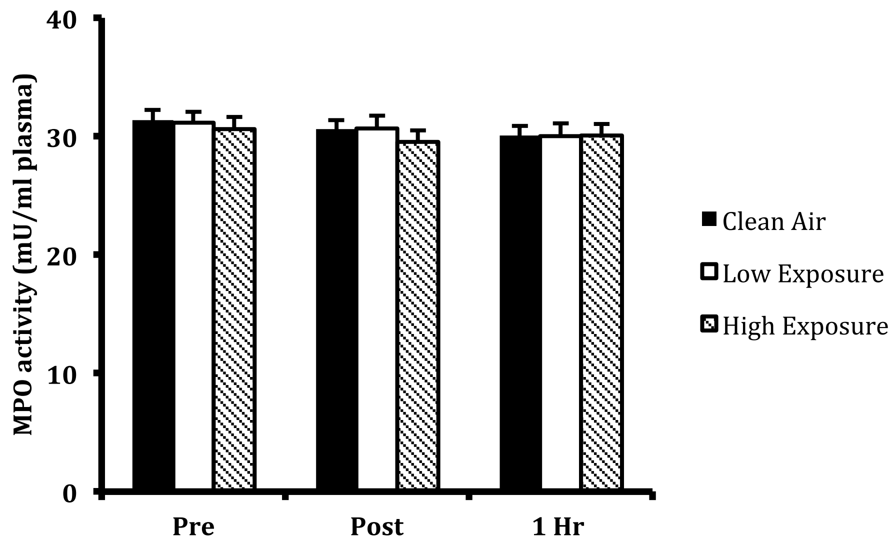

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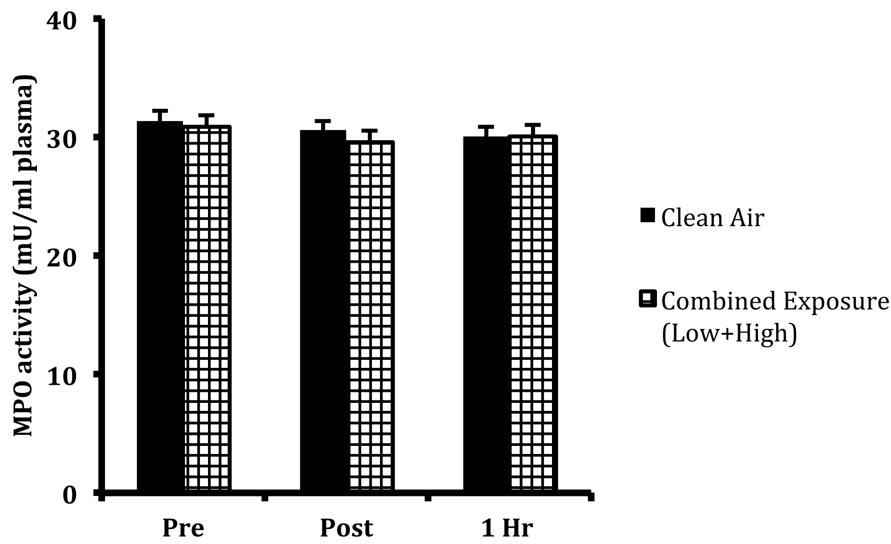

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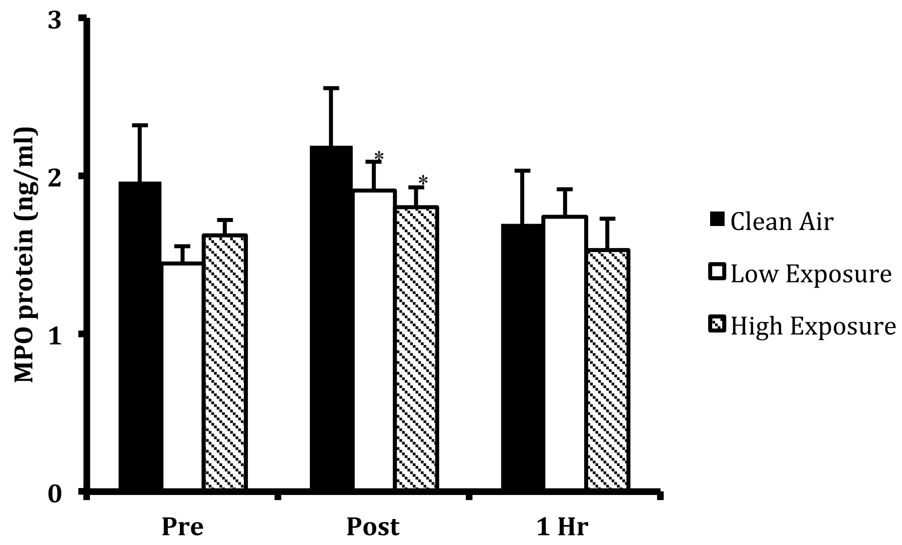

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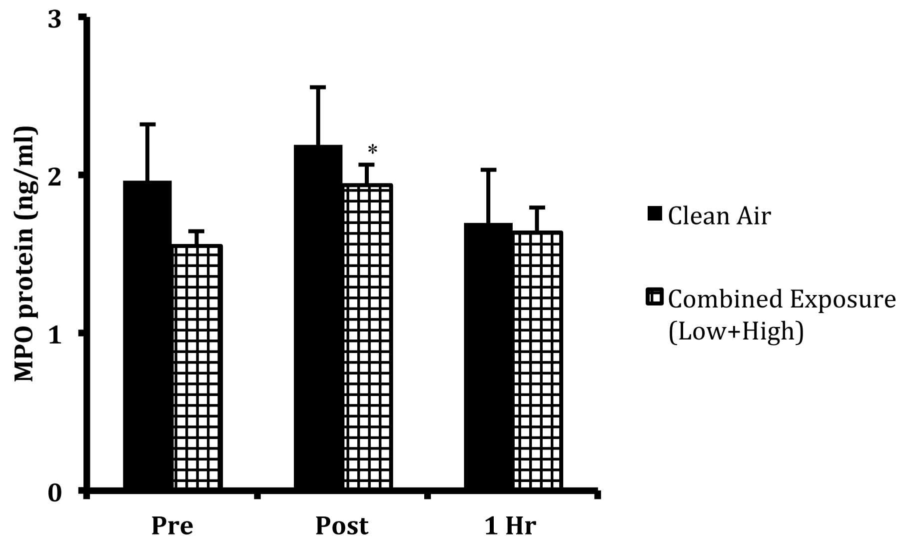

Figure Legends

Figure 1. Study Design. Participants were exposed once to Clean Air, Low Exposure PM 250 μg/m^3, or High Exposure PM 500 μg/m^3 wood smoke $PM_{2.5}$, Trials were performed in a randomized order with one week between each trial. All exposures matched to a common bout of treadmill exercise designed to simulate the intensity of wildland firefighting.

Figure 2. Plasma uric acid values. A. Uric acid values are expressed as Uric Acid equivalents (μM) for Clean Air (*black bars)*, Low Exposure (*white bars)*, and High Exposure (*striped bars)*. **B.** Uric acid values are expressed as Uric Acid equivalents (μM) between Clean Air (*black bars)* and Combined (Low/High Exposure Average, *checkered bars*); values are expressed as Uric acid equivalents (μM). Means are expressed± SEM. *significantly different from Pre; $^{\#}$ significantly different from Post.

Figure 3. Plasma trolox equivalent antioxidant capacity. A. Trolox equivalent antioxidant capacity values are expressed as Trolox equivalent antioxidant capacity equivalents (μmol/L) for Clean Air (*black bars*), Low Exposure (*white bars*), and High Exposure (*striped bars*). **B.** Trolox equivalent antioxidant capacity between Clean Air (*white bars*) an Combined (Low/High Exposure Average, *checkered bars*), values are expressed as Trolox equivalent antioxidant capacity equivalents (μmol/L) Means are expressed ± SEM. *significantly different from Pre; $^{\#}$ significantly different from Post.

Figure 4. Plasma lipid hydroperoxides. A. Lipid hydroperoxides are expressed as lipid hydroperioxide equivalents (μM) for Clean Air (*black bars*), Low Exposure (*white bars*), and High Exposure (*striped bars*). **B.** Lipid hydroperoxide between Clean Air (*white bars*) and Combined (Low/High Exposure Average, *checkered bars*), values are expressed as lipid hydroperioxide equivalents (μM). Means are expressed ± SEM. *significantly different from Pre; $^{\#}$ significantly different from Post.

Figure 5. Plasma 8-Isoprostanes. A. 8-isoprostanes values are expressed in standard comparison to 8-isoprostanes protein content (pg/ml), for Clean Air (*black bars*), Low Exposure (*white bars*), and High Exposure (*striped bars*). **B.** 8-Isoprostanes between Clean Air (*white bars*) and Combined (Low/High Exposure Average, *checkered bars*), are expressed in standard comparison to 8-isoprostanes protein content (pg/ml). Means are expressed ± SEM. *significantly different from Pre; $^{\#}$ significantly different from Post.

Figure 6. Plasma Protein Carbonyls. A. Protein carbonyl values are expressed in standard comparison to Protein Carbonyl equivalents (µM) for Clean Air (*black bars*), Low Exposure (*white bars*), and High Exposure (*striped bars*). **B.** Protein carbonyl values between Clean Air (*white bars*) and Combined (Low/High Exposure Average, *checkered bars*), are expressed in standard comparison to Protein Carbonyl equivalents (µM). Means are expressed ± SEM.

Figure 7. Plasma Nitrotyrosine. Nitrotyrosine values are expressed in standard comparison to Nitrotrysine protein content (µg/ml) for Clean Air (*black bars*), Low Exposure (*white bars*), and High Exposure (*striped bars*). **B.** Nitrotyrosine values between Clean Air (*white bars*) and Combined (Low/High Exposure Average, *checkered bars*) are expressed in standard comparison to Nitrotrysine protein content (µg/ml). Means are expressed ± SEM. *significantly different from Pre; # significantly different from Post.

Figure 9. Plasma Myeloperoxidase Activity. A. Myeloperoxidase activity values are expressed in standard comparison amount of myeloperoxidase activity (mU/ml plasma) for Clean Air (*black bars*), Low Exposure (*white bars*), and High Exposure (*striped bars*). **B.** Myeloperoxidase activity between Clean Air (*white bars*) and Combined (Low/High Exposure Average, *checkered bars*) are expressed in standard comparison amount of myeloperoxidase activity (mU/ml plasma). Means are expressed ± SEM.

Figure 10. Plasma Myeloperoxidase Protein Content. Myeloperoxidase activity values are expressed in standard comparison amount of myeloperoxidase protein content (ng/ml plasma) for Clean Air (*black bars*), Low Exposure (*white bars*), and High Exposure (*striped bars*). **B.** Myeloperoxidase activity between Clean Air (*white bars*) and Combined (Low/High Exposure Average, *checkered bars*) are expressed in standard comparison amount of myeloperoxidase protein content (ng/ml plasma). Means are expressed ± SEM. *significantly different from Pre; # significantly different from Post.

APPENDIX A: Institutional Review Board Study Protocol

SUBJECT INFORMATION AND INFORMED CONSENT

STUDY TITLE: EVALUATION OF EXECUTIVE FUNCTION AND MARKERS OF PULMONARY AND CARDIOVASCULAR STRESS FOLLOWING EXPOSURE TO WOOD SMOKE

SPONSOR: Center for Environmental Health Sciences (CEHS) Pilot Grant Program (*pending approval*)

INVESTIGATORS:
Tony Ward, PhD (406) 243-4092
Charles Dumke, PhD
Erin Semmens, PhD
Joe Domitrovich, PhD
Charles Palmer, PhD

Please read the following information carefully and feel free to ask questions. This consent form may contain words that are new to you. Only sign the final page when you are satisfied procedures and risks have been sufficiently explained.

REQUIREMENTS

This research study requires that you meet the following criteria:

- You must be male, between the ages of 18 and 40, not a regular smoker of any substance, with no signs of cardiovascular risk factors, or pre-existing chronic lung disease, or be exposed to smoke frequently at home or work, and have a maximal oxygen consumption of > 40 ml/kg/min.

PURPOSE OF THE STUDY

This research study is designed to understand the risk of breathing wood smoke on pulmonary, cardiovascular and executive function risk factors.

STUDY PROCEDURES:

You will be asked to participate in three trials, each one occurring one week apart (i.e., Day 2, Day 3, and Day 4). You will be exposed once to either clean air, 250 μg/m^3 or 500 μg/m^3 wood smoke particles in random order. Day 1 of the study will be used to determine eligibility for Days 2-4. Prior to Day 1, you will be called by phone to verify that you have not developed a respiratory infection or other event that would change your health status from the time of enrollment. You will also be reminded to fast for 3 hours before arriving on Day 1. The phone call to check for respiratory infections or other events will be repeated before each day of the study. If you report a change of health status, the visits will be rescheduled.

Day 1: (McGill Hall, ~1.25 hrs) You will be administered oral and written informed consent. You will then be asked to complete a personal information questionnaire and the Physical Activity Readiness Questionnaire (PARQ). You will then undergo a test to determine your maximum oxygen uptake (VO_2 max). This will be used to determine if you are eligible for the study. This test consists of walking and running on a treadmill, which will gradually increase in intensity until you reach a maximal effort. You will be encouraged to continue to walk/run until volitional fatigue. During the entire testing session on

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the treadmill, you will wear a nose clip and headgear that will support a mouthpiece to allow us to measure the amount of oxygen used during the exercise. Heart rate will be measured using an elastic chest strap that is worn under a shirt. You will be asked to fast (not eat) for at least 3 hours prior to this test. This test will take approximately 45 minutes of which 10-20 minutes will be on the treadmill. Also on Day 1, your percentage of body fat will be determined via an underwater weighing test. Your height and weight will first be recorded in a bathing suit. The underwater weight requires that you submerse in our weighting tank (similar to a hot tub) and maximally exhale as much air as possible while underwater and hold your breath for ~4 seconds while we record the weight. This procedure will be repeated until ~3 consistent measurements have been obtained. A nose clip will be provided upon request. This test will take approximately 30 minutes. Following Day 1, if you meet all the inclusion criteria, scheduling will be completed for Days 2, 3, and 4.

Day 2: (Skaggs, ~3 hrs) You will be asked to provide pre-trial samples and undergo baseline tests, consisting of a 1) urine sample, 2) exhaled breath condensate sample, 3) exhaled nitric oxide (eNO), 4) a blood sample, 5) a baseline executive function test, and 6) baseline lung function measurements (see below for details on each type of sample).

Following these pretest measurements you will be exposed to clean air or wood smoke at a concentration of 250 or 500 μg/m^3 for 1.5 hours in a controlled setting. Experimental trials and sample collection will be done in the IPPC facility of the Skaggs Building, rooms 061B and 061C. During exposure, wood smoke will be delivered directly via a modified mask respirator to make it more comfortable. You will be asked to walk on a treadmill at a set rate and incline (3.5 mph and 5.7% grade, <57% of maximum) for 1.5 hours. You will be allowed to ingest water throughout the trials.

Measurements will be done within the exposure chamber to monitor the concentration of wood smoke particles delivered to the modified mask during exposure. Concentrations of smoke will be monitored continuously using a DustTrak II (Model 8530). Temperature, humidity, carbon monoxide, and carbon dioxide in the exposure chamber and the exercise room will be monitored with a Q-Trak. Immediately after the 1.5 hour experimental trials, you will be asked to provide another urine, blood, exhaled breath, and eNO sample, undergo lung function testing, and repeat the executive function test. One hour after completing the trial a third set of biological samples will be collected. After the trial and both sample collection times, the study activities will be complete for the visit.

Days 3 and 4: (Skaggs, ~3 hrs each) You will be asked to arrive at the Skaggs building no less than the seventh day after Day 2 when you will be administered the same protocol as Day 1 at a different randomized exposure, and again on the seventh day (Day 4) after Day 3.

The total time commitment will be approximately 1.5 hours on Day 1 of the study, and approximately 3 hours each on Days 2, 3 and 4. These visits will be carried out over a 4-6 week period. No time will be required outside of what occurs at the study site, except for a brief phone call to verify health status.

Tests 1-5 below will be completed immediately before, after, and 1 hour after the exposure to clean air and wood smoke on Days 2-4.

1) Urine Samples
Urine samples will be collected and stored in laboratories within the Skaggs Building (Center for Environmental Health Sciences, or CEHS) prior to the analysis at the University of Montana for indicators of oxidative stress and creatinine. For this collection you will urinate into a sterile plastic cup.

2) Exhaled Breathe Condensate (EBC)
For collection of EBC, you will be asked to breathe normally into a cold tube for 10–15 minutes. When you breathe into this tube the exhaled breath will condense and we will be able to collect the

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condensate. EBC samples will be analyzed at the University of Montana (CEHS) for indicators of respiratory inflammation, including cytokines.

3) Exhaled Nitric Oxide (eNO)
Exhaled NO will be measured using a NIOX Mino instrument. During eNO measurements, you will be asked to inhale and then exhale for 10 seconds into the instrument.

4) Blood Samples
Approximately 30 milliliters (or 3 tablespoons) of blood will be taken from a vein in your arm under the direction of Dr. Charles Dumke into 3 separate tubes at each time point for a total of 90 ml per visit. Blood samples will be stored in CEHS laboratories prior to analysis of markers of systemic inflammation and cardiovascular risk factors, including blood counts, CRP, clotting factors and cytokines.

5) Lung Function
To obtain this data, you will be asked to forcibly exhale into a device called a spirometer to collect measurements of lung function.

6) Executive Function Tests
You will be asked to complete a computerized executive function test before and after each of the three trials (n=6). The test will be administered via touch screen computer using proprietary software from Cambridge Cognition. The Cambridge Cognition tests consist of four subtests: Attention Switching Task, Reaction Time, Rapid Visual Information Processing, and Spatial Working Memory. Time to complete these tests is approximately 20-25 minutes.

Payment for Participation:
If you complete all aspects of the study, you will be given $300. If you choose not to complete the entire study for any reason, you will be paid $100 for each experimental trial initiated (Days 2,3,4).

Risks/Discomforts:
For this study, we will use clean air, low (250 μg/m^3), and high (500 μg/m^3) wood smoke levels. The lower level of wood smoke particulate matter exposure in this study (250 μg/m^3) is comparable to levels recorded when wood is burned for cooking or heating purposes in homes without sufficient ventilation (200-500 μg/m^3). This lower level of 250 ug/m^3 is also consistent with several other human exposure wood smoke exposure studies that have been reported in the literature. The higher level of exposure (500 μg/m^3) is comparable to human exposure studies where subjects were exposed to an average concentration of 485 ug/m^3 over a two hour period. It should be noted that even higher levels of wood smoke exposure have been used in other wood smoke exposure studies (1500 μg/m^3 for 2 hours). The Forest Service has collected wood smoke exposure data the last four years, with over 300 subjects in 17 States during actual wild land firefighting activities. Their data is showing average PM exposure to be 300-900 ug/m^3, depending on the operational task on the fire (unpublished data).

A wood smoke exposure study reported that the discomfort from smoke was generally weak with exposure at 250 μg/m^3, with the most prevalent symptom being a mild increase in eye irritation in 10 of 13 subjects. A slight increase in nose irritation was reported by 5 of 13 subjects, and 6 subjects reported the smell of wood smoke to be somewhat unpleasant. These subjects were exposed to similar concentrations of particulate matter for 4 hours, more than double the time proposed in this study, so the discomfort experienced should be comparable. The modified mask used in the proposed study covers just the nose and mouth, and would therefore not result in eye irritation. A research assistant will be constantly monitoring both the particulate matter content and your signs of discomfort during all trials.

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To our knowledge no previous literature has assessed changes in executive function following wood smoke exposure in humans. Research suggests that exposure to <300 ug/m^3 does not alter lung function, however little is known about exposures at concentrations that resemble those of wild land firefighters. Firefighters following a shift where they were exposed to 6 hours at wood smoke levels of >1000 µg/m^3 reported 11% difficulty breathing, 13% sore throats and headaches, 20% nasal congestion, 24% cough, 29% sputum production, and 36% were asymptomatic. Dr. Paul Smith (Community Hospital) will be our medical consultant on this project. Dr. Smith is one of only two pediatric pulmonologists in the state and the only pediatric intensive care physician residing in Montana. At his recommendation, if you experience an adverse reaction to wood smoke exposure, the mask will be immediately removed and you will be allowed to rest while breathing clean air. If symptoms persist, you will be escorted to the Curry Health Center for examination and treatment. If necessary, you will be referred to Community Medical Center Emergency room where you will be seen by Dr. Paul Smith.

You may experience discomfort when providing an exhaled breath sample due to the breathing requirements of this technique. When blood samples are collected for this study, you may feel a slight sting or "pinch" in your arm, you may suffer a small bruise, and there is a very slight possibility of infection. Should you notice unusual redness, bruising, or swelling at the blood sampling site you should seek medical attention and contact the study director, Tony Ward.

Benefits:
You will receive no direct benefit from participation in this study. You will receive information on your physical fitness (VO_{2max}), and body composition. However, this research will provide useful information about how exposure to smoke can affect acute changes in decision making and memory and other general health indices. This will be particularly useful for wild land firefighters, who are asked to perform strenuous physical activities while being exposed to wood smoke. Results from this study will also provide additional information on the health effects to the general public following wood smoke exposure.

Confidentiality:
You will be randomly assigned an identification number at the start of the study. All samples, questionnaire responses, physiological measurements, and other data collection forms will be labeled with this number. Personal information will also be collected and linked to this ID number. Data will be recorded on the Data Collection Forms and entered in an electronic database that will contain your ID number and no other personal identifiers. Signed informed consent forms and the key linking the identification number to your name and contact number will be under the control of the principal investigator (Ward) and kept separate from data collection materials. After completion of the study protocol, the key linking identifying personal information to their respective study ID number will be destroyed. A separate sheet containing only the contact information with no identification numbers will be kept until data analysis is complete.

Compensation for Injury:
Although we believe that the risk of taking part in this study is minimal, the following liability statement is required in all University of Montana consent forms: In the event that you are injured as a result of this research you should individually seek appropriate medical treatment. If the injury is caused by the negligence of the University of Montana or any of its employees, you may be entitled to reimbursement or compensation pursuant to the Comprehensive State Insurance Plan established by the Department of Administration under the authority of M.C.A., Title 2, Chapter 9. In the event of a claim for such injury, further information may be obtained from the University's Risk Manager (406-243-2700; kathy.krebsbach@umontana.edu) or the Office of Legal Counsel (406-243-4742; legalcounsel@umontana.edu). (Reviewed by University Legal Counsel, May 9, 2013)

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APPENDIX B: APPROVED CONSENT FORM

SUBJECT INFORMATION AND INFORMED CONSENT

STUDY TITLE: EVALUATION OF EXECUTIVE FUNCTION AND MARKERS OF PULMONARY AND CARDIOVASCULAR STRESS FOLLOWING EXPOSURE TO WOOD SMOKE

SPONSOR: Center for Environmental Health Sciences (CEHS) Pilot Grant Program (*pending approval*)

INVESTIGATORS:
Tony Ward, PhD (406) 243-4092
Charles Dumke, PhD
Erin Semmens, PhD
Joe Domitrovich, PhD
Charles Palmer, PhD

Please read the following information carefully and feel free to ask questions. This consent form may contain words that are new to you. Only sign the final page when you are satisfied procedures and risks have been sufficiently explained.

REQUIREMENTS
This research study requires that you meet the following criteria:

- You must be male, between the ages of 18 and 40, not a regular smoker of any substance, with no signs of cardiovascular risk factors, or pre-existing chronic lung disease, or be exposed to smoke frequently at home or work, and have a maximal oxygen consumption of > 40 ml/kg/min.

PURPOSE OF THE STUDY
This research study is designed to understand the risk of breathing wood smoke on pulmonary, cardiovascular and executive function risk factors.

STUDY PROCEDURES:
You will be asked to participate in three trials, each one occurring one week apart (i.e., Day 2, Day 3, and Day 4). You will be exposed once to either clean air, 250 μg/m^3 or 500 μg/m^3 wood smoke particles in random order. Day 1 of the study will be used to determine eligibility for Days 2-4. Prior to Day 1, you will be called by phone to verify that you have not developed a respiratory infection or other event that would change your health status from the time of enrollment. You will also be reminded to fast for 3 hours before arriving on Day 1. The phone call to check for respiratory infections or other events will be repeated before each day of the study. If you report a change of health status, the visits will be rescheduled.

Day 1: (McGill Hall, ~1.25 hrs) You will be administered oral and written informed consent. You will then be asked to complete a personal information questionnaire and the Physical Activity Readiness Questionnaire (PARQ). You will then undergo a test to determine your maximum oxygen uptake (VO_2 max). This will be used to determine if you are eligible for the study. This test consists of walking and running on a treadmill, which will gradually increase in intensity until you reach a maximal effort. You will be encouraged to continue to walk/run until volitional fatigue. During the entire testing session on

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the treadmill, you will wear a nose clip and headgear that will support a mouthpiece to allow us to measure the amount of oxygen used during the exercise. Heart rate will be measured using an elastic chest strap that is worn under a shirt. You will be asked to fast (not eat) for at least 3 hours prior to this test. This test will take approximately 45 minutes of which 10-20 minutes will be on the treadmill. Also on Day 1, your percentage of body fat will be determined via an underwater weighing test. Your height and weight will first be recorded in a bathing suit. The underwater weight requires that you submerse in our weighting tank (similar to a hot tub) and maximally exhale as much air as possible while underwater and hold your breath for ~4 seconds while we record the weight. This procedure will be repeated until ~3 consistent measurements have been obtained. A nose clip will be provided upon request. This test will take approximately 30 minutes. Following Day 1, if you meet all the inclusion criteria, scheduling will be completed for Days 2, 3, and 4.

Day 2: (Skaggs, ~3 hrs) You will be asked to provide pre-trial samples and undergo baseline tests, consisting of a 1) urine sample, 2) exhaled breath condensate sample, 3) exhaled nitric oxide (eNO), 4) a blood sample, 5) a baseline executive function test, and 6) baseline lung function measurements (see below for details on each type of sample).

Following these pretest measurements you will be exposed to clean air or wood smoke at a concentration of 250 or 500 μg/m^3 for 1.5 hours in a controlled setting. Experimental trials and sample collection will be done in the IPPC facility of the Skaggs Building, rooms 061B and 061C. During exposure, wood smoke will be delivered directly via a modified mask respirator to make it more comfortable. You will be asked to walk on a treadmill at a set rate and incline (3.5 mph and 5.7% grade, <57% of maximum) for 1.5 hours. You will be allowed to ingest water throughout the trials.

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The total time commitment will be approximately 1.5 hours on Day 1 of the study, and approximately 3 hours each on Days 2, 3 and 4. These visits will be carried out over a 4-6 week period. No time will be required outside of what occurs at the study site, except for a brief phone call to verify health status.

Tests 1-5 below will be completed immediately before, after, and 1 hour after the exposure to clean air and wood smoke on Days 2-4.

1) Urine Samples
Urine samples will be collected and stored in laboratories within the Skaggs Building (Center for Environmental Health Sciences, or CEHS) prior to the analysis at the University of Montana for indicators of oxidative stress and creatinine. For this collection you will urinate into a sterile plastic cup.

2) Exhaled Breathe Condensate (EBC)
For collection of EBC, you will be asked to breathe normally into a cold tube for 10–15 minutes. When you breathe into this tube the exhaled breath will condense and we will be able to collect the

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condensate. EBC samples will be analyzed at the University of Montana (CEHS) for indicators of respiratory inflammation, including cytokines.

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Exhaled NO will be measured using a NIOX Mino instrument. During eNO measurements, you will be asked to inhale and then exhale for 10 seconds into the instrument.

4) Blood Samples
Approximately 30 milliliters (or 3 tablespoons) of blood will be taken from a vein in your arm under the direction of Dr. Charles Dumke into 3 separate tubes at each time point for a total of 90 ml per visit. Blood samples will be stored in CEHS laboratories prior to analysis of markers of systemic inflammation and cardiovascular risk factors, including blood counts, CRP, clotting factors and cytokines.

5) Lung Function
To obtain this data, you will be asked to forcibly exhale into a device called a spirometer to collect measurements of lung function.

6) Executive Function Tests
You will be asked to complete a computerized executive function test before and after each of the three trials (n=6). The test will be administered via touch screen computer using proprietary software from Cambridge Cognition. The Cambridge Cognition tests consist of four subtests: Attention Switching Task, Reaction Time, Rapid Visual Information Processing, and Spatial Working Memory. Time to complete these tests is approximately 20-25 minutes.

Payment for Participation:
If you complete all aspects of the study, you will be given $300. If you choose not to complete the entire study for any reason, you will be paid $100 for each experimental trial initiated (Days 2,3,4).

Risks/Discomforts:
For this study, we will use clean air, low (250 μg/m^3), and high (500 μg/m^3) wood smoke levels. The lower level of wood smoke particulate matter exposure in this study (250 μg/m^3) is comparable to levels recorded when wood is burned for cooking or heating purposes in homes without sufficient ventilation (200-500 μg/m^3). This lower level of 250 ug/m^3 is also consistent with several other human exposure wood smoke exposure studies that have been reported in the literature. The higher level of exposure (500 μg/m^3) is comparable to human exposure studies where subjects were exposed to an average concentration of 485 ug/m^3 over a two hour period. It should be noted that even higher levels of wood smoke exposure have been used in other wood smoke exposure studies (1500 μg/m^3 for 2 hours). The Forest Service has collected wood smoke exposure data the last four years, with over 300 subjects in 17 States during actual wild land firefighting activities. Their data is showing average PM exposure to be 300-900 ug/m^3, depending on the operational task on the fire (unpublished data).

A wood smoke exposure study reported that the discomfort from smoke was generally weak with exposure at 250 μg/m^3, with the most prevalent symptom being a mild increase in eye irritation in 10 of 13 subjects. A slight increase in nose irritation was reported by 5 of 13 subjects, and 6 subjects reported the smell of wood smoke to be somewhat unpleasant. These subjects were exposed to similar concentrations of particulate matter for 4 hours, more than double the time proposed in this study, so the discomfort experienced should be comparable. The modified mask used in the proposed study covers just the nose and mouth, and would therefore not result in eye irritation. A research assistant will be constantly monitoring both the particulate matter content and your signs of discomfort during all trials.

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To our knowledge no previous literature has assessed changes in executive function following wood smoke exposure in humans. Research suggests that exposure to <300 ug/m^3 does not alter lung function, however little is known about exposures at concentrations that resemble those of wild land firefighters. Firefighters following a shift where they were exposed to 6 hours at wood smoke levels of >1000 μg/m^3 reported 11% difficulty breathing, 13% sore throats and headaches, 20% nasal congestion, 24% cough, 29% sputum production, and 36% were asymptomatic. Dr. Paul Smith (Community Hospital) will be our medical consultant on this project. Dr. Smith is one of only two pediatric pulmonologists in the state and the only pediatric intensive care physician residing in Montana. At his recommendation, if you experience an adverse reaction to wood smoke exposure, the mask will be immediately removed and you will be allowed to rest while breathing clean air. If symptoms persist, you will be escorted to the Curry Health Center for examination and treatment. If necessary, you will be referred to Community Medical Center Emergency room where you will be seen by Dr. Paul Smith.

You may experience discomfort when providing an exhaled breath sample due to the breathing requirements of this technique. When blood samples are collected for this study, you may feel a slight sting or "pinch" in your arm, you may suffer a small bruise, and there is a very slight possibility of infection. Should you notice unusual redness, bruising, or swelling at the blood sampling site you should seek medical attention and contact the study director, Tony Ward.

Benefits:
You will receive no direct benefit from participation in this study. You will receive information on your physical fitness (VO_{2max}), and body composition. However, this research will provide useful information about how exposure to smoke can affect acute changes in decision making and memory and other general health indices. This will be particularly useful for wild land firefighters, who are asked to perform strenuous physical activities while being exposed to wood smoke. Results from this study will also provide additional information on the health effects to the general public following wood smoke exposure.

Confidentiality:
You will be randomly assigned an identification number at the start of the study. All samples, questionnaire responses, physiological measurements, and other data collection forms will be labeled with this number. Personal information will also be collected and linked to this ID number. Data will be recorded on the Data Collection Forms and entered in an electronic database that will contain your ID number and no other personal identifiers. Signed informed consent forms and the key linking the identification number to your name and contact number will be under the control of the principal investigator (Ward) and kept separate from data collection materials. After completion of the study protocol, the key linking identifying personal information to their respective study ID number will be destroyed. A separate sheet containing only the contact information with no identification numbers will be kept until data analysis is complete.

Compensation for Injury:
Although we believe that the risk of taking part in this study is minimal, the following liability statement is required in all University of Montana consent forms: In the event that you are injured as a result of this research you should individually seek appropriate medical treatment. If the injury is caused by the negligence of the University of Montana or any of its employees, you may be entitled to reimbursement or compensation pursuant to the Comprehensive State Insurance Plan established by the Department of Administration under the authority of M.C.A., Title 2, Chapter 9. In the event of a claim for such injury, further information may be obtained from the University's Risk Manager (406-243-2700; kathy.krebsbach@umontana.edu) or the Office of Legal Counsel (406-243-4742; legalcounsel@umontana.edu). (Reviewed by University Legal Counsel, May 9, 2013)

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Voluntary Participation/Withdrawal:
It is important that you realize that you are free to withdraw from the study at any time. If you chose to withdraw please notify Tony Ward (contact info below) as soon as possible. As mentioned above, even if you decide to drop out of the study, you will receive compensation for all the test sessions you complete. A copy of this consent form will be provided for you at your request. In addition, the data collected during this study will be done at no cost to you.

Questions:
You may wish to discuss this with others before you agree to take part in this study. If you have any questions about the research now or during the study contact Dr. Tony Ward, PhD at (406) 243-4092 (tony.ward@umontana.edu). If you have any questions regarding your rights as a subject, you may contact the chair of the IRB through the University of Montana Research Office at (406) 243-6672.

Statement of Your Consent:

I have read the above description of this research study. I have been informed of the risks and benefits involved, and all my questions have been answered to my satisfaction. Furthermore, I have been assured that any future questions I may have will also be answered by a member of the research team. I voluntarily agree to take part in this study.

____
Printed Name of Subject

______________________________ ____________________
Subject's Signature Date

STATEMENT OF CONSENT TO BE PHOTOGRAPHED DURING DATA COLLECTION

During the study, I understand that pictures may be taken. I provide my consent to having my picture taken during the course of the research study. I provide my consent that my picture may be used in some presentations related to this study. If pictures are used at any time for presentation, names and physiological data will not be associated with them.

Signature ______________________________ Date __________

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APPENDIX C: Physical Activity Readiness Questionnaire (PAR-Q) and You Document

Physical Activity Readiness Questionnaire (PAR-Q) and You

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly:

YES	NO		
☐	☐	1.	Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?
☐	☐	2.	Do you feel pain in your chest when you do physical activity?
☐	☐	3.	In the past month, have you had chest pain when you were not doing physical activity?
☐	☐	4.	Do you lose your balance because of dizziness or do you ever lose consciousness?
☐	☐	5.	Do you have a bone or joint problem that could be made worse by a change in your physical activity?
☐	☐	6.	Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?
☐	☐	7.	Do you know of any other reason why you should not do physical activity?

If you answered:

YES to one or more questions

Talk to your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want – as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:

- Start becoming much more physically active – begin slowly and build up gradually. This is the safest and easiest way to go.
- Take part in a fitness appraisal – this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively.

Delay becoming much more active:

- If you are not feeling well because of a temporary illness such as a cold or a fever – wait until you feel better; or
- If you are or may be pregnant – talk to your doctor before you start becoming more active.

Please note: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed use of the PAR-Q: Reprinted from ACSM's Health/Fitness Facility Standards and Guidelines, 1997 by American College of Sports Medicine

APPENDIX D: BIOCHEMICAL ASSAYS STEPWISE PROCEDURES

Uric Acid Assay

Modified by: Bridget Peters and Hayden Hyatt (5.21.2013)
References: Kovar KA, el Bolkiny MN Rink R, Hamid MA. An enzymatic assay for the colorimetric and fluorimetric determination of uric acid in sera. Arch Pharm (Weinheim). 1990 Apr;323(4):235-7

Solutions:

- **Uricase** (MP biochemcials ;**Cas no. 9002-12-4**)
- **DMAB** (3-(Dimethylamino) benzoic acid; **Cas no. 99-64-9**):
  - 750mg + 150 mL PBS (pH 6.5)
    20 mL aliguots
  - Store at -20°C
- **MBTH** (3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate; **Cas no. 38894-11-0**):
  - 28 mg + 200 ml PBS (pH 4.5)
- **Peroxidase** (from Horseradish; **Cas no. 900-3-99-0**)
  - 5 mg + 142.86 ml PBS (pH 8)
  - 1ml aliquot store at -80
- **Final Uric Acid Work Solution:**

Solution	Volume
MBTH	8mL
DMAB	8mL
Peroxidase	0.9 mL

-Test: 10 mL PBS (pH 8.0) + 1 mL Uricase 1.30/mL
-Blank: 10mL PBS (pH 8.0)

Standards:

- Uric Acid Stock Solution: 17.1 mg Uric Acid + 150 mL dH2O
- Serial dilute to obtain different concentrations

[Uric Acid] (uM)	Amount of solution (mL of uM)	dH20 (mL)
600	0	0
300	5	5
150	5	5
75	5	5
0	0	5

Trolox-Equivalency Antioxidant Capacity (TEAC) Assay

Modified by: Lindsey Miller (1-26-10), Christopher Ballmann (5-9-12)

References: Re et al. 1999, Villano 2004

Solutions:

- 2.5 mM Trolox Solution
  - 31.1 mg Trolox + 50 mL 10 mM PBS
  - Needs to be made first due to length of time it takes to go into solution
  - Will be used to later make STDs
- 50 mM Glycine Buffer
  - LIGHT SENSITIVE (cover beaker with foil)
  - 600 mL dH2O + 2.25 g Glycine
  - Adjust to pH of 4.4
- 211 ug/ 550 mL peroxidase
  - 5.2 mg peroxidase + 3 mL 50 mM Glycine Buffer
  - Extract 122 uL of Glycine-peroxidase mixture and add to the 597 mL of 50 mM Glycine buffer.
- ABTS Solution
  - Weigh 490.0 mg ABTS
  - Add to your ~597 mL of Glycine Buffer peroxidase solution and stir.
- 22 mM H2O2
  - 20 ul 30% (8.8 mM) H2O2 + 8000 uL dH2O
  - Add 375 uL 22 mM H2O2 to ~597 mL of Glycine Buffer peroxidase ABTS solution
  - **Color change from green to blue! (Final Working Solution!)**

Standards:

- Make 1.0 mM Trolox solution from 2.5 mM Trolox solution
  - 7.5 mL 10 mM PBS + 5 mL 2.5 mM Trolox = 1.0 mM Trolox solution
  - Use 1.0 mM Trolox solution to make STD curve
  - Serial dilute to obtain different concentrations

[STD]	PBS (uL)	1.0 mM Trolox (ul)
200	800	200
100	900	100
50	950	50
25	975	25
0	1000	0

- Add 20 uL of STD to 250 uL of Final Working Solution
  - 20 uL STD from pre-plate!
- Incubate 2 mins
- Read @ 414 nM
- Record in Excel

Samples:

- Plasma should be diluted 1:5 for best results
    - 20 uL Plasma + 80 uL 10 mM PBS
- Plasma readings should fall within range of STDs.

Reagent Info:

- Glycine (Sigma # G-8890)
- Horseradish peroxidase Type VI-A (Sigma # P6782)
- ABTS (Sigma # A1888)
- H_2O_2 30% 8.8 mM (Sigma # 216763)
- Trolox (Sigma # 23, 881-3)

FOX assay for blood plasma, serum

Modified by: John Quindry – 6-13-02

References: Nourooz-Zadeh, et al. 1995. Elevated levels of authentic plasma hydroperoxides in NIDDM. *Diabetes,* 44; 1054-1058

See also: Nourooz-Zadeh, et al. 1994 Measurement of plasma hydroperoxide concentrations by the ferrous oxidation-xylenol orange assay in conjunction with triphenylphosphine. *Analytical Biochemistry*, 220:403-409.

Wolf, S. 1994. Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement in hydroperoxides. *Methods in Enzymology*, 233; 182-189.

Stock solutions, reagents:

TCEP / reducing agent **– tris(2-carboxyethyl) phosphine HCl, mw=286.65 (20mM) Mix 57.3 mg into 10ml dH_2O. Make aliquots of 1ml and store at –80^oC**

Xylenol orange / chromogen– o-cresolsulfonpthalein-3,3 bis(methytimmodiacetric acid), mw=760.6

Butylated hydroxytoluene – mw=220.36 (4mM) 79.2mg into 90ml MeOH
H_2SO_4 - mw=98 (250 mM) - 6.8ml into 493ml dH_2O

Ferrous ammonium sulfate - ($Fe(NH_4)_2(SO_4)_2*6H_2O$), mw=392.1 (2.5mM) – 9.8mg into 10 ml H_2SO_4. The solution is stable for at least 10 days under argon at 2-8^oC in dark.
Methanol (HPLC grade)

Cumene hydroperoxide - (mw=152.2) – Make stock solution of 1mM. To make stock sol. dissolve 18µl (Aldrich brand 80%, #**24,750-2**) into 100ml methanol.

Work solution: make during first incubation

1. **Combine ferrous ammonium sulfate (10ml) with BHT (90ml)**
2. **Add 7.6 mg xylenol orange to this solution**
   Final concentrations are: 250µM ferrous ammonium sulfate, 100µM xylenol orange, 25mM H_2SO_4, 3.6mM BHT.

Procedures:

- Use freshly separated plasma or plasma stored at -80^oC. Freeze–thaw of samples should be avoided. NOTE - Untreated plasma (chelators, antioxidants, etc.) produces the best results.

1. Add 10µl **TCEP** ("BLANK") or 10µl methanol ("TEST") to 1.5ml microcentrifuge vials.
2. Add 90µl sample to 1.5ml microcentrifuge vials.
3. Cap vials, vortex, and incubate at room temperature for 30 minutes (make work solution)

4. Add 900μl **work solution** to each vial, vortex and incubate for 45 minutes at room temperature
5. Centrifuge at 12K x g for 15 minutes
6. Transfer 830 μl supernatant to cuvette for absorbance reading at 560.

Bradford Assay

1. Make Standards:
   Standard stock solution: 15 mg Bovine Serum Albumin (Sigma A- 7906) + 12 mL 10 mM PBS = 1.25 mg/mL
   Serial dilution from stock solution

[BSA] (mg/ml)	**Amount of solution (mL)**	**PBS (mL)**
1.25	7	0
0.625	7	7
0.312	7	7
0.156	14	7
0	0	7

2. Dilute plasma samples 1:100 in PBS

   -990 µl 10 mM PBS + 100 µl plasma

3. Add 285 µl of Bradford reagent +15 µl standard/sample to a 96 well plate
4. Incubate in dark at room temperature for 20 minutes
5. Read absorbance at 595 nm

8-Isoprostane ELISA Kit

1. Prepared standards
    a. 100 μl 50 ng/ml 8-isoprostane + 900 μl deionized water= 5ng/ml (Stock standard)
    b. Serial dilute from stock solution

[8-ISO] (pg/ml)	Amount of solution (μL)	EIA buffer (μL)
200	500	750
80	500	750
32	500	750
12.8	500	750
5.1	500	750
2.0	500	750
0.8	500	750

2. Prepared 8-isoprostane AchE tracer
    a. 6 ml of EIA buffer +100 dtn 8-isoprostane AchEtracer
    b. Add 60 μl of dye to 6 ml of tracer
3. Made 8-isoprostane EIA Antiserum
    a. 6ml of EIA buffer +100 dtn 8-isoprostane antiserum
    b. Add 60 μl of dye to 6 ml of tracer
4. Diluted plasma by adding 40 μl sample to 200 μl deionized water
5. Loaded plate
    a. Added 100 μl EIA buffer to NSB and 50 μl EIA to B_0 wells
    b. Add 50 μl diluted plasma to each well
    c. Add 50 μl of AchE tracer to each well except TA and Blank wells
    d. Add 50 μl EIA Antiserum to each well except TA, NSB, and Blank wells

6. Incubate for 18 hours at 4°C
7. Reconstitute Ellman's reagent
    a. 100 dtn vial Ellman's reagent with 20 ml of deionized water
    b. Light sensitive, cover vial with foil
8. Aspirate wells and wash plate 5 times with 200 μl of wash buffer
9. Add 200 μl of Ellman's reagent to each well
10. Add 5 μl of AchE tracer to TA wells
11. Incubate on circular shaker for 90 minutes in dark
12. Read at 405 nm

Protein Carbonyl ELISA Kit

1. Oxidize standards overnight by adding 25μl if deionized water to each tube
    a. Vortex
    b. Leave on benchtop at room temperature
2. Diluted plasma according to Bradford assay results
3. Derivatisation step
    a. Add 200 μl diluted- DNP to each tube
    b. Add 50 μl diluted sample to each tube + standards
4. Incubate for 45 minutes
5. Reaction Step
    a. Add 1 ml of EIA buffer to each a set of 1.5 ml tubes
    b. Add 5 μl of derivatisation sample/standard to each tube
6. Add 200 μl of each sample/standard to plate
7. Incubate overnight
8. Wash plate 5 times with 300 μl of EIA buffer
9. Add 250 μl of diluted blocking buffer to each well on plate
10. Incubate at room temperature for 30 minutes
11. Repeat wash step, Step 8
12. Add 200 μl of diluted anti-DNP antibody to each well
13. Incubate at 37 °C for 1 hour
14. Repeat wash step, Step 8
15. Add 200 μl of diluted streptavidin-HRP to each well
16. Incubate at room temperature for 1 hour
17. Repeat wash step, Step 8
18. Ass 200 μl chromatin reagent to each well
19. Read absorbance at 650 nm

Nitrotyrosine ELISA Kit

1. Make standards
   a. Suspend 1000X BSA in 1 ml 1X wash buffer = 1.25 standard
   b. Add 600 μl 1X wash buffer to tubes 2-7

[3NT] (pg/ml)	Amount of solution (μL)	1X Wash Buffer (μL)
2000	300	200
666.7	300	200
222.2	300	200
74.1	300	200
24.1	300	200
8.2	300	200
2.7	300	200

2. Diluted plasma according to Bradford assay results
   a. 1:100 10 μl plasma :1000 μl PBS +.1 EDTA(1mM)
3. Load 50 μl diluted standard/sample
4. Add 50 μl 2X HRP Detector Antibody to each well (standards/wells)
5. Incubate for 2 hours at room temperature on shaker
6. Aspirate and wash plate with 300 μl of 1 X wash buffer
7. Add 100 μl HRP Development Solution to each well
8. Immediately record results at 600 nm.

Myleoproxidase Activity Optimization Practice

1. Bring all MPO Buffer to room temperature
   a. Vortex
   b. Leave on benchtop at room temperature
2. Make MPO substrate solution (light sensitive)
   a. 50 µl of MPO substrate into 300 µl of deionized water
3. Made standards
   a. Add appropriate MPO buffer to each well
   b. Note: TNB Reagent will be added later in experiment (See Step 14)

MPO Buffer Solution (µl)	TNB Reagent (µL)
150	0
130	20
120	30
110	40
100	50

4. Made Reaction Mixes (Did not do blank wells)
   a. Sample Mix
      i. 640 µl of MPO buffer +160 µl MPO substrate
5. Loaded differing amounts of plasma 50, 25, 12.5, 6.25, 3.13 µl to each well
6. Brought sample to total volume of 50 µl with MPO Buffer
7. Add 50 µl Reaction Mix with Substrate to each Sample well (Sample Mix)
8. Incubate for 30 minutes in dark at room temperature
9. Reconstitute Stop Solution
   a. Add 200 µl of deionized water + 20 µl Stop Mix
10. Add 2 µl Stop Solution to Samples (Do not add to standards)
11. Add 50 µl TNB Reagent to sample
12. Read at 412 nm
13. Incubate for 60 minutes in dark at room temperature
14. Add 2 µl Stop Solution to Samples (Do not add to standards)
15. Add 50 µl TNB Reagent to sample
16. Read at 412 nm
17. Incubate for 120 minutes in dark at room temperature
18. Add 2 µl Stop Solution to Samples and blanks (Do not add to standards)
19. Add 50 µl TNB Reagent to sample
20. Bring Standards to a total volume of 150 µl with TNB Reagent
21. Incubate for 10 minutes in dark
22. Read at 412 nm

Myleoproxidase Activity ELISA Kit

1. Bring all MPO Buffer to room temperature
2. Make MPO standards
    a. Reconstitute MPO standard with 1.0 ml of deionized
    b. Reconstitution brings the stock solution to 100 ng/ml
3. Made standards
    a. Add appropriate MPO buffer to each well
    b. Note: TNB Reagent will be added later in experiment (See Step 14)

MPO Buffer Solution (µl)	TNB Reagent (µL)
150	0
135	15
110	40
100	50
75	75
50	100
30	120
10	140

4. Made Reaction Mixes (doubled amounts)
    a. Sample Mix
        i. 2040 µl of MPO buffer +510 µl MPO substrate
    b. Blank Mix
        i. 1040 µl of MPO buffer + 260 µl deionized water
5. Loaded 37.5 µl to each well
6. Loaded plasma (12.5 µl) to appropriate sample well
7. Add 50 µl Reaction Mix with Substrate to each Sample well (Sample Mix)
8. Add 50 µl of Reaction Mix with deionized water (Blank Mix) to each well
9. Incubate for 120 minutes in dark at room temperature
10. Reconstitute Stop Solution
    a. Add 200 µl of deionized water + 20 µl Stop Mix
11. Add 2 µl Stop Solution to Samples and blanks (Do not add to standards)
12. Incubate in dark for 10 minutes
13. Add 50 µl TNB Reagent to sample/blank wells
14. Add TNB Reagent to Standards according to the Standards chart (See Step 3).
15. Incubate for 10 minutes in the dark
16. Read at 412 nm

Myleoperoxidase Protein ELIZA Kit

1. Bring all reagents to room temperature
2. Made MPO standard (100ng/ml)
    a. Reconstitute MPO standard with 1.0 ml of deionized water
3. Made MPO standards
    a. Add 900 µl of Calibrator Diluent RD6-8 into 10 ng/ml standard (See Step 2)
    b. Add 500 µl of Calibrator Diluent into remaining tubes including 0
    c. Serial dilute according to chart below

[MPO] (ng/ml)	MPO Standard (µL)	Calibrator Diluent (µL)
10	100	500
5	500	500
2.5	500	500
1.25	500	500
0.625	500	500
0.313	500	500
0.156	500	500
0	0	500

4. Prepared 25 X Wash Buffer
    a. Diluted 20 ml of Wash Buffer concentrate into deionized water to prepare 500 ml of Wash Buffer
5. Diluted plasma samples 10-fold
    a. 20 µl sample + 180 µl of Calibrator diluent RD6-58
6. Add 100 µl of Assay Diluent to each well
7. Add 50 µl standard/sample to appropriate well
8. Incubate at room temperature for 2 hours on orbital rocker
9. Aspirate and wash wells with 300 µl of Wash Buffer 4 times
10. Add 200 µl MPO Conjugate to each well
11. Incubate at room temperature for 2 hours on orbital shaker
12. Repeat wash step (See Step 9)
13. Made Substrate Solution (make within 15 minutes of use)
    a. Mixed Color Reagent A and Reagent B
14. Add 200 µl Substrate Solution to each well
15. Incubate for 30 minutes at room temperature on bench top (light sensitive)
16. Add 50 µl of Stop Solution to each well
17. Read at 450 nm