A Report from the University of Vermont Transportation Research Center

Biological Inflammatory and Metabolic Effects of Petro- and Bio-diesel Exhaust Particulate Matter Emissions from a Light-Duty Diesel Engine

TRC Report 15-009

June 2015

Fukagawa, Holmen

UVM TRC Report # 15-009

FINAL TECHNICAL REPORT

TRC Project #026904

Biological Inflammatory and Metabolic Effects of Petro- and Bio-diesel Exhaust Particulate Matter Emissions from a Light-Duty Diesel Engine

University of Vermont Transportation Research Center

June 16, 2015

Prepared by:

Naomi K. Fukagawa, MD PhD, Principal Investigator
Professor Emerita of Medicine
UVM College of Medicine

Britt A. Holmén, PhD, Co-Principal Investigator
Professor, Civil and Environmental Engineering
UVM School of Engineering

Transportation Research Center
Farrell Hall
210 Colchester Avenue
Burlington, VT 05405

Phone: (802) 656-1312
Website: www.uvm.edu/trc

ABSTRACT

Sustainability of our transportation system depends on making well-informed choices on vehicle energy sources for human and goods mobility. Motor vehicles operating on fossil fuels are a significant source of air pollution risk and challenge the ability of humans to mitigate climate change. Biodiesel is a low carbon fuel substitute for petroleum diesel, but relatively little is known about how exposure to biodiesel combustion particles affects chronic diseases such as asthma and type II diabetes mellitus (T2DM). This study examined the effect of particulate matter (PM) generated by the combustion of commercially available petrodiesel and soybean biodiesel fuels on inflammation and metabolic dysfunction using two types of *in vivo* experiments. To evaluate inflammation response, oropharyngeal 3-day exposures of females to B20 (20% biodiesel/80% petrodiesel by volume) and B0 (100% petrodiesel) particles from a light-duty diesel engine operating on a semi-transient cycle were followed by lung fluid and tissue biochemical analysis. Prenatal exposure effects on offspring growth and metabolism were evaluated by maternal exposure during gestational days 9-17 (delivery 18-20 days) and monitoring offspring for 12 months. Prenatal exposure to exhaust PM from petrodiesel fuel combustion had different metabolic effects in male vs. female offspring. For B20 exhaust PM, the number of offspring was too low to detect differences. The results of this study suggest that males and females may have differential risk for metabolic dysfunction after prenatal exposure during gestation. Future studies should be conducted to quantify sex-specific effects on exposure to vehicle exhaust PM from biodiesel fuel blends.

Acknowledgements

This work would not have been possible without the dedication and efforts of the following members of the UVM Transportation Air Quality (TAQ) Laboratory who assisted in development and implementation of the CM-12 exhaust emissions sampling system: Tyler Feralio, Jim Dunshee, Karen Sentoff, Phil Cannata and John Kasumba are gratefully acknowledged for engine emissions testing and sample collection and analysis. U.S. DOT support to the PIs under Project TRC024 via the UVM TRC is gratefully acknowledged.

Disclaimer

The contents of this report reflect the views of the authors, who are responsible for the facts and the accuracy of the data presented herein. The contents do not necessarily reflect the official view or policies of the UVM Transportation Research Center. This report does not constitute a standard, specification, or regulation.

Table of Contents

1.0 Overview

This final report summarizes the results of investigations of the biological effects associated with exposure to particles from light-duty diesel engine exhaust. Particles were generated by the co-PI via tailpipe emissions testing on the light-duty diesel engine in the UVM Transportation Air Quality Laboratory with two fuels: petroleum diesel (petrodiesel) and a 20% blend of soybean-derived biodiesel (B20). The particles from these exhaust streams were provided to the PI for the experiments described here.

More information on the detailed procedures associated with engine emissions testing and particle characterization can be found in the UVM Transportation Research Center reports TRC #14-008 and #14-009.

2.0 Introduction and Project Objective

A significant public health issue is the nature of how chronic exposure to environmental toxins such as vehicle exhaust may predispose individuals to chronic diseases such as asthma and type II diabetes mellitus (T2DM). Recent concerns about air quality, climate change, the health effects of petroleum diesel particulate matter (PM) as well as reducing dependence on foreign sources of petroleum have drawn attention to the use of biofuels. Understanding how the products of the combustion of different fuel sources (petro- and bio-diesel) affect susceptibility to chronic disease will have broad implications. For example, technology solutions such as new engines or exhaust systems to reduce harmful products of combustion may be developed; or new therapies targeted at specific inflammatory pathways and/or based on nutritional intervention may be identified.

The outcomes of this work have implications for the UTC theme of “sustainable systems and advanced technologies for northern communities” because of the necessity to transport food and fuel to northern New England communities and the potential for the development of novel local sources of biofuels (e.g. sunflowers, algae, grasses, waste grease) that may prove to be sustainable and economically viable, thus creating independence from petroleum or soy-based biodiesel from large, commodity-based farming enterprises. The impact of emissions on health has broad implications for the people living in and beyond New England communities.

This study directly addresses the health effects of exposure to tailpipe emissions and the yet unexplored area of the effects of emissions on inflammatory and metabolic responses. Specifically, the research objective was to compare and contrast the effects of inhalation of exhaust generated by the combustion of commercially available petrodiesel and soybean biodiesel fuels on inflammation (lung and systemic) and metabolic outcomes associated with risk for glucose intolerance/reduced insulin sensitivity.

3.0 Research Approach

The overall plan was to expose a commonly used animal model to exhaust generated by the combustion of petro- and bio-diesel fuels and to compare and contrast biological

responses. Concurrently, the emissions were characterized for particle size and composition as described in UVM TRC Report #14-009.

3.1 PM Generation and Exposures

Animal Model: C57BL6 mice were used because many published studies have utilized this strain and they represent the background strain for several knockout models that may be useful in future studies (e.g. Nrf2 knockout).

Exposure to Petrodiesel and Biodiesel Exhaust: In early experiments, mice were placed in glass inhalation chambers and diluted exhaust from a diesel engine operating on petro- or biodiesel fuel was flowed through the chamber at equivalent particle number concentrations for 1.5 h/day, three to five days a week, mimicking exposure by humans commuting to work. Real-time monitoring of particle size and concentration were conducted using a TSI Engine Exhaust Particle Sizer (EEPS) and Electrical Aerosol Detector such that known particle sizes were administered to the mice. The particles were also collected on filters for chemical characterization. Control mice were similarly exposed to the dilution air only (i.e., engine OFF) on each day of exposure. The particle number and size of the combustion exhaust and dilution air were monitored during the exposures. The biodiesel fuel was soy-based because it is commercially available and ASTM-grade. The engine was a light-duty, 1.9 L Volkswagen diesel engine outfitted on an engine dynamometer to enable realistic loading of the engine.

Unfortunately, the exposures conducted in the glass inhalation chambers did not induce the expected inflammatory responses in 3 separate experiments. Possible reasons for the negative results included:

1. Behavioral responses of the mice to reduce their inhalation of exhaust (huddling in the chamber or changing respiratory parameters);
2. Possible inconsistent delivery of exhaust to the chambers because of intermittent obstruction or gas/particle dynamics in the transfer lines;
3. Unknown properties of the fuels testing that led to less “toxic” exhaust, possibly due to raw exhaust dilution conditions.

Consequently, after year 2, the oropharyngeal route of administration of particulate matter was used as we have previously reported (Fukagawa et al., 2013) using the side-by-side impinger samples (ethanol as the collection fluid) of raw exhaust.

Exhaust Particle Size Distributions and Air Toxic Emissions for Biodiesel Blends: Number-based particle size distributions were measured simultaneously with gaseous air toxics emissions using state-of-the-art real-time particle sizing (TSI, Inc. Model 3090 Engine Exhaust Particle Spectrometer,EEPS) and Fourier transform infrared (MKS MultiGas HG-2030 HS) instruments in Dr. Holmén’s laboratory. Emissions measurements were collected at 1-sec resolution during each test cycle repetition to accurately characterize peak emissions events associated with transient engine operation. Particle number distributions and 32 air toxic gas (i.e., acetaldehyde, benzene, formaldehyde, etc.) concentration were compared and total particle concentrations in various size-fractionated bins were recorded to normalize exposure study results. All particle and gas sampling were conducted using QA/QC procedures routinely used in the Holmén research laboratory. The quality control program ensured the reliability and

validity of data through validation of sampling and analysis equipment, involving blank, duplicate and standard measurements. Pre- and post-sampling verification of the EEPS and SMPS using particles of known size were conducted in Dr. Holmén's laboratory. Daily operational checks for the particle instruments included zero check, leak check, and background scanning. Data were archived on a daily basis and examined prior to the next test day to ascertain any testing anomalies. These data were combined into a database that can be used to develop quantitative relationships between measured exhaust particle number distributions and (a) engine operating conditions and (b) biologic response. To provide further insight into the mechanisms of biologic responses, a subset of exhaust particulate matter samples were collected for detailed organic speciation via thermal desorption-GCMS techniques used in the Holmén laboratory to quantify individual nonpolar (alkanes, PAHs) and polar (aldehydes, ketones, carboxylic acids, quinones) compounds.

Preparation of Particle Stock Suspensions for oropharngeal aspiration: Raw exhaust particles collected in ethanol impingers were concentrated via gentle N_2 blowdown to generate stock suspensions of approximately 1 mg/ml. PM concentrations of impinger suspensions were determined in triplicate using gravimetric analysis (Cahn C-31 microgram balance, 0.001 mg sensitivity) of 100uL aliquots prepared prior to, during, and after dilution of the stock suspension with Milli-Q water to obtain aqueous solutions with final ethanol concentrations less than 10% v/v to avoid cell death (Castilla et al., 2004).

In Vivo Particle Exposures: *In vivo* experiments used female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, n=6 per group) exposed to B20 and B0 particles (~84 µg/treatment) or vehicle control (50 µl 8% ethanol) administered via the oropharyngeal (OP) aspiration (Fukagawa et al, 2013). Dose was based on previous petrodiesel studies (Lewis et al., 2007; Yoshizaki et al., 2010: Nemmar et al., 2009). For the OP treatments, mice were anesthetized with inhaled isoflurane (to effect) and suspended by their incisors on a 60° incline board. The tongue was gently extended and 50 µl of the stock solution particles delivered into the distal part of the oropharynx and aspirated into the lower respiratory tract. Mice were allowed to aspirate the material for approximately 15 seconds, after which they were returned to the cage and monitored until *fully* recovered. The dose of particles delivered was determined as the mean gravimetric mass of triplicate 50 µl aliquots of the stock suspension on the day of use. Mice were euthanized after 3 consecutive days of OP exposure and lungs were lavaged for evaluation of cell counts and biological measures as previously described (Lakatos et al., 2006; Fukagawa et al., 2008). Protein was isolated from the right lung lobes using standard techniques. These experiments evaluated whether particles from the combustion of either fuel resulted in unique responses associated with development of pulmonary inflammation.

For experiments examining the effects of prenatal exposure, pregnant mice were exposed during gestational days 9-17 and allowed to deliver between gestational days 18-20. Offspring was followed for 12 months to determine whether the nature of the exposure influenced growth and metabolic parameters.

3.2 Biological Assays

Animal bronchoalveolar lavage fluid (BALF) and lung tissue proteins were examined for inflammatory mediators and specific proteins related to disease pathogenesis.

Cytokines: Bronchoalveolar lavage fluid (BALF) obtained from the mice at necropsy were frozen at -80° C until analysis. Lung tissue lysates were prepared by T-PER Tissue Protein Extraction Reagent complemented with Halt Protease and Phosphatase Inhibitor (Thermo Scientific). Cytokines and chemokines in BALF and lung tissue lysates were analyzed using both the Bio-Plex Pro™ Assay (Bio-Rad) and Milliplex Map® Assay (Millipore). Mouse samples were analyzed using the Milliplex-22 murine cytokine kit. All samples were prepared according to the manufacturer's instructions and ran on the Bio-Plex suspension array system (Bio-Rad). Standard curves were calculated and samples were analyzed using the Bio-Plex Manager Software Version 6 (Bio-Rad).

Western-blot: The antibodies for total or phospho-ERK1/2, caspase-1, total or phospho-EGFR (Tyr1068) were purchased from Cell Signaling; the antibody for TLR4 and Nrf2 was from Santa Cruz; the antibodies for GCLC were from Abnova and β-actin was from Abcam Inc. 20 μg of each total protein was electrophoresed on 10% or 7.5% Mini-PROTEAN® TGX™ precast gels (Bio-Rad) and then electroblotted onto nitrocellulose or PVDF membranes. After blocking of membranes with 1% BSA blocking/dilution buffer, the membranes were incubated with the appropriate primary antibody at the recommended concentration overnight with shaking at 4° C and then rinsed. After incubation with corresponding secondary antibodies, the protein bands were visualized using SuperSignal™ West Pico Trial Kit (PIERCE) and exposed to radiographic films. The images and densities were captured with a GS-700 Imaging Densitometer (Bio-Rad, Richmond, CA) and analyzed with Quantity One Software Version 4.2 (Bio-Rad, Richmond, CA). The membranes were stripped and then reimmunoblotted with another antibody or β-actin antibody as a loading control.

4.0 Results and Discussion

4.1 Preliminary Studies

Preliminary studies in preparation for this study culminated in two publications (abstracts provided):

Fukagawa NK, Li M, Poynter ME, Palmer BC, Parker EP, Kasumba J, Holmén B. Soy Biodiesel and Petrodiesel Emissions Differ in Size, Chemical Composition and Stimulation of Inflammatory Responses in Cells and Animals. Environ Sci Technol 2013; 47:12496-12504. dx.doi.org/10.1021/es403146c |PMID:24053625.

Debate about the biological effects of biodiesel exhaust emissions exists due to variation in methods of exhaust generation and biological models used to assess responses. Because studies in cells do not necessarily reflect the integrated response of a whole animal, experiments were conducted in two human cell lines representing bronchial epithelial cells and macrophages and female mice using identical particle suspensions of raw exhaust generated by a Volkswagen light-duty diesel engine using petrodiesel (B0) and a biodiesel blend (B20: 20% soy

biodiesel/80% B0 by volume). Tailpipe particle emissions measurement showed B0 generated two times more particle mass, larger ultrafine particle number distribution modes, and particles of more nonpolar organic composition than the B20 fuel. Biological assays (inflammatory mediators, oxidative stress biomarkers) demonstrated that particulate matter (PM) generated by combustion of the two fuels induced different responses in in vitro and in vivo models. Concentrations of inflammatory mediators (Interleukin-6, IL-6; Interferon-gamma-induced Protein 10, IP-10; Granulocyte stimulating factor, G-CSF) in the medium of B20-treated cells and in bronchoalveolar lavage fluid of mice exposed to B20 were 20– 30% higher than control or B0 PM, suggesting that addition of biodiesel to diesel fuels will reduce PM emissions but not necessarily adverse health outcomes.

Traviss N, Li M, Lombard M, Thelen BA, Palmer BC, Poynter ME, Mossman BT, Holmén BA, Fukagawa NK. Petrodiesel and Waste Grease Biodiesel (B20) Emission Particles: Characterization and Effects on Lung Epithelial Cells and Macrophages. Air Quality, Atmosphereand Health 2014; 7:59-70. DOI 10.1007/s11869-013-0231-x.

Diesel engine emissions are an important source of ultrafine particulate matter (PM) in both ambient air and many occupational settings. Biodiesel is a popular 'green' alternative to petroleum diesel fuel, but little is known about the impact of 'real-world' biodiesel combustion on workplace PM concentrations and particle characteristics including size, morphology, and composition; or on biological responses. The objectives of the present work were to characterize PMworkplace concentrations and tailpipe emissions produced by the combustion of commercially purchased low sulfur petrodiesel and a waste grease B20 blend (20 % biodiesel/80 % petrodiesel by volume) in heavy duty diesel (HDD) nonroad equipment operating in a 'realworld' rural recycling center. Furthermore, we assessed the in vitro responses of cell lines representing human lung epithelial cells (BEAS-2B) and macrophages (THP-1) after 24 h of exposure to these real-world particles. Compared to petroleum diesel, use of B20 in HDD equipment resulted in lower mass concentrations of PM2.5, PM<0.25 (particle diameter less than 2.5 and 0.25 μm, respectively), and elemental carbon. Transmission electron analysis of PM showed that primary particle size and morphology were similar between fuel types. Metals composition analysis revealed differences between fuels, with higher Fe, Al, V, and Se measured during B20 use, and higher As, Cd, Cu, Mn, Ni, and Pb concentrations measured during petrodiesel use. In vitro responses varied between fuels but data supported that waste grease B20 particles elicited inflammatory responses in human macrophages and lung epithelial cells comparable to petrodiesel particles. However, the effects were more pronounced with B20 than petrodiesel at the same mass concentration. Since the primary particle size and morphology were similar between fuels, it is likely that the differential results seen in the in vitro assays points to differences in the composition of the PM. Future research should focus on the organic carbon and metals speciation and potential impact of real-world particles on reactive oxygen species generation and mechanisms for differences in the cellular inflammatory responses.

The initial experiments were plagued by technical difficulties and no detectable physiological responses were identified in mice exposed to exhaust when compared to no exhaust. These included protein expression of signaling pathways of interest. In addition, pulmonary function could not be assessed because of technical issues related to the size of the mice. In order to focus on other outcome measures that may be affected by inhalation of exhaust, the decision was made to determine the differential effects on metabolic parameters associated with risk for diabetes mellitus and obesity.

4.2 Metabolic Dysfunction in Offspring of Mothers Exposed to B0 and B20 Particles

To test the hypothesis that prenatal exposure to particulate matter from petrodiesel combustion leads to alterations in weight (WT) gain and insulin sensitivity, pregnant C57BL/6J mice were exposed daily to <10% ethanol (control, CON) or to ~80 ug of PM suspended in <10% ethanol, mimicking daily ambient exposure, via oropharyngeal aspiration from gestational day 9-17 and allowed to deliver. Experiments were carried out for PM from petrodiesel (B0) and B20 soybean biodiesel combustion. Mice were fed standard chow after weaning. Weekly body WT, food intake (FI), and bi-weekly fasting glucose levels were measured for 44 wks. While FI was similar between the control and treated groups, female offspring (F) of mothers exposed to B0 gained less WT than CON ($p<0.001$) whereas male offspring (M) began gaining more WT than CON after ~24 wks of age (Figure 1A and 1B) below:

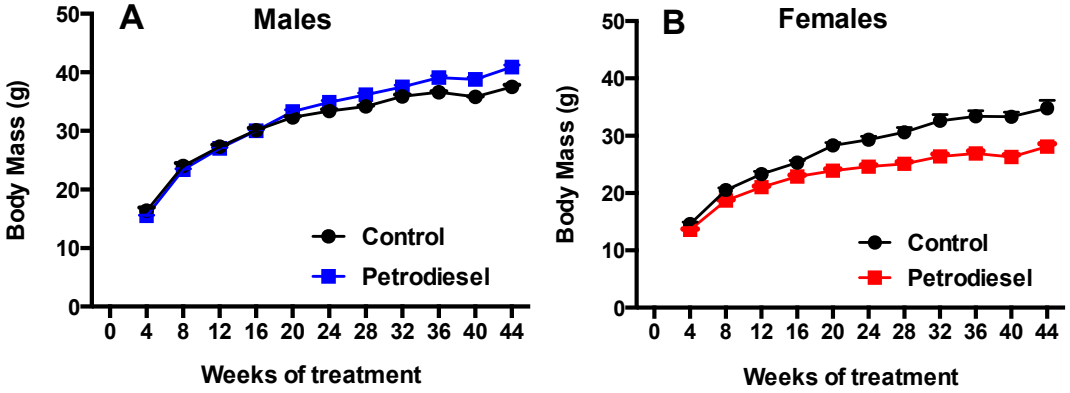

Figure 1. Body mass of petrodiesel offspring over 44 weeks. Although no differences were observed in food consumption, B0 females gained *less* weight than controls ($p<0.001$) whereas B0 males gained *more* weight over a period of 44 wks.

As shown in Figures 2 and 3 on the next page, males (n=10) were also hyperinsulinemic and insulin resistant (homeostatic model assessment of IR, HOMA-IR) compared to CON (n=3) at 24 wks (1.8±0.2 vs 0.9±0.09 ng/ml; HOMA-IR 15±2 vs. 7±1, respectively; $p<0.05$, mean±SE) despite a similar degree of fasting glycemia (135±5 vs 137±5 mg/dl). In contrast, females remained normoglycemic with no difference in HOMA-IR between CON (n=4) and B0-exposed (n=9).

Males **Females**

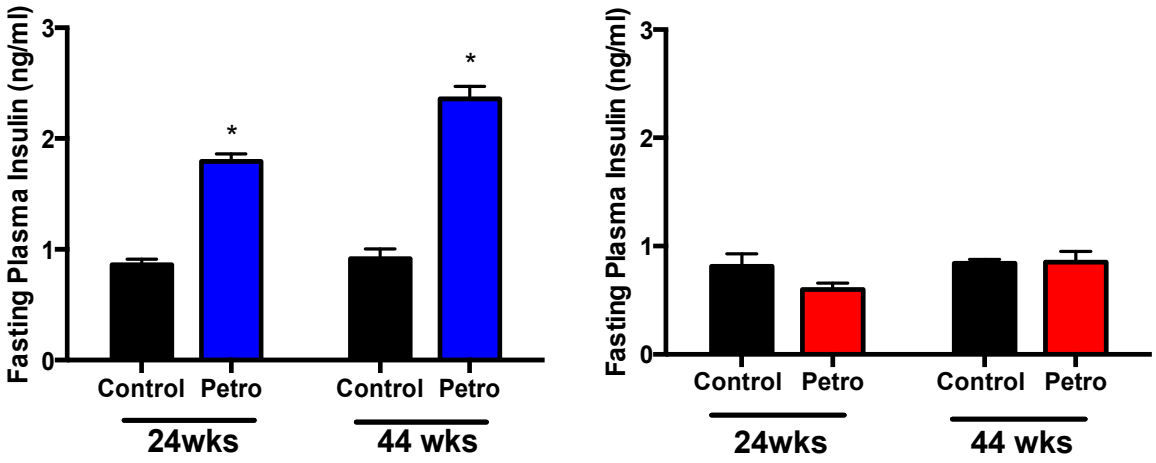

Figure 2. In contrast to female mice (right panel), prenatally B0-exposed male mice (left panel) exhibited progressive increases in fasting plasma insulin level from 24 wks up to 40 wks compared to control mice (*p<0.05).

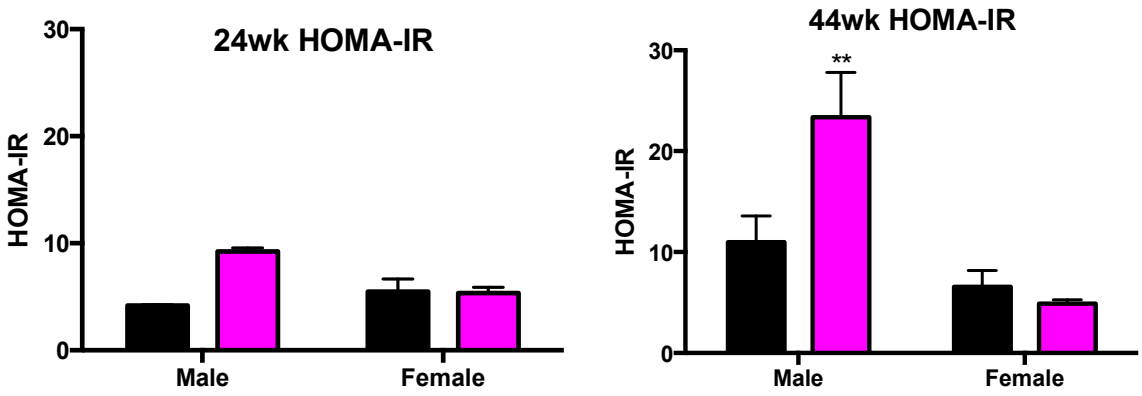

Figure 3. Using the homeostasis model assessment of insulin sensitivity, HOMA-IR scores at 24 and 44 wks were elevated in petrodiesel-exposed (B0) male offspring compared to controls (**p<0.01) whereas they remained similar in female offspring indicating insulin resistance in male mice. The higher HOMA-IR scores in control and males at 44 compared to 24 wks reflect the influence of aging *per se* on insulin resistance. Control = black; B0-exposed=pink bars.

These data suggest that prenatal exposure to exhaust PM from petrodiesel fuel combustion has different metabolic effects in male compared to female offspring that portend differential risk for metabolic dysfunction and may influence future reproductive capacity.

Comparison of offspring born to mothers exposed to B20 biodiesel exhaust PM suggests that males were heavier whereas female offspring were similar in their responses to

petrodiesel (data not shown). However, the number of offspring for mothers exposed to biodiesel exhaust particles was small and hence there was not sufficient power to detect significant effects of the B20 fuel.

5.0 Conclusion

The data collected from the offspring lay the groundwork for future studies on the effects of prenatal exposure to engine exhaust/particulate matter on metabolic outcomes. These initial data suggest a sexually dimorphic response, which may have implications for the sex-related differences in the onset of metabolic syndrome in humans. These data are supportive of reports of sex-specific effects of environmental exposures (Bolton et al, 2012; Allen et al., 2014). Futures studies will focus on the mechanisms for the sex-specific effects and on possible ways to attenuate the adverse health outcomes.

6.0 References Cited

Allen JL, Liu X, Weston D, Prince L, Oberdorster G, Finkelstein JN, Johnston CJ and Cory-Slechta DA. Developmental Exposure to Concentrated Ambient Ultrafine Particulate Matter Air Pollution in Mice Results in Persistent and Sex-Dependent Behavioral Neurotoxicity and Glial Activation. *Toxicol Sci* 2014, 140(1), 160-178.

Bolton JL, Smith SH, Huff NC, Gilmour MI, Foster WM, Auten RL and Bilbo SD. Prenatal air pollution exposure induces neuroinflammation and predisposes offspring to weight gain in adulthood in a sex-specific manner. *FASEB J* 2012, 26: 4743-4754, 2012.

Castilla, R.; Gonzalez, R.; Fouad, D.; Fraga, E.; Muntane, J., Dual effect of ethanol on cell death in primary culture of human and rat hepatocytes. *Alcohol and alcoholism (Oxford, Oxfordshire)* **2004,** *39*, (4), 290-6.

Fukagawa, N. K.; Li, M.; Sabo-Attwood, T.; Timblin, C. R.; Butnor, K. J.; Gagne, J.; Steele, C.; Taatjes, D. J.; Huber, S.; Mossman, B. T. Inhaled asbestos exacerbates atherosclerosis in ApoE-deficient mice via CD4+ T cells. *Environ. Health Perspect.* 2008, 116 (9), 1218−1225.

Fukagawa NK, Li M, Poynter ME, Palmer BC, Parker EP, Kasumba J, **Holmén B.** Soy Biodiesel and Petrodiesel Emissions Differ in Size, Chemical Composition and Stimulation of Inflammatory Responses in Cells and Animals. *Environ Sci Technol* 2013; 47:12496-12504. dx.doi.org/10.1021/es403146c |PMID:24053625.

Lakatos, H. F.; Burgess, H. A.; Thatcher, T. H.; Redonnet, M. R.; Hernady, E.; Williams, J. P.; Sime, P. J. Oropharyngeal aspiration of a silica suspension produces a superior model of silicosis in the mouse when compared to intratracheal instillation. *Exp. Lung Res.* 2006, 32 (5), 181−199.

Lewis, J. A.; Rao, K. M.; Castranova, V.; Vallyathan, V.; Dennis, W. E.; Knechtges, P. L. Proteomic analysis of bronchoalveolar lavage fluid: Effect of acute exposure to diesel exhaust particles in rats. *Environ. Health Perspect.* 2007, 115 (5), 756−763.

Nemmar, A.; Al Salam, S.; Dhanasekaran, S.; Sudhadevi, M.; Ali, B. H. Pulmonary exposure to diesel exhaust particles promotes cerebral microvessel thrombosis: Protective effect of a cysteine prodrug l-2-oxothiazolidine-4-carboxylic acid. *Toxicology* 2009, 263 (2−3), 84−92.

Traviss N, Li M, Lombard M, Thelen BA, Palmer BC, Poynter ME, Mossman BT, Holmén BA, Fukagawa NK. Petrodiesel and Waste Grease Biodiesel (B20) Emission Particles: Characterization and Effects on Lung Epithelial Cells and Macrophages. *Air Quality, Atmosphere and Health* 2014; 7:59-70. DOI 10.1007/s11869-013-0231-x.

Yoshizaki, K.; Brito, J. M.; Toledo, A. C.; Nakagawa, N. K.; Piccin, V. S.; Junqueira, M. S.; Negri, E. M.; Carvalho, A. L.; Oliveira, A. P.; Lima, W. T.; Saldiva, P. H.; Mauad, T.; Macchione, M. Subchronic effects of nasally instilled diesel exhaust particulates on the nasal and airway epithelia in mice. *Inhal. Toxicol.* 2010, 22 (7), 610−617.