School of Public Health

The effect of diesel exhaust emission on blood-brain barrier integrity and function

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material, which has been accepted for the award of any other degree or diploma in any University.

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Abstract

Many epidemiological studies have demonstrated an association between air pollution and a broad range of detrimental chronic health conditions. Diesel exhaust has been identified as one of the most serious contributors to air pollution as a result of its complex mixture of toxic substances either in gas phase or particulate matter (PM). Diesel exhaust particulate (DEP), particles produced by diesel engines, are the main component of fine (PM2.5, diameter <2.5μm) and ultrafine PM (UFPs, diameter <0.1μm) in urban areas and a major constituent of inhaled air pollution. Fine and ultrafine DEPs are considered more detrimental to health than coarser PM, because of a greater propensity for lung deposition and extra-pulmonary effects. Moreover, the greater surface-to-volume ratio associated with smaller particles enhances the possibility for absorption via lung epithelial cells and systemic (blood) delivery.

Diesel particulate matter describes the fine elemental carbon particles or 'soot' which makes up 70%-90% of the DEP mass. Diesel particulate matter has the potential to adsorb volatile organic compounds such as polycyclic aromatic hydrocarbons. About 40 polycyclic aromatic hydrocarbons have been associated with DEP but it is estimated that greater than 300 polycyclic aromatic hydrocarbons may be present within DEPs. Several lines of evidence suggest that volatile organic compounds exacerbate the health risks associated with diesel particulate matter exposure, although the magnitude of this effect is difficult to delineate because of the transient presence of volatile organic compounds.

Traditionally associated with an increased risk of pulmonary and cardiovascular morbidity and mortality, diesel exhaust and specifically DEP exposure has recently been suggested to be associated with neurodegenerative disorders such as Alzheimer's disease, vascular dementia and Parkinson's disease. Cerebral and systemic inflammation concomitant with heightened oxidative stress is considered the likely mechanism for the detrimental physiological effects of DEP and adsorbed volatile organic compounds in diesel particulate matter.

Inhaled DEPs that enter the circulation may distribute to other organ systems such as liver, kidneys, testis and lymph nodes and thereafter have tissue-specific

effects. Several lines of evidence suggest that diesel exhaust might be a significant pro-inflammatory stimulus of the central nervous system (CNS) and by extension may contribute to disease onset and progression of neurodegenerative disorders. Moreover, absorption of PM along the olfactory nerve (via intranasal uptake), reaching the trigeminal nerves, brainstem and hippocampus offers a direct route of cerebral tissue uptake. However, the putative association between diesel exhaust and neurodegenerative diseases has largely been overlooked in the literature to date. The mechanism for this putative association is thought to be through pathways that principally describe compromised cerebrovascular integrity and function.

Cerebral capillary vessels provide an extraordinary neurovascular interface. Characterized by unique structural properties commonly described as the blood-brain barrier (BBB), the pivotal function of these vessels is to strictly regulate the kinetics of solutes and macromolecules into the brain parenchyma. In addition, astroglial cells, which surround the brain capillary vessels, serve as inflammatory phagocytes when breach of the BBB occurs. Several *in vitro* studies have indicated that diesel exhaust is likely to affect cerebral vascular function and capillary permeability by altering endothelial tight junction protein expression and promoting glial cell activation. The purpose of this study was principally to investigate an *in vivo* animal model to assess the hypothesis that sub-chronic diesel exhaust inhalation exposure compromises BBB integrity and function.

Groups of 12 mice were randomized for treatment in environmental chambers. The treatment regimen was to HEPA filtered air or diesel exhaust, regulated to deliver as a mixture with air 20 mg/m^3 DEP or 30 mg/m^3 DEP. The exposure pattern was for four consecutive days, followed by 3 days with no treatment, then another four days of treatment. The duration of each treatment was for two consecutive hours. Mice were sacrificed at 6 and 24 hours post the final exposure to DEP. The 8h-time weighted averages (TWA8h) on days when exposures occurred were 5 mg/m^3 and 7.7 mg/m^3 (for the 20 and 30 mg/m^3 groups, respectively). Over the duration of the study, the TWA concentrations were 1.2 and 1.8 mg/m^3 for the two groups. The TWA were within the ranges reported from several heavy industry settings such as mines**.** The levels of exhaust gases were constantly monitored. Diesel soot was collected and analysed for polycyclic aromatic hydrocarbons, volatile organic compounds, elemental and thermo gravimetric composition.

Immunofluorescent quantitative analysis was applied to assess the presence of any BBB disturbances and inflammation in the CNS. The functional integrity of BBB was determined by considering brain parenchymal abundance of immunoglobulin G (IgG) within the hippocampal formation (HPF) and cortex (CTX). Neurovascular inflammation was expressed as the abundance of glial fibrillar acidic protein (GFAP). Mice were euthanized with ketamine/xylazine (1:20 respectively) at 0.1ml/g body weight. Brain tissues were immersion-fixed in 4% paraformaldehyde for 24 hours, cryoprotected in 20% sucrose for 3 days, frozen in isopentane/dry ice and stored at –80°C for immunofluorescence microscopy. Immunofluorescent micrographs were quantitatively analysed in 3-dimensional as we have previously established and published. Briefly, immunofluorescent micrographs were captured with an mRM digital camera (Zeiss) attached to AxioVert 200M. At a magnification of 200x (20x Zeiss Plan-Neofluar objective with mRM camera), a minimum of five images per section were captured from randomly selected areas of the CTX and HPF in the approximate stereotaxic areas of 1.7 mm interaural and -2.1 mm Bregma. Each 3-dimensional image consisted of 12 Z-stack 2-dimensional images, and the distance between the Z-stack images were 1.225 μm which were optimized by Nyquist overlap theory. The voxel intensity of the fluorescent signal was then analysed with the Volocity 6.2 image analysis software (PerkinElmer, UK). Means of total fluorescent intensities of all the images in the CTX or HPF regions were calculated within each animal.

Mice exposed to diesel exhaust had substantially greater abundance of parenchymal IgG compared to control mice not exposed to diesel exhaust. There was exaggerated IgG extravasation in both the CTX and HPF of the 24h group exposed to the higher level of diesel exhaust concentration, indicating the clear possibility of a dose-dependent effect. Increased parenchymal GFAP at 24h post diesel exhaust exposure suggested heightened neurovascular inflammation. These findings are proof-of-concept that inhalation of diesel exhaust can compromise BBB function and support the broader contention that diesel exhaust exposure may contribute to neurovascular disease risk.

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Statement of Contribution

In this section, the contributions of co-authors for the primary publication included in this thesis are clearly stated. All co-authors have read and approved the final version of this manuscript before submission.

Sayeh Heidari Nejad was responsible for undertaking the literature review, primary writing of the manuscript, data collation and analysis. Ryusuke Takechi was involved in supervision of brain tissue collection and immunofluorescent quantitative analysis and manuscript appraisal. Corey Giles assisted in data collation, data statistical analysis and generating figures. Alexander N. Larcombe contributed in data collation. Benjamin J Mullins, Dean Bertolatti and Krassi Rumchev contributed to preparation of the diesel engine, diesel exhaust particle genesis, standardisation and diesel exhaust data interpretation. Satvinder Dhaliwal assisted with statistical analysis of the data. John Mamo contributed to data interpretation and critical appraisal of the manuscript.

Abbreviations

BBB	Blood brain barrier
CNS	Central Nervous System
$PM_{10-2.5}$	Coarse particle
CTX	Cortex
DNA	Deoxyribo nucleic acid
DEP	Diesel exhaust particulate
$PM_{2.5}$	Fine particle
FEV_1	Forced expiratory volume in one second
$FEV_{0.75}$	Forced expiratory volume in time 75 hundredths of a second
GFAP	Glial fibrillary acidic protein
HPF	Hippocampal formation
IgG	Immunoglobulin-G
IFN-γ	Interferon-γ
IL-1α	Interleukin-1alfa
IL-1β	Interleukin-1betta
IARC	International Agency for Research on Cancer
μm	Micrometer
NADPH-oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase-oxidase
PM	Particulate matter
RNA	Ribo nucleic acid
TW	Time weighted averages
TNF-α	Tumor necrosis factor alpha
PM0.1	Ultrafine particle
UFP	Ultrafine particle

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CHAPTER 1

CHAPTER 1:

Literature review

1. Air pollution

1.1 Air pollution definition, composition and sources

Air pollution is defined as the presence of any substance in the atmosphere that causes discomfort, morbidity or mortality to living organisms. Activities that release pollutants into the air are regarded as the source of air pollution **(**http://www.environment.gov.au). Air pollution sources can be classified in to natural, also called biogenic, and human-generated or anthropogenic. In general, human-generated sources are considered to be more serious air pollution sources. Some examples of biogenic sources are: volcanoes by driving out air pollutants into the atmosphere, lightning strikes contributing to forest fires resulting in expelling of many air pollutants in to the air, trees and other vegetation by releasing great amounts of spores and pollen **(**http://www.environment.gov.au)**.** Anthropogenic sources of air pollution are mainly generated by transport such as automobiles, residential such as domestic devices and industrial activity (Calderón-Garcidueñas et al., 2008; Pope III, 2000).

Air pollutants constitute a mixture of particles, gases, metals, and organic compounds. Major man-made air pollutants include: sulphur oxides, Nitrogen oxides, carbon monoxide, carbon dioxide, volatile organic compounds, persistent free radicals, toxic metals such as lead, cadmium and copper, ammonia, Chlorofluorocarbons, radioactive pollutants and particulate matters (PM)s (Calderón-Garcidueñas et al., 2008).

1.2 Ambient air quality standards

Air pollutants are undoubtedly detrimental to human-health. Tremendous air pollution episodes in the 1930s-1950s were associated with substantially elevated cardiopulmonary morbidity and mortality. Evidence of serious health effects caused by these episodes raised a growing apprehension about air pollution in many countries (Pope III, 2000; Pope III et al., 2004). A series of legislative efforts were made with the aim to control air pollution in the 1950s through early 1970s by United States Environmental Protection Agency. A measure called 'ambient air quality standard' was developed in consultation with health professionals, environmental groups and the community for every nation to improve air quality. The measure sets air quality standards for some air pollutants that are relatively common and widespread but may reasonably be anticipated to endanger public health. Six pollutant components considered in assessing ambient air quality (criteria pollutants) are PM, sulphur dioxide, nitrogen dioxide, carbon monoxide, ozone and lead (http://www.epa.gov).

Strategies to improve air quality have been adopted in many countries, however in some industry settings and urban environments, exposure commonly exceeds generally acceptable safety standards (Akimoto, 2003). Different air pollutants have been investigated broadly by both epidemiological and toxicological studies for long-term adverse health consequences to humans in urban areas (Götschi, Heinrich, Sunyer, & Künzli, 2008; Valavanidis, Fiotakis, & Vlachogianni, 2008). A number of large epidemiological studies have shown that of the criteria pollutants, ozone (Craig et al., 2008; Mills et al., 2009) and PM have a significant and widespread association with adverse health effects (Dockery et al., 1993; Hoek et al., 2001 May; Katsouyanni et al., 2001; Pope et al., 1992; Pope III et al., 1995; Schwartz, 1999; Zanobetti et al., 2000).

1.3 Particulate matter composition and classification

Particulate air pollution or PM consists of a diverse mixture of solid and liquid particles, dispersed in the air as an aerosol. Particles have a variety of morphological, chemical and thermodynamic properties. They originate from different anthropogenic and natural sources (Srimuruganandam & Shiva, 2012).

The chemical composition of particles includes elemental carbon, organic materials including polycyclic aromatic hydrocarbons and some trace inorganics such as sulphur, nitrates, carbon, lipopolysaccharides, metals, ammonium, hydrogen ions, and water (Craig et al., 2008; Nemmar et al., 1999; Oberdörster et al., 1996). Particulate matter is normally expressed as the mass of particles within a cubic meter of air (micrograms per cubic meter, $\mu g/m^3$). In addition, PM is considered relative to aerodynamic diameter. Commonly PMs are categorized as either ultrafine $PM_{0.1}$ (particles with an aerodynamic diameter, $d_a < 100$ nm), fine $PM_{2.5}$ ($d_a < 2.5$ μm), coarse $PM_{10–2.5}$ ($d_a \geq 2.5$ μm and $d_a < 10$ μm) or PM_{10} ($d_a < 10$ μm) (Craig et al., 2008) (**Fig. 1**).

Ambient PM levels, specifically PM_{10} and $PM_{2.5}$ concentrations, in many cities of the developing nations routinely exceed World Health Organization safety standards by a factor of three or more (Srimuruganandam & Shiva Nagendra, 2012).

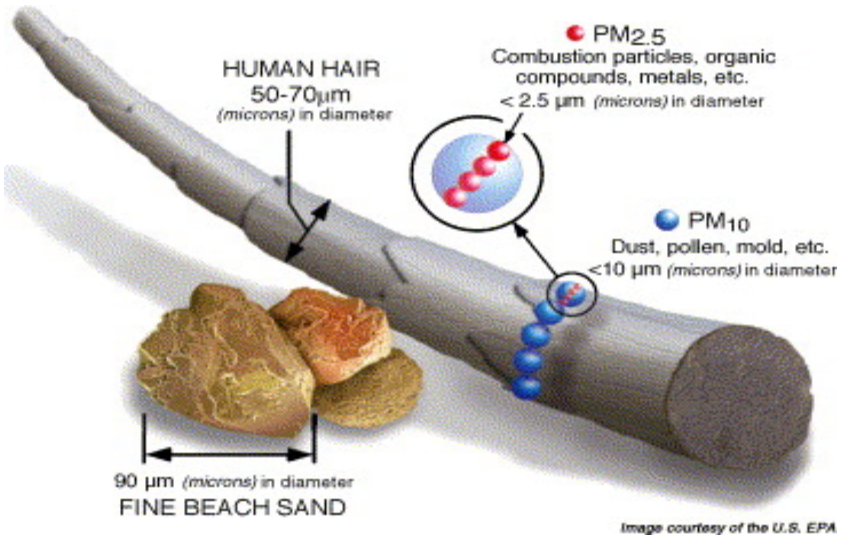

Fig. 1. Various sizes of particulate matter in comparison with a strand of human hair and grains of fine beach sand (Image courtesy of the U.S. EPA)

Fine and ultrafine PMs are considered more detrimental to health than coarser PMs, because of their propensity for lung deposition, lung penetration and extra pulmonary effects (Mühlfeld et al., 2008; Rothen-Rutishauser et al., 2008; Valavanidis et al., 2008). Moreover, the greater surface-to-volume ratio associated with smaller particles enhances their penetration into the cell membrane (Geiser et al., 2005) and the possibility for absorption via lung epithelial cells, systemic (blood) delivery and broader physiological effects thereafter (Nemmar et al., 1999; Oberdörster et al., 1996). The large surface area of smaller PMs increases the probability of adsorption for greater amounts of toxic organic and inorganic compounds (Schuetzle, 1983a; Strandell et al., 1994b; Vouk & Piver, 1983). Some of the more physiologically toxic organic substances adsorbed on to the particles include polycyclic aromatic hydrocarbons, nitro-polycyclic aromatic hydrocarbons, and oxidized polycyclic aromatic hydrocarbons derivatives (Yokota et al., 2009). Polycyclic aromatic hydrocarbons are a group of substances that result from incomplete burning of substances such as coal, oil, gas, wood or other organic chemicals. One of the most common sources of release of polycyclic aromatic

hydrocarbons in to the air is vehicle exhaust. Among more than one hundred polycyclic aromatic hydrocarbons identified with PM, seventeen are indicated as particularly hazardous **(Table 1)** (Liu, Liu, Lin, Tang, & Hayakawa, 2006).

Table 1. Identified hazardous polycyclic aromatic hydrocarbons

1. Acenaphthene
2. Acenaphthylene
3. Anthracene
4. Benz[a]anthracene
5. Benzo[a]pyrene
6. Benzo[e]pyrene
7. Benzo[b]fluoranthene
8. Benzo[j]fluoranthene
9. Benzo[g,h,i]perylene
10. Benzo[k]fluoranthene
11. Chrysene
12. Dibenz[a,h]anthracene
13. Fluoranthene
14. Fluorene
15. Indeno[l,2,3-c,d]pyrene
16. Phenanthrene
17. Pyrene

1.4 Diesel exhaust and diesel exhaust particulates

Diesel exhaust is an increasingly abundant air pollutant in urbanised communities and considered one of the most serious air pollutants due to its deleterious health effects (Yokota et al., 2009). Diesel exhaust is a heterogeneous mixture of particulate matter, gases and volatile chemicals. The gaseous components

of diesel exhaust include carbon dioxide, oxygen, nitrogen, water vapour, carbon monoxide, nitrogen compounds, sulphur compounds, and low-molecular weight hydrocarbons. The diesel exhaust particles, characterized predominantly as fine and ultrafine particles, are composed of elemental carbon and adsorbed toxic organic substances such as polycyclic aromatic hydrocarbons, which comprise about 1% of the DEP mass and inorganic substances such as sulphate, nitrate, metals, and other trace elements (Yokota et al., 2009).

Diesel exhaust is identified as a significant source of PM air pollution due to the recent major increase in the number of diesel engine-powered vehicles which emit one hundred times more particles than gasoline engines with contemporary emission-control devices (Franck, Herbarth, Wehner, Wiedensohler, & Manjarrez, 2003; Levy, Dumyahn, & Spengler, 2002).

Diesel exhaust is exceedingly heterogeneous since it is the outcome of the incomplete combustion of hydrocarbons. The quantity of different elements in diesel exhaust depends largely on engine technology, fuel type, and operation techniques. The composition of diesel exhaust has changed significantly since 1988 owing to regulatory requirements by governing bodies for emission standards for DEP and nitrogen oxides, two main toxic elements in diesel exhaust. In USA the permissible level of DEP was lowered to 99% below the pre-1988 level by the year 2007 (Hesterberg et al., 2009). Similar regulatory requirements for nitrogen oxides were indicated. In the literature, pre-1988 diesel exhaust, 1988-2006 diesel exhaust and post-2006 diesel exhaust are commonly referred to as ‘traditional diesel exhaust’, ‘transitional diesel exhaust’ and ‘new technology diesel exhaust’, respectively (Hesterberg et al., 2009) (**Fig. 2**).

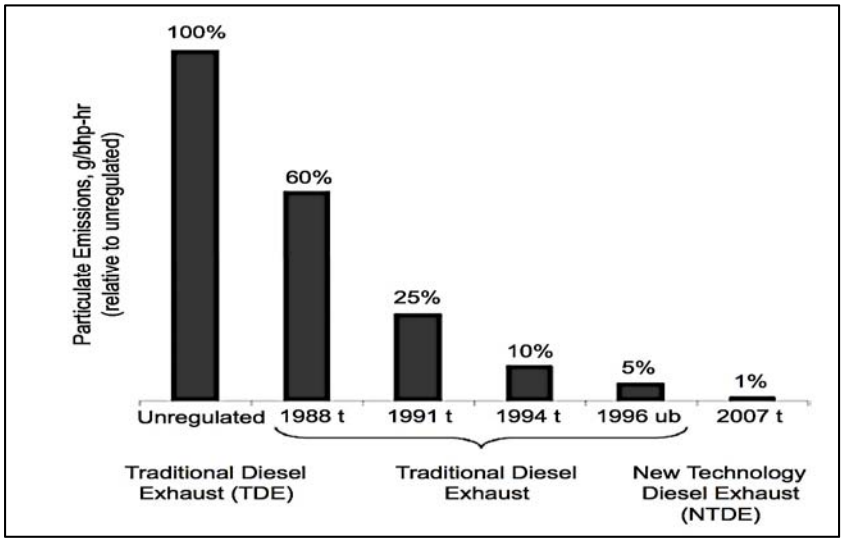

Fig. 2. Reduction in US EPA standards for particulate emissions from heavy-duty diesel trucks (t) or urban buses (ub) (Hesterberg et al., 2009)

Several recent publications discuss differences between transitional and new technology diesel exhaust (Hesterberg et al., 2006; Hesterberg, Bunn, McClellan, Hart, & Lapin, 2005). They have demonstrated declined levels of toxic substances, such as total hydrocarbons, non-methane hydrocarbons, formaldehyde, ethylene, benzene, acetaldehyde PM, carbon monoxide, and total polycyclic aromatic hydrocarbons (**Fig. 3-4**).

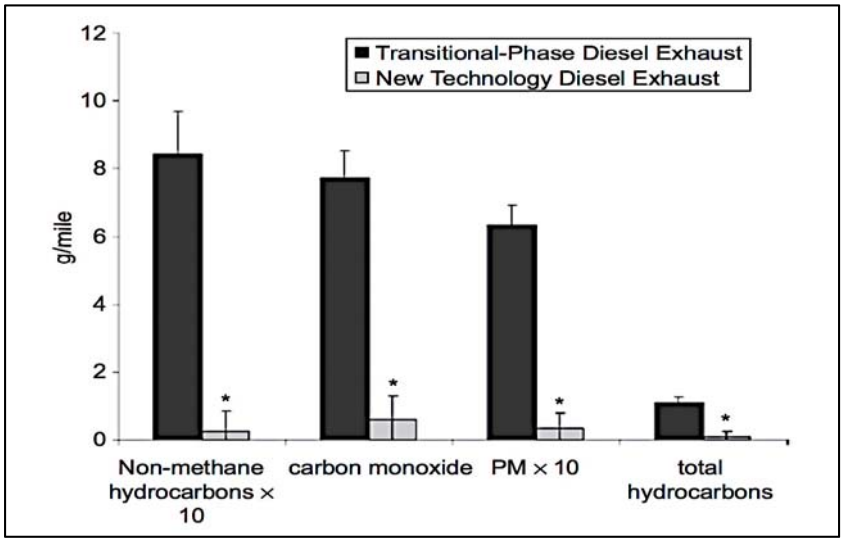

Fig. 3. Reduction in various regulated emissions from transit buses. Asterisks indicate a significant difference at $p<0.05$. (Hesterberg et al., 2009)

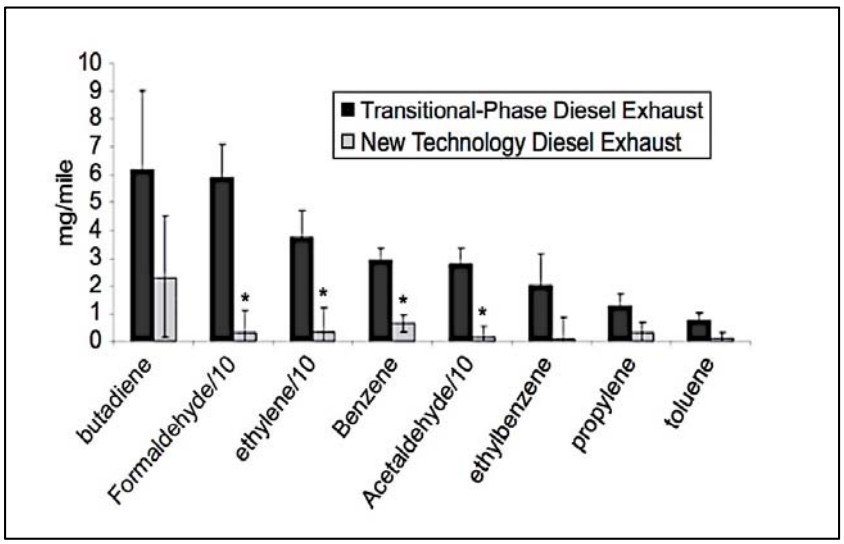

Fig. 4. Reduction in various volatile organic chemical emissions from transit buses. Asterisks indicate a significant difference at $p<0.05$ (Hesterberg et al., 2009)

1.5 Diesel exhaust and health effects

The broad range of adverse health effects of diesel exhaust has resulted in an increase in toxicological studies focusing on this prevalent air pollutant and its components. In reviewing these toxicological studies, some have investigated several diesel exhaust components such as DEP, gaseous elements (e.g. nitrogen monoxide, nitrogen dioxide, sulphur dioxide, carbon monoxide), and polycyclic aromatic hydrocarbons (e.g. benz[a]anthracene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene) while others have investigated the whole diesel exhaust. It is speculated that the main toxic health effect of diesel exhaust is through exposure to DEP fine and ultrafine particles (Hesterberg et al., 2009). However, the toxic effects of other components of diesel exhaust should not be underestimated. It is interesting to note that a considerable number of recent studies continue to report the potential health effects of traditional diesel exhaust in spite of considerable qualitative alterations of diesel exhaust during recent decades. Therefore, further consideration regarding diesel exhaust pollutants from contemporary engine technologies is warranted.

1.5.1 Diesel exhaust and foetal, infantile development

Maternal exposure to particulate air pollution, which mainly originates from diesel exhaust, is associated with adverse effects on foetal development including placental function and morphology (Veras et al., 2008); intrauterine growth retardation (Bernstein, Horbar, Badger, Ohlsson, & Golan, 2000; Dejmek, Selevan, Beneš, Solanský, & Šrám, 1999); low birth weight (Parker, Woodruff, Basu, & Schoendorf, 2005); and pre-term birth (Huynh, Woodruff, Parker, & Schoendorf, 2006; Ritz, Yu, Chapa, & Fruin, 2000). A growing body of literature has documented the association between ambient air pollution exposure during pregnancy and infant mortality (Bobak, 2000; Woodruff, Grillo, & Schoendorf, 1997). A significant association was observed between diesel exhaust gases including nitrogen dioxide,

sulphur dioxide, carbon monoxide and intrauterine mortality in a time period from January 1991 to December 1992 in the city of São Paulo, Brazil (Pereira et al., 1998). In addition to infant mortality, stillbirth has been revealed as one of the consequences of diesel exhaust exposure during pregnancy (Bobak, 2000; Faiz et al., 2012; Mavalankar, Trivedi, & Gray, 1991).

Animal studies have also supported the evidence of the deleterious effects induced by prenatal diesel exhaust exposure on reproductive system and the CNS. Prenatal diesel exhaust-exposed adult mice had compromised spermatogenesis and significant decrease in expression of male gonadal differentiating factors such as mallerian inhibiting substance and steroidogenic factor-1 (Yokota et al., 2009). Maternal exposure to diesel exhaust may also influence the development of central dopaminergic system and leading to behavioural disorder in mice offspring (Yokota et al., 2009).

1.5.2 Diesel exhaust and cancers

The majority of studies about diesel exhaust exposure in humans generally support the link between diesel exhaust and lung cancer with an overall greater relative risk of approximately 1.3 (Yokota et al., 2009). There has also been a positive dose-response relationship between the duration and dose of exposure to diesel exhaust and cancer incidence (Yokota et al., 2009). Epidemiologic studies evaluating the association of diesel exhaust exposure and cancers among humans before 1989 were reviewed by the International Agency for Research on Cancer (IARC) Volume 46 (IARC 1989). This group has indicated the positive association of lung cancer and diesel exhaust exposure in results of only one cohort study which analysed the link between incidence of cancer and diesel exhaust exposure rate on 55,407 US railroad workers in 1959 (Garshick et al., 1988). Therefore, they concluded that there was limited evidence to identify the carcinogenicity of diesel exhaust in humans. In 1989 and later, several published cohort studies explored the association between the prevalence of cancer and diesel exhaust exposure in humans (Hansen, Raaschou-

Nielsen, & Olsen, 1998; Hayes et al., 1989; Marie Swanson et al., 1993; Steenland, Deddens, & Stayner, 1998). All studies reported statistically significant association between lung cancer incidence and diesel exhaust exposure. In addition to human studies, animal experiments have also supported the carcinogenicity of whole diesel exhaust (Heinrich, Pott, & Rittinghausen, 1986; Pepelko & Peirano, 1983; Stöber, 1986). Furthermore, the association between lung cancer and individual components of diesel exhaust including sulphur dioxide, carbon monoxide, nitrogen dioxide, nitric oxide and PM has been demonstrated (Choi, Inoue, & Shinozaki, 1997; Katanoda et al., 2011; Næss, Nafstad, Aamodt, Claussen, & Rosland, 2007; Nafstad et al., 2004; Nyberg et al., 2000).

There was a positive association between bladder cancer and diesel exhaust exposure in eight of eleven studies assessed by the IARC group in 1989 (Coggon, Pannett, & Acheson, 1984; Hoar & Hoover, 1985; Howe et al., 1980; Iscovich et al., 1987; Jensen, Wahrendorf, Knudsen, & Sorensen, 1987; Silverman, Hoover, Albert, & Graff, 1983; Steenland, Burnett, & Osorio, 1987). There also has been some evidence associating several other types of cancers such as testicular cancer (Garland, Gorham, Garland, & Ducatman, 1988), multiple myeloma (Flodin, Fredriksson, & Persson, 1987) and chronic lymphocytic leukaemia (Bender et al., 1989) due to diesel exhaust exposure.

1.5.3 Diesel exhaust and respiratory effects

Epidemiological evidence has indicated an association between elevated hospitalizations, emergency visits, and clinic/outpatient visits for respiratory distress/disorders and exposure to diesel exhaust (Pope III, 2000). Exaggerated rates of asthma, coughing, and decline in pulmonary function measures such as forced expiratory volume in the first 0.75 second ($FEV_{0.75}$), forced expiratory volume in 1 second (FEV_1), or peak expiratory flow are some of the reported respiratory adverse effects of acute exposure to ultrafine and fine particulate air pollution, such as DEPs

(Choudhury, Gordian, & Morris, 1997; Dockery et al., 1982; Hoek et al., 1998; Neas, Dockery, Koutrakis, Tollerud, & Speizer, 1995)

Chronic exposure to diesel exhaust has been shown to induce a significant inflammatory response in the airways (Pourazar et al., 2004) resulting in both restrictive pattern of lung function, including increased stiffness of the lung along with lower lung volumes, total lung capacity and vital capacity, and an obstructive pattern (Hesterberg et al., 2009). The animal experimental studies have demonstrated the pathological changes of respiratory tract in response to diesel exhaust (McClellan, 1987; White & Garg, 1981). Some of the observed pathological changes in respiratory tracts include: an increase in number and size of alveolar macrophages, an increase in number and size of type-2 pneumocytes that contain DEP-laden macrophages, elevation in the number of polymorphonuclear and aggregation of DEP in macrophages of alveoli, alveolar interstitial, peribronchial and perivascular interstitium, lymphatic channels, histocytes, Type-1 epithelial cells, and eosinophils (McClellan, 1987; White & Garg, 1981).

1.5.4 Diesel exhaust and cardiovascular effects

It has also been observed that the peaks of world urban air pollution are associated with adverse cardiovascular conditions (Peters, Döring, Wichmann, & Koenig, 1997; Peters et al., 2000; Peters et al., 1999). Indeed, the mortality rate of cardiovascular diseases has been even greater than pulmonary diseases during such episodes of high pollution (Pope III et al., 1995, Peters, et al., 2000).

Several landmark epidemiological studies have indicated that particulate air pollution contributes significantly to cardiovascular dysfunction (Dockery et al., 1993; Pope et al., 1992; Pope III et al., 1995; Schwartz, 1999). Exposure to PM leads to increases in conditions associated with cardiovascular morbidity and mortality such as acute myocardial infarction (Burnett, Smith-Doiron, Stieb, Cakmak, & Brook, 1999; Hoek et al., 2001 May; Ichinose, Takano, Miyabara, & Sagai, 1998;

Zanobetti et al., 2000); exercise-induced myocardial ischemia (Mills et al., 2007) and development of atherosclerosis by inducing production of atherosclerotic plaques (Künzli et al., 2005); platelet aggregation (Mills et al., 2007) and high blood pressure (Ibald-Mulli, Stieber, Wichmann, Koenig, & Peters, 2001). Diesel exhaust also affects the parameters of cardiovascular and systemic vascular function (Hirano, Furuyama, Koike, & Kobayashi, 2003; Mills et al., 2005). Diesel exhaust induces vasoconstriction via the increased release of endothelium-dependent endothelin-1 and consequently may exacerbate the risk of hypertension (Hirano et al., 2003). Exposure to diesel exhaust has been shown to impair endogenous fibrinolytic processes in humans with coronary heart disease, which causes plaque formation in systemic vasculature (Mills et al., 2007).

The rise in occurrence of ischemic incidents in the vascular system of the brain such as ischemic stroke in response to inhalation exposure of DEP has been demonstrated by a number of epidemiologic studies as well (Hong, Lee, Kim, & Kwon, 2002; Lisabeth et al., 2008; Lokken et al., 2009). The possible explanation for such observation is suggested to be the activation of prothrombotic events in the venous and arterial circulation triggered by DEP (Nemmar, Al-Salam, Dhanasekaran, Sudhadevi, and Ali, 2009) provided the first experimental evidence of DEP accelerating platelet activation and subsequently generating dose-dependent (platelet-rich) venous thrombus in injured vessels over 40-minutes interval following intra tracheal instillation of DEPs.

1.5.5 Diesel exhaust and central nervous system effects

Air pollution may rank as one of the most widespread environmental origins of CNS function impairment (Craig et al., 2008). Although the historical target for epidemiological and toxicological studies of exposure to various air pollutants has been the cardiopulmonary system, it has recently been indicated that inhaled ultrafine PMs such as DEPs, can pass through the lungs and enter the circulation where they

distribute to other organ systems such as the brain potentially causing a range of adverse physiological and pathological effects (Kreyling et al., 2002; Oberdörster et al., 2004a; Takenaka et al., 2001).

In a pivotal study conducted by Calderón-Garcidueñas, Mora-Tiscareño, et al. (2008) healthy children dwelling in the highly polluted Mexico City showed considerable cognitive impairments in areas such as working memory when compared to socio-economically-matched children residing in a low pollution environment. Working memory is a memory system that is centrally involved higher cognitive tasks such as abstract reasoning, general fluid intelligence (Engle, Laughlin, Tuholski, & Conway, 1999) and reading (Turner & Engle, 1989). The children also elucidated hyper intense white matter prefrontal lesions in brain magnetic resonance image, which may provide an explanation for working memory deficit in them. Simultaneously, these children's dogs exhibited similar brain lesions as well as significant up regulation of several important inflammatory genes: cyclooxygenase-2, interleukin-1 beta (IL-1β) and glial fibrillary acidic protein (GFAP) in the site of brain lesions compared to age-matched control dogs from a low pollution city. Interleukin-1 beta plays an important role in modulating cerebral function in inflammatory conditions (Strauss, 2008). Cyclooxygenase-2 contributes to free radical mediated brain cellular damage and cerebrovascular dysfunction (Strauss, 2008), which are both crucial for neurodegenerative processes (Strauss, 2008) and GFAP gene overexpression is a marker of neuroinflammation (Li et al., 2011). Moreover, the presence of ultrafine particles in the cerebral vessels of the frontal lobe of the canine brain lesions as well as diffused vascular pathology and perivascular astroglial activation might suggest that UFPs contribute to neuroinflammation and brain lesion by imposing disruption of the cerebral vascular system that is called blood brain barrier (BBB).

The first epidemiological study investigating the association between air pollution and cognitive function in adults reported reduced neurobehavioral and neurocognitive performance in 1764 adults in the United States exposed to higher levels of ozone and PM_{10} from 1988 to 1991 (J. C. Chen & Schwartz, 2009). In

several other human studies, the brain s electrical activity was constantly monitored by electroencephalograph at different sites on the scalp during diesel exhaust exposure which showed some activity changes in the frontal cortex of brain within 30 minutes of exposure that was interpreted to be related to DEPs that either penetrate into the brain or the influence neurophysiologic signalling (Hesterberg, Long, Lapin, Hamade, & Valberg, 2010). Although there was not enough evidence to link cognitive impairment with such activity changes in the frontal cortex, several previous studies have associated these changes to frontal brain cortex stress and inflammation (Hoek et al., 2010). The latter observations are similar to some psychopathological and neurological disorders.

The stated studies led to a series of animal experiments, which aimed to confirm the potential linkage of PM, and diesel exhaust as the main source of PM in the environment, on CNS and investigate the putative mechanisms. Gerlofs-Nijland et al. (2010) analysed the relationship between diesel exhaust and neuroinflammation by measuring established pro inflammatory markers such as tumour necrosis factor alpha (TNF-α) and interleukin-1alpha (IL-1α) in different regions of the rat brain using a nose-only exposure chamber. The study provided evidence of neuroinflammatory responses in a region-specific manner to diesel exhaust exposure. Levesque, et al. (2011) underscored the inflammatory reaction of the rat brain in response to diesel exhaust inhalation exposure and explained that the activation of microglial cells, the brain's primary immune cells, during the inflammatory phase might lead to an up-regulation of oxidative stress in the CNS. The underlying interacting mechanisms of diesel exhaust-induced neuropathology are not yet delineated. However, systemic and neuroinflammation (activated microglial cells and astrocytes), altered redox state (oxidative stress) and capillary dysfunction (BBB) are implied as major mediators of diesel exhaust-induced CNS damage (Block & Calderon-Garciduenas, 2009; Block et al., 2004).

Neuroinflammation, BBB damage and oxidative stress are central to a number of cerebrovascular-based neurodegenerative disorders such as Alzheimer's disease, vascular dementia and Parkinson's disease (Calderón-Garcidueñas, Mora-Tiscareño,

et al., 2008; Grammas, Martinez, & Miller, 2011; Persidsky, Ramirez, Haorah, & Kanmogne, 2006). It's commonly suggested that chronic exposure to diesel exhaust, or components of diesel exhaust may exacerbate risk factors for CNS disorders, however, there is a paucity of studies that have investigated this hypothesis directly.

1.6 Diesel exhaust/particle and central nervous system

1.6.1 Diesel exhaust/particle mechanisms of action on central nervous system

Although inflammation and oxidative stress have been suggested as the common mechanisms of diesel exhaust/particle-induced injury in pulmonary and cardiovascular system disorders (Mills et al., 2009; Muhlfeld et al., 2008; Riedl, 2008; Simkhovich, Kleinman, & Kloner, 2008), few studies have investigated this in the context of diesel exhaust -CNS effects.

1.6.1.1 Inflammatory response induced by diesel exhaust/particle

Inhaled DEPs move their way along the airways to reach the alveolar spaces and lung tissue. Alveolar macrophages have an important phagocytic function and clear the lungs of microorganisms and environmental particles. The airway and alveolar epithelium have physiochemical properties, which facilitate the alveolar macrophage phagocytic process. The deposition of DEPs in the airspace activates two pathways of inflammation via alveolar macrophages and lung epithelial cells. Diesel exhaust particles stimulate the synthesis and secretion of pro-inflammatory cytokines and chemokines promoting macrophage infiltration at the site of deposition (Boylen, Sly, Zosky, & Larcombe, 2011; Fujii, Hayashi, Hogg, Vincent, & Van Eeden, 2001; Mukae et al., 2001; Terashima, Wiggs, English, Hogg, & Van Eeden, 1997). Cytokines also promote the activation of the macrophages resulting in the

generation of a respiratory burst and degradation of the phagocytized particulate matter (Mukae, Hogg, English, Vincent, & Van Eeden, 2000; Mukae et al., 2001; Pope III, Dockery, Kanner, Villegas, & Schwartz, 1999). It is suggested that the alveolar-cytokine axis may have synergistic effects on the systemic inflammatory pathways triggered by diesel exhaust exposure and diesel exhaust/particle-induced systemic inflammation may have direct effects on CNS function and integrity (Fujii et al., 2001; Ishii et al., 2005; Jimenez et al., 2000; P. R. Mills, Davies, & Devalia, 1999).

There are two hypotheses for how diesel exhaust/particle exposure induces a systemic inflammatory response: 1) the bioactive cytokines originating from the pulmonary system overflow into the circulation triggering a cascade of inflammatory reactions in the circulation and other organs (Goto, Hogg, et al., 2004; Goto, Ishii, et al., 2004; Mukae et al., 2000; Terashima et al., 1997) and 2) extra-pulmonary translocation of ultrafine particles into blood may directly trigger an inflammatory response in the circulation as well as other organs (Nemmar et al., 2001).

Plasma inflammatory biomarkers such as IL-1β, tumour necrosis factor-α (TNF-α) and granulocyte-macrophage colony-stimulating factor, which are generated from the interplay between alveolar macrophages, airway epithelial cells and DEPs are observed in the blood and may influence other tissues such as the liver or CNS (Goto, Hogg, et al., 2004; Goto, Ishii, et al., 2004; Ishii et al., 2005; Mukae et al., 2000; Terashima et al., 1997).

Nemmar et al. (2001) initially provided the evidence for extra-pulmonary translocation of inhaled air UFPs to the systemic circulation. They reported a considerable proportion of intra-tracheally instilled radioactively labelled albumin nanocolloid particles (diameter ≤80 nanometer) in the systemic circulation of hamsters within 5 minutes of instillation. Diesel exhaust particles were elucidated to be able to diffuse through the physiological alveolar-blood barrier and move to other peripheral organs such as the liver (Oberdörster et al., 2004a), cardiovascular system and the CNS (Block & Calderon-Garciduenas, 2009). Systemically delivered DEP may intensify the cascade of inflammatory reactions induced via the

alveolar/alveolar macrophages axis. Ultimately, all the components of this exacerbated systemic inflammatory cascade, i.e. cytokines, are capable of penetrating the BBB and entering the brain parenchyma (Banks, Plotkin, & Kastin, 1995; Gutierrez, Banks, & Kastin, 1993).

Although peripheral circulating inflammatory cytokines can translocate into the brain parenchyma and impose CNS inflammatory and cytotoxic effects directly, they can also stimulate the synthesis of inflammatory biomarkers from the innate immune cells of the brain (Peters et al., 2006). A study by Qin et al. (2007) demonstrated that a single intraperitoneal injection of lipopolysaccharide, a cell wall component of gram-negative bacteria, as a pro-inflammatory stimulus causes a systemic inflammatory reaction as evidenced by elevated levels of plasma TNF-α. The latter led to increased levels of inflammatory markers within the brain and subsequent chronic neuroinflammation and neurodegeneration that continued for months after the systemic inflammation subsided. The activation of microglial cells, the innate immune cells of the brain, seemed to be the predominant process through which systemic inflammation instigated neuroinflammation and neuropathology (McGeer, Klegeris, & McGeer, 2005; Polazzi & Contestabile, 2002). These findings moved the attention towards the intrinsic sources of inflammation in the brain following systemic inflammation.

1.6.1.2 Oxidative stress induced by diesel exhaust/particle

The extra pulmonary translocation of ultrafine particles provides a plausible explanation for the hypothesis suggesting that ambient particles are capable of causing direct adverse effects on critical organs such as the brain independent of systemic and local inflammatory reactions (Khandoga et al., 2004; Nemmar, Hoylaerts, Hoet, Vermylen, & Nemery, 2003; Peters et al., 2001).

Oxidative stress, the imbalance in favour of generating free radicals, i.e. reactive oxygen species and reactive nitrogen species, is suggested to be the central mechanism for direct damage to cells afflicted by UFP (Prahalad, Inmon, Ghio, &

Gallagher, 2000; Upadhyay, Panduri, Ghio, & Kamp, 2003). Ultrafine DEP has an intrinsic ability to induce oxidative stress via several pathways by directing the generation of free radicals from the surface soluble compounds (i.e. transition metals, polycyclic aromatic hydrocarbon) (Gilmour et al., 1996; Jimenez et al., 2000); altering function of the brain capillary mitochondria or nicotinamide adenine dinucleotide phosphate-oxidase-oxidase (NADPH-oxidase) (Castell et al., 1989; Cermak et al., 1993) and stimulating reactive oxygen species and reactive nitrogen species formation by inflammation mediated cells residing in the brain parenchyma (Bermudez, Rifai, Buring, Manson, & Ridker, 2002; Cheng & Kang, 1999). Systemic inflammation has synergistic effects on heightened oxidative stress generated directly by particulate air pollution (Prasad et al., 2013; Risom, Møller, & Loft, 2005). The gaseous components and the volatile compounds usually present together with particles in diesel exhaust have been shown to heighten measures of oxidative stress *in vivo* (Sørensen, Skov, Autrup, Hertel, & Loft, 2003; Tuo, Loft, & Poulsen, 1999; Vestergaard, Loft, & Møller, 2002). Oxidative stress can potentially damage biomolecules including lipids, proteins, carbohydrates, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), leading to cell damage (Sies, 1993). Furthermore, oxidative stress is associated with many chronic disorders and is pivotal to the progression of some CNS pathologies such as neurodegeneration (Risom et al., 2005).

1.6.2 Passage of diesel exhaust/particle to the central nervous system

In addition to the lung-blood barrier, the sinusoidal endothelial cells (Khandoga et al., 2004), the alveolar–capillary barrier (Gumbleton, 2001), the BBB (Kreuter, 2012) and the olfactory bulb (Oberdörster et al., 2002) are several other potential passages that UFPs might cross and enter the blood circulation or some other organs. The last two passages are suggested to be the prominent pathways that enable inhaled ultrafine DEP travel to the CNS (Block & Calderon-Garciduenas, 2009).

1.6.2.1 Peripheral pathway

Following their translocation into the blood circulation through the lung-blood barrier, inhaled DEPs access the blood–brain barrier (BBB). The functional and structural properties of the BBB serve as the main barrier for UFPs to reach the brain. Although it might be realized that the structural and biochemical properties of the BBB which forms a tight impenetrable complex that should not be easily accessible to UFPs in the blood circulation, the systemic inflammatory reaction triggered by inhaled UFPs may impair BBB integrity and facilitate the passage of UFPs (Sano & Kanda, 2013). In addition, there are some regions of the BBB with less tight epithelial junctions such as the blood-cerebrovascular fluid barrier, which include the choroid plexus, ventricles, brainstem centres, and hypothalamus (Sano & Kanda, 2013; Ueno et al., 2000), and are highly susceptible to damage by DEPs.

1.6.2.2 Direct pathway

In addition to peripheral circulation, which mediates UFP translocation to the cerebral vessels and subsequently to the brain parenchyma, a direct pathway was recently demonstrated that allows DEPs to bypass the BBB and directly influence the CNS function along the nasal olfactory pathway. Following deposition on the olfactory mucosa, UFPs are taken up by the sensory nerve endings located in the outermost cell layer of olfactory epithelium called olfactory cells. They move via the filaments of the olfactory nerve to reach the olfactory bulb. Afterwards, UFPs cross the synapses in the olfactory glomerulus to reach dendrites of the mitral cell layer of olfactory bulb and ultimately the bundle of axons, which make olfactory tract. The olfactory tract transfers particles from the olfactory bulb to certain regions of the frontal lobe of the brain (Oberdörster et al., 2004a). The mucociliary portion of the respiratory mucosa is designing to prevent such neuronal uptake of environmental toxins, however, it has been well demonstrated that air pollution causes considerable

nasal epithelial injury (Calderón-Garcidueñas, Osnaya, Rodríguez-Alcaraz, & Villarreal-Calderón, 1997; Calderon-Garciduenas, Osorno-Velazquez, Bravo-Alvarez, Delgado- Chavez, & Barrios-Marquez, 1992; Calderon-Garciduenas et al., 1998). Interestingly, impaired olfaction and hyposmia are considered some of the early manifestations of several neurodegenerative diseases (Hock et al., 1998; Kovács, Cairns, & Lantos, 1999). Ultrafine particles can also be transmitted to the trigeminal nerves, brainstem and hippocampus through the nasal olfactory pathway since some of the trigeminal sensory nerve endings in the nasal epithelium also have connections in the olfactory bulb (Oberdörster et al., 2004a). The nasal olfactory pathway can lead UFPs to the cerebrovascular fluid as well as systemic blood stream. Afterwards, the UFPs in the systemic circulation might reach the cerebral vessels and cross the BBB that is already impaired by the UFP-imposed systemic inflammatory reactions (Illum, 2000).

1.6.3 Brain cellular mediators of diesel exhaust/particle-induced central nervous system pathology

Dysfunction of the cerebral vascular structure, the BBB, situated at the interface between peripheral blood circulation and the CNS, is regarded as one of the main potential mechanisms that allow DEPs to affect the CNS adversely as well as activating CNS resident cells, neurons and glial cells, which are responsible for innate brain immunological processes (Block & Calderon-Garciduenas, 2009).

1.6.3.1 Diesel exhaust/particle and the blood brain barrier

The blood brain barrier is a tightly sealed barrier recognized as the boundary between the cerebral blood flow and brain tissue and it is regarded as the main obstacle preventing DEP passage to the brain parenchyma. In addition, the BBB

restricts the entry of blood components such as leukocytes, red blood cells, hydrophilic substances and most lipid-soluble molecules into the cerebral tissue. Indeed, lipid-soluble molecules with a molecular weight less than 400 Dalton are the only molecules to succeed in crossing the BBB unassisted through lipid-mediated diffusion along concentration gradients (Pardridge, 2007; Zlokovic, 2008) and all the other molecules can only move across the BBB through specific transport systems. For instance, substances such as glucose and amino acids enter the brain via carrier-mediated transport, whereas the exchange of larger molecules - including transferrin, insulin and leptin- is mediated by active efflux transporters (Zlokovic, 2008).

Air pollution, in particular PM, has been associated with BBB disruption and subsequent neurotoxicity through inflammation and oxidative stress. Lockman, Koziara, Mumper, and Allen (2004) first provided evidence of UFPs crossing the BBB. In a study by Calderón-Garcidueñas, et al. (2008) post mortem investigations demonstrated significant accumulation of UFPs within red blood cells of frontal lobe vessels of children and young adults living in high-pollutant regions of Mexico who died suddenly. Translocation of UFPs from red blood cells to endothelial cells and to perivascular macrophage-like cells in frontal capillaries of these children and young adults was another important finding that supports the hypothesis that UFPs might be able to damage the BBB and cause cerebrovascular leakage.

The blood brain barrier consists of a monolayer of endothelial cells, a basement membrane, pericytes and astrocytes. The blood brain barrier endothelial cells have distinctive features including a lack of fenestrations, increased mitochondrial content, a greater number of junction complexes and sparse pinocytic transport activity compared to capillary endothelial cells in general (**Fig. 5**) (Ballabh, Braun, & Nedergaard, 2004). These features cause a highly restrictive paracellular flux through BBB endothelial cells (Liebner, Czupalla, & Wolburg, 2011). Therefore, the quantification of large macromolecules such as albumin or IgG in the brain parenchyma can be used as an indicator of the tightness of interendothelial cell junctions and the BBB integrity (Enciu, Gherghiceanu, & Popescu, 2013). Cerebrovascular endothelial cells are tightly connected by junction complexes

formed by tight junction and adheren junction proteins. The tight junction complexes, which are composed of integral membrane proteins, cytoplasmic accessory proteins and actin cytoskeleton, appear as a set of continuous intramembranous fibrils sealing the apical intercellular space. Adheren junctions complement the functions of tight junction proteins (Liebner et al., 2011).

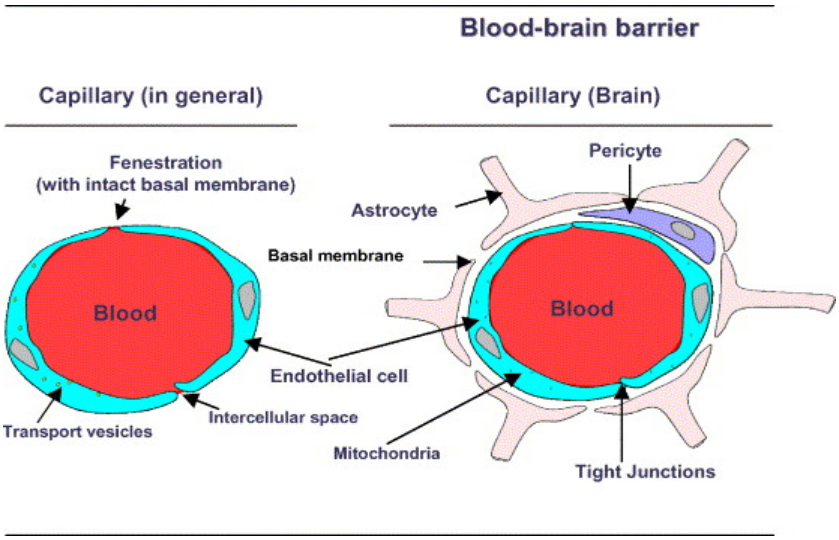

Fig. 5. Comparison of brain capillary with other capillaries in general (Liebner et al., 2011)

The increased permeability of the BBB endothelial cells via a decrease in mitochondrial content, increased pinocytosis, collagen accumulation and under expression of tight junction proteins are observed in several neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Claudio, 1996). Damage to the BBB is regarded as one of the underlying pathologies of these diseases (Block & Calderon-Garciduenas, 2009; Calderón-Garcidueñas et al., 2002; Grammas, 2000; Grammas, Botchlet, Moore, & Weigel, 1997). Ultrafine particles are also able to impose damage to the BBB by triggering oxidative stress in endothelial cells and under expression or disruption of various tight junction proteins (Hartz et al., 2008). In addition to the direct effects of UFPs on BBB endothelial cell

structure and function, UFPs may directly trigger a systemic inflammatory response, exacerbating BBB dysfunction. Different cytokines such as TNFα and IL-1β can stimulate the overexpression of inflammatory mediators inside the BBB endothelial cells, which may disrupt the BBB integrity.

It is strongly believed that all components of the BBB are crucial for maintaining the structural and physiological characteristics of this configuration and they all may be targeted by UFPs to increase BBB permeability. The blood brain barrier basement membrane located between endothelial cells and neighbouring glial cells, consists of extracellular matrix structural proteins such as collagen IV, laminins and proteoglycans as well as pericytes, known as immune cells of the microvessels, which are embedded within the basement membrane (Del Zoppo & Milner, 2006). Pericytes are key cells in sustaining the stability of the BBB both mechanically and also via matrix deposition (Armulik, Abramsson, & Betsholtz, 2005), as well as secreting a number of various growth factors that adjust microvascular permeability, remodelling, and angiogenesis (Dore-Duffy & LaManna, 2007). Astrocyte glial cells surround the outer surface of the BBB through numerous “foot processes”. Pericytes and astrocytes are two types of glial cells, which contribute considerably in mediating CNS inflammatory reactions induced by diesel exhaust (section 1.6.1.1) (Piehl & Lidman, 2001).

Owing to its unique and highly specific structural and functional properties, the BBB provides the most suitable environment for brain function and development. However, disrupted BBB endothelial cells cannot prevent the passage of normally excluded substances such as toxins, inflammatory cytokines, blood cells and plasma constituents through the barrier, which could induce the glial cells to produce neurotoxic substances (Abbott, Rönnbäck, & Hansson, 2006; Hawkins, O'Kane, Simpson, & Viña, 2006). Inflammatory substances such as TNF-α and IL-1β have been shown to be able to pass through BBB endothelial cells and stimulate the production of additional inflammatory and neurotoxic substances by glial cells (Nguyen, Julien, & Rivest, 2002; Pan & Kastin, 2001; Rivest, 2001).

1.6.3.2 Diesel exhaust and glial cells

Glial cells are non-neuronal cells of the CNS with several well-established roles including maintenance of the neuronal microenvironment with respect to nutrients, ions, nerve signal transmission, synaptic action modulation, and also activating and modulating the systemic immune responses to different nervous injuries (Piehl & Lidman, 2001). There are three classes of glial cells in the CNS: oligodendrocytes, astrocytes and microglia. Astrocytes and microglial cells are thought to be more important in mediating CNS inflammatory reactions to diesel exhaust (Piehl & Lidman, 2001).

Microglial cells are the primary innate immune cells of the brain and are also known as CNS macrophages. They are subdivided into two main cell populations: one inhabits the brain parenchyma and the other, referred to as pericytes, reside within the BBB. Microglia become activated in response to any kind of brain damage by undergoing morphological alterations such as hypertrophy or an increase in motile branches (Nimmerjahn, Kirchhoff, & Helmchen, 2005) as well as functional changes including hyper-secretion of inflammatory cytokines, reactive oxygen species and nitric oxide. Microglial activation is usually beneficial; however, it can impose neurotoxic effects depending on the severity of injury and the pathological state and subsequently initiate or exacerbate neuronal damage (Nimmerjahn et al., 2005). Microglial cells produce excessive number of inflammatory factors [TNF-α, Prostaglandin E2 and IFN-γ (interferon-γ)] and reactive oxygen species ($^{\bullet}$NO, H_2O_2, $O_2^{\bullet-}$ and $ONOO^-/ONOOH$) in an uncontrolled state termed microgliosis. Microgliosis contributes to a number of CNS diseases. The mechanisms that regulate microglial activation and prevent it from becoming deleterious for neurons may not be clear yet, however, some stimuli have been found to predispose microglia to react excessively, become more sensitive to previous inoffensive stimuli and remain active for longer period of time even when the triggering stimuli is already attenuated (Block & Hong, 2007). Exposure to air pollutants and some environmental toxins are proven to cause microgliosis. Indeed, microgliosis is one of the fundamental events

mediating CNS damage in response to exposure to components of air pollution such as UFP (Blasko et al., 2004; Sama et al., 2007). Block et al. (2004) demonstrated that DEP exposure could trigger microgliosis directly, or via stimulating peripheral inflammatory cytokines that can reach the brain through the blood vessels.

Astroglial cells have a pivotal role in maintaining the normal function of neuronal cells (Kimelberg, 2010). Astrocytes are non-excitable cells surrounding the outer surface of CNS blood vessels through their abundant processes as well as nervous synapses. A number of studies have suggested astrocytes as the cells playing an important role in inducing the unique barrier properties on the brain endothelial cells including controlling tight junction protein expression and angiogenesis, which are vital for maintaining endothelial structural integrity (Balabanov & Dore-Duffy, 1998; Zlokovic, 2005). Some other tasks of astrocytes include active uptake and metabolism of neurotransmitters such as glutamate, maintenance of ionic homeostasis in brain and control of CNS blood flow. Astrocytes, neurons and endothelial regulatory functions are interdependent. Astrocyte stimulation triggers vasodilation via up-regulation of cyclooxygenase, which increases prostaglandin levels and influences BBB permeability (Ambrosini et al., 2005; Maragakis & Rothstein, 2006; Tanji et al., 2003; Yang, Zhao, Dong, Chen, & Yu, 2008; Zonta et al., 2003). Astroglial activation or astrogliosis is the term used for the astrocyte reaction to any CNS insult, which takes place after microglia cells activation. The activated astrocytes are recognized by morphological alterations such as proliferation and hypertrophy as well as concomitant accumulation of glial fibrillary acidic protein (GFAP) and secretion of growth factors or chemokines, which causes recruitment of leukocytes to the injured site (Li et al., 2011). The overproduction and release of proinflammatory factors such as IL-1, TNF-α from astrocytes and also the attraction of leukocytes across the BBB are both demonstrated to be involved in neuroinflammation as the main underlying pathogenic mechanism of CNS damage following exposure to air pollution and in neurodegenerative diseases (Maccioni, Rojo, Fernández, & Kuljis, 2009; Maragakis & Rothstein, 2006; Wirenfeldt et al., 2007). Undeniably, air pollution components are considered as a major stimulant

associated with astrogliosis in the CNS as shown by the presence of astrogliosis as indicated by overexpression of GFAP and microgliosis (Calderón-Garcidueñas et al., 2004; Calderón-Garcidueñas et al., 2008), which are involved in neuroinflammation and are also considered as early triggers that lead to progression of neurodegenerative diseases (Maccioni et al., 2009; Simpson et al., 2010).

Introduction and structure of thesis

1. Introduction

The possible association between air pollution and detrimental health conditions was initially realized by the substantial rise in cardiopulmonary morbidities and mortalities during severe air pollution episodes between the 1930s and 1950s (Pope III, 2000). Air pollution is a heterogeneous mixture of PM, gases and chemicals with PM consistently implicated as one of the most harmful components to health (Hoek, Brunekreef, Fischer, & van Wijnen, 2001 May; Katsouyanni et al., 2001; Pope, Schwartz, & Ransom, 1992; Pope III et al., 2004; Zanobetti, Schwartz, & Dockery, 2000). Particulate matter consists of liquid and solid particles dispersed in a gas phase as an aerosol, originating from natural or human-generated sources. Particulate matter is generally classified into several groups with respect to the particle aerodynamic diameter: coarse particles (PM2.5–PM10, diameter 2.5–10μm); fine particles (PM2.5, diameter <2.5μm); and ultrafine particles (UFPs, diameter <0.1μm) (Nemmar et al., 1999; Oberdörster et al., 1996). Particles that are fine and ultrafine are found to be more harmful to health than the coarser ones because of their high propensity to pass through the lung-capillary barrier after inhalation, thus increasing the risk of extra pulmonary adverse effects (Nemmar et al., 1999; Oberdörster et al., 1996). Diesel exhaust, largely generated by diesel engines, is a major source of fine and ultrafine particles. Diesel exhaust particles are composed of elemental carbon with toxic adsorbed organics such as polycyclic aromatic hydrocarbons, and inorganic substances such as sulphates, nitrates, metals, and other trace elements. In addition to the particulate constituent, diesel exhaust is also composed of some toxic gaseous components such as carbon dioxide, carbon monoxide, nitrogen compounds and volatile chemicals (Yokota et al., 2009). Although DEP is not the only noxious constituent of diesel exhaust, it has been enormously applied in toxicological studies analysing the fine and ultrafine

associated health effects. Several toxicological studies have demonstrated the adverse effects of diesel exhaust and its individual components on different organs such as the lungs, cardiovascular system, liver and CNS (Kreyling et al., 2002; Oberdörster et al., 2004a; Takenaka et al., 2001). In comparison with the toxicological studies investigating the effects of DEP on the cardiopulmonary system, there are fewer reports of CNS effects (Block & Calderon-Garciduenas, 2009). The specific mechanisms for diesel exhaust-induced adverse effects on the CNS are unclear. However, a study by Chao et al. (2011) demonstrated significant vascular endothelial dysfunction caused by diesel exhaust exposure. It is my contention that diesel exhaust-induced cerebrovascular endothelial dysfunction may be the preliminary mechanism for the adverse effects of diesel exhaust on the CNS. The blood brain barrier describes the hallmark features of the cerebrovascular structure that are essential for regulation of bi-directional blood-brain delivery of molecules and electrolytes preserving proper function of the CNS.

The blood brain barrier is comprised of a monolayer of endothelial cells and a basement membrane with embedded pericytes surrounded by astrocyte processes. The endothelial cells of the BBB possess more extensive junction complexes and lack fenestrations and pinocytosis compared to the rest of the vascular endothelial cells (Abbott, Patabendige, Dolman, Yusof, & Begley, 2010; H. C. Bauer et al., 2011). The tightly interconnected endothelial cells of the BBB protect the brain parenchyma from neurotoxic and inflammatory substances in the circulation whilst allowing passage of small hydrophobic molecules (oxygen, carbon dioxide), and active transport of metabolic products (amino acids, glucose and vitamins) by specific protein transporters (B. T. Hawkins & Davis, 2005)**.** The barrier properties of BBB endothelial cells are not intrinsic to these cells and are induced by other components of the BBB neighbouring them. Astrocytes and the basement membrane with embedded pericytes maintain the structural integrity of BBB endothelial cells, contribute to BBB endothelial cell differentiation, cerebral blood flow auto regulation and angiogenesis (Dehouck et al., 1997; Van Zwieten, Ravid, Swaab, &

Woude, 1988). The blood brain barrier is the main obstacle for different toxic components of diesel exhaust to pass through and reach the brain parenchyma.

The systemic circulation and the nasal olfactory passage are two proposed pathways of transmission of diesel exhaust/particle to the CNS. It is possible that the inhaled diesel exhaust/particle penetrate the lungs and transfer via the systemic circulation to the BBB, which is compromised by the systemic inflammatory effects of diesel exhaust/particle exposure which might facilitate transmission of diesel exhaust components as well as the circulatory inflammatory cytokines to the CNS resulting in neurotoxic consequences. The diesel exhaust particle can also translocate to BBB then CNS or directly to CNS along the olfactory nerve (Block & Calderon-Garciduenas, 2009).

The post exposure diesel exhaust/particle inflammatory mechanisms in the blood and brain parenchyma may be responsible for BBB endothelial cell disruption. In addition, the innate toxic properties of diesel exhaust/particle may directly induce cytotoxicity altering functional and structural properties of BBB endothelial cells leading to subsequent neuroinflammation and neurotoxicity (Block & Calderon-Garciduenas, 2009; Levesque, Surace, McDonald, & Block, 2011). The mechanisms through which DEP navigate across the BBB are still unidentified. However, a few *in vitro* studies have demonstrated a decrease in vascular cell viability, attenuation of mitochondrial function, reduced expression of intercellular tight junctions, adherens (Chen, Yokel, Hennig, & Toborek, 2008) and transporter proteins (Hartz, Bauer, Block, Hong, & Miller, 2008) in addition to oxidative stress (Hartz et al., 2008) after exposure to diesel exhaust/particle which may be responsible for increased BBB endothelial permeability to DEP (Block & Calderon-Garciduenas, 2009).

Neuroinflammation is determined by the augmented level of inflammatory mediators in the CNS and has also been reported in neurodegenerative diseases. The latter might result either from translocation of systemic circulatory inflammatory biomarkers to the brain due to a compromised BBB or from inflammatory neurotoxic biomarkers secreted by innate immune cells of the CNS including microglial cells and astrocytes (Calderón-Garcidueñas et al., 2008). Diesel exhaust gaseous

components and particles are considered some main factors stimulating hyper expression of microglial and astroglial cells, a condition that occurs at the early stages of some neurodegenerative diseases (MohanKumar, Campbell, Block, & Veronesi, 2008). In addition, oxidative stress biomarkers and tissue injury have also been reported in the brain of mice exposed to high concentration of UFP, which provides another evidence for hyper secretion of reactive oxygen species by stimulated astroglial and microglial cells (Campbell et al., 2005).

Calderón-Garcidueñas, Solt, et al. (2008) suggested air pollution, including UFP, as a possible risk factor for Alzheimer's disease and Parkinson's disease. They reported cerebral accumulation of Amyloid-beta-42 and α-synuclein in the brain of dogs residing in the polluted Mexico City. Alzheimer's disease is one of the most common cerebrovascular-based neurodegenerative diseases and Amyloid-beta-42 and α-synuclein accumulation are two characteristic neuropathological features of Alzheimer's disease. In addition, a number of other pathological similarities were observed between subjects exposed to chronic air pollution and some neurodegenerative diseases such as oxidative DNA damage and Cyclooxygenase-2 over expression as indicators of chronic neuroinflammation and oxidative stress (Ho et al., 2001; Nunomura et al., 2001).

Although the underlying mechanisms responsible for the positive association between diesel exhaust/particle and neurodegenerative diseases are unresolved, diesel exhaust-induced BBB breakdown might be considered the initial triggering factor for neuroinflammation and neurodegeneration. It is a recent contention that diesel exhaust-induced BBB dysfunction may be associated with the development and/or progression of cerebrovascular-based neurodegenerative diseases such as Alzheimer's disease, vascular dementia or Parkinson's disease (Block & Calderon-Garciduenas, 2009).

2. Hypothesis

The central hypothesis of my thesis was:

Sub-chronic diesel exhaust inhalation exposure will compromise BBB integrity and function. The alteration in structural and functional properties of BBB imposed by exaggerated inhalation exposure to diesel exhaust is the primary mechanism by which diesel exhaust increases the risk of neurodegenerative diseases.

3. Objectives

Objective 1: The primary objective of this study was to determine whether inhalation exposure to, diesel exhaust could cause any alterations in BBB integrity in a wild-type mouse model, randomised to be exposed in an approved environmental chamber to either filtered air (control) or diesel exhaust (either TWA-1.2 mg/m^3 or TWA-1.8 mg/m^3) (treatment groups: low diesel exhaust concentration or high diesel exhaust concentration) for 2 consecutive hours per day, 4 days per week for 2 weeks. Cerebral capillary integrity was investigated by determining the abundance of the plasma-derived protein (IgG) in the brain parenchyma. The approach utilized for this objective was 3-dimensional immunofluorescent microscopy.

Objective 2: A secondary objective of this study was to qualitatively assess astroglial cell activation following diesel exhaust exposure, a surrogate marker of neurovascular inflammation. Parenchymal GFAP was determined by immunofluorescence microscopy.

Objective 3: Another specific objective of this study was to provide evidence for whether disturbances in cerebrovascular integrity explain any positive association between diesel exhaust inhalation exposure and neurodegenerative disease risk.

4. Thesis outline

This thesis consists of four chapters. The first chapter includes the literature review, which explored the key elements that led us to the hypothesis that was presented in the thesis. The literature review is followed by an introduction, hypothesis, objectives, and thesis outline.

The second chapter provides the detailed description of the study design and experiments that were conducted to assess the hypothesis presented in the thesis.

The third chapter provides the first-author manuscript that was published in the Journal of Applied Toxicology. The paper demonstrates the findings of this study with tables and figures summarizing the results.

The candidate had lead responsibility for the study design, experimental execution, data interpretation and genesis of the manuscript. The contribution of co-authors is detailed in "**Statement of Contribution"** section of the present thesis.

The thesis then is concluded with a general discussion, chapter four, and limitations of the study in investigating the original hypothesis. The prospective studies to strengthen the findings presented in this thesis and opportunities for extending the results into clinical context for recognizing underlying mechanisms leading to neurodegenerative diseases are discussed as well.

CHAPTER 2

CHAPTER 2

Study Design

1. Animals

Eight-week-old (n=60), male and female BALB/c mice were purchased from the Animal Resources Centre (www.arc.wa.gov.au), (Murdoch, W.A., Australia). The animals were housed in filter-top cages (22 Litres) at the Research Centre of the Telethon Institute for Child Health Research (TICHR). They were acclimatized for approximately one week under controlled air pressure, temperature (23 ± 2^0C), and relative humidity (35±10%) on a 12-h light/dark cycle and maintained on standard "Rat and Mouse Cubes" (Specialty Feeds, Glen Forrest, W.A., Australia) supplied *ad libitum* with water until the end of experiment.

This experimental animal study was approved by National Health and Medical Research Council of Australia-accredited Animal Ethics Committee (Telethon Institute for Child Health Research animal ethics approval number #221).

2. Diesel exhaust genesis and exposure

Mice were randomised into five groups of 12 mice for inhalation exposure and loaded into new Perspex exposure chambers ($30 \times 30 \times 30$ cm) as previously described (Larcombe et al., 2014). Plastic dividers separated the mice and the temperature of the chamber was maintained between 22-25^{0}C. Mice and the environmental measures were continuously monitored during the exposure periods.

For the mice in diesel exhaust inhalation groups, diesel exhaust was generated by a light medium duty Euro 1 diesel engine (Isuzu 4BD1-T, 3.9L) operated at 1500 rpm and 30% duty on Australian ultra-low sulphur diesel fuel based on the methods described by Ichinose et al. (1998). Exhaust was prepared using standard methods as previously described (Burtscher, Kunzel, Huglin, 1998). The animals were exposed

to HEPA filtered clean air, or whole diesel exhaust, which was diluted with HEPA filtered air to a target particle concentrations of either 20mg DEP/m^3 (low dose) or 30mg DEP/m^3 (high dose) prior to the exhaust entering the exposure chambers. The flow through the exposure chambers was 10 litres per minute (lpm) total flow. This included exhaust and clean air. To achieve the required concentrations, the low dose exposure was approximately 1 lpm of exhaust and 9 lpm of HEPA filtered air, and the high dose was approximately 5 lpm exhaust and 5 lpm HEPA filtered air. Doses were maintained by manually altering flow of HEPA filtered air. The exposure pattern was for two consecutive hours per day for four succeeding days: 3-days no-exposure; followed by, another four days of 2h treatment periods (Larcombe et al., 2014).

The dose of diesel exhaust was considered in the context of estimating the number of particles an adult BALB/c mouse would likely inhale in each 2 hours of exposure. At ventilation rate of 35 mL/min in an adult BALB/c mouse and an inhalation capacity of 4200 mL for each 2-hour exposure, approximately 0.084 and 0.126 mg of particles would be inhaled per exposure for 20 and 30 mg/m^3 diesel exhaust groups respectively.

Time-weighted average (TWA) is a calculated determinant used to consider and compare overall exposure rates in different industry settings. For the 20 and 30 mg/m^3 treatment groups indicated in this study the 8h-time weighted average (TWA8h) on days were 5 mg/m^3 and 7.7 mg/m^3, respectively. Over the duration of the study, the TWA concentrations were estimated to be 1.2 and 1.8 mg/m^3 for the two groups. The assessed treatment effects of DEP are greater than what typically occurs in urbanised communities, but are comparable to reports in some industry settings with poor regulation or monitoring arrangements (Van Niekerk et al., June 2002 and YuXu (1987)). Furthermore, industry estimates commonly report elemental carbon, which underestimates total diesel particulate by approximately one third (Lippmann, 2009). YuXu (1987) reported TWA worker exposures over 1.7 mg/m^3 elemental carbon. In this study, the two exposure groups are referred to as TWA-1.2 and TWA-1.8 mg/m^3 respectively.

The exhaust entering the exposure chamber was constantly monitored using a DustTrak DRX 8533 (TSI, Shoreview, MN). Detailed measurements were generated using a TSI scanning mobility particle sizer (SMPS), which consisted of a 3081 differential mobility analyser (DMA) and 3775 condensation particle counter (CPC). SMPS data were processed to obtain mass data (Liu et al., 2012). Exhaust gas levels including carbon monoxide, sulphur dioxide, nitrogen dioxide and nitrogen monoxide were continually monitored in the chambers (ENERAC, model 3000E).

At the conclusion of each 2-hour exposure, a section of stainless steel pipe work immediately after the exhaust was brushed clean of diffusion-deposited soot, collected in a glass container with minimal air space, and stored at 40 °C temp prior to analysis. Soot samples taken on each day were combined and the soot thoroughly mixed. The pooled soot sample was analysed for polyaromatic hydrocarbons, volatile organic compounds, elemental and thermo gravimetric composition (relative proportions of volatiles, non-volatiles and inorganics) (Mullins et al., 2013; Larcombe et al., 2014). Identification of the polycyclic aromatic hydrocarbons was carried out by extraction (from the soot samples) using organic solvent, followed by pre-concentration and analysis by gas chromatography–mass spectrometry GC/MS (Agilent 6890 Gas Chromatograph (GC) coupled to a 5973 Mass Spectrometer) operating in the selected ion-monitoring mode (Bezemer et al., 2011; Van Niekerk et al., June 2002) as previously described (Mullins et al., 2013; Larcombe et al., 2014). Control and laboratory blank samples were analysed concurrently with the soot samples.

Samples were spiked with a known amount of surrogate standard (ChemService Product CSS8250-1JM) and placed in the ASE for further extraction with 4:1 mixture of hexane and ethylacetate. The sample extracts were combined and concentrated down, a known amount of internal standard (ChemService Product PP-HC8JM) was added prior to the sample being made up to volume with equal volumes of ethylacetate and hexane. Sub-samples of the extract were analysed by GC/MS.

For the duration of this study an Agilent 6890 Gas Chromatograph (GC) coupled to a 5973 Mass Spectrometer (MS) operating in the selected ion monitoring (SIM) mode was used to carry out the analyses (Mullins et al., 2013; Larcombe et al., 2014). The GC was fitted with a HP-5MS capillary column (60m x 0.25mm x 0.25µm). The injector was operated in the pulsed splitless mode and was held at 300˚C with a target pulse pressure of 50 psi. After the run had been initiated the oven was held at 50˚C for 2 minutes, the column was then heated to 240˚C at 12˚C/min and then to 310˚C at 3˚C/min, the column was then held at this temperature for 16.5 minutes. The GC eluate was introduced into the MS via a transfer line maintained at 300˚C. The MS operated with a start delay of 10 minutes and positive electron ionization (EI) of 70 eV. To improve sensitivity the system was set to monitor five groups of ions ranging from 82 to 172 atomic mass units (amu) with a dwell time of 40 milli seconds (ms) in Group 1, to 138 to 300 amu with a 40 ms dwell time in Group 5. The GC/MS system was calibrated using five calibration standards, plus a zero point (Mullins et al., 2013; Larcombe et al., 2014).

The elemental analysis for the sample of particles including nitrogen, carbon, hydrogen, sulphur and oxygen was determined using a Thermo Finnigan EA 1112 Series Flash Elemental Analyser as previously described (Bredin et al., 2011).

3. Brain tissue collection and immunofluorescent quantitative analysis of Immunoglobulin G and glial fibrillar acidic protein

Six and twenty-four hours after the final exposure, mice were sacrificed. Mice were euthanized with ketamine/xylazine (1:20 respectively) at 0.1ml/g body weight. Brain tissues were isolated and immersion-fixed in 4% paraformaldehyde for 24 hours, cryoprotected in 20% sucrose for 3 days, frozen in isopentane/dry ice and stored at –80°C.

A novel methodology, which was established and developed in our laboratory previously, was implemented to evaluate the hypothesis that sub-chronic diesel

exhaust inhalation exposure may compromise BBB integrity and function. This highly specific immunofluorescent labelling method uses polyclonal antibodies that are produced in the same species and conjugated with labels such as fluorochromes, prior to their application on brain tissue.

Immunoglobulin-G is a high molecular weight protein abundant in plasma that is widely utilized as a surrogate marker of cerebral capillary permeability. (Kittelson et al. 2006; ElAli et al., 2011). Parenchymal abundance of IgG was determined based on established methods detailed in publications from our laboratory (Takechi et al., 2012; Takechi, Pallebage-Gamarallagea, Lam, Giles & Mamo, 2010; Takechi, Galloway, Pallebage-Gamarallage, Johnsen, Mamo, 2008). Briefly, 20 μm thick cryosections from random brain hemispheres of frozen brain tissues were prepared onto polysine-coated slides and dried under a fume hood for an hour. After rehydrating the slides with PBS (150-200 μL per slide) for 10 minutes, non-specific binding sites were blocked with 10 % goat serum for 30 minutes at 20°C. Brain sections were incubated with a primary antibody of goat anti-mouse IgG conjugated to Alexa 488 (1:50, Invitrogen) for 20 hours at 4°C. The nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (1:1000) at 20°C for 5 minutes and mounted with antifade mounting medium.

The cortex and hippocampal expression of glial fibrillary acidic protein (GFAP), representing the astrocyte activation, was determined as well by utilizing quantitative immunofluorescent microscopy based on the methods established previously (Takechi, Pallebage-Gamarallagea, Lam, Giles & Mamo, 2013; Takechi et al., 2012). Sections were prepared as for IgG detection. Following blocking with 10% goat serum, sections were incubated with primary antibody of rabbit anti-mouse GFAP (1: 500; Abcam, UK) for 20 hours at 4°C. After a washing procedure to remove excess primary antibodies not bound to antigen, secondary antibodies of anti-rabbit IgG conjugated to Alexa 546 (1:200, Invitrogen) was added to the section for 1 hour, and the sections were counterstained with DAPI.

The other important methodological approach in this study was to analyse abundance and distribution quantitatively in three dimensions utilizing newly

released microscopy hardware and software (Zeiss's ApoTome optical sectioning technologies and AxioVision image analysis software). Immunofluorescent micrographs were captured with a mRM digital camera (Zeiss) attached to AxioVert 200M. At a magnification of 200x (20x Zeiss Plan-Neofluar objective with 10x mRM camera), a minimum of five three-dimensional structured illumination images (Zeiss ApoTome) per section were captured from randomly selected areas of the cortex (CTX) and hippocampal formation (HPF) respectively in the approximate stereotaxic areas of 1.7 mm interaural and -2.1 mm bregma. Each 3-dimensional image consisted of 12 2-dimensional images collected in the axial plane at 1.225 μm and the distance was optimized by Nyquist overlap theory. All 3-dimensional images were analysed using Volocity 6.2 image analysis software (PerkinElmer, UK). For IgG quantitation, in three-dimensions, capillary lumen were manually masked/excluded and the extravascular voxel intensity of fluorescent signal was analysed, corresponding to the extravascular presence of IgG (a principally blood only marker). For GFAP quantitation, the densitometric sum of fluorescent pixel intensity was analysed. For all images analysed within the CTX or HPF region, the mean of the total fluorescent intensities were calculated within each animal.

4. Statistical analysis

A prior power analysis was conducted based on coefficients of variation of key measures obtained in previous studies (Pallebage-Gamarallage et al., 2011). Treatment groups contained 12 mice provide sufficient power, which was 80%, at an alpha of 0.05. Furthermore, this number of mice far exceeds studies from other laboratories reporting similar outcomes. A minimum of 60 2-dimensional images was taken from each mouse brain region and in total more than 1440 images per group for the quantitative immunomicroscopy. All values are expressed as mean ± standard error (SE). Animal mean densitometric sum of immunofluorescent data was analysed using Kruskal-Wallis. Mann-Whitney U test was used, following a significant Kruskal-Wallis test, to compare groups based on diesel exhaust treatment

and sacrifice time post final exposure. Statistical analysis was done using the statistical package SPSS (version 18). P-values less than 0.05 were considered as statistically significant.

CHAPTER 3

CHAPTER 3

Results

This following manuscript titled “The effect of diesel exhaust exposure on blood-brain barrier integrity and function in a murine model” has been published in the journal of Applied Toxicology. It describes the study results in detail.

Research Article

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The effect of diesel exhaust exposure on blood–brain barrier integrity and function in a murine model

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ABSTRACT: Epidemiological studies indicate that exposure to diesel exhaust (DE) is associated with vascular-based disorders. To investigate the effect of DE on blood–brain barrier (BBB) function and integrity, 8-week-old BALB/c mice were randomized to DE in a cyclical treatment regimen over a 2-week period. Functional integrity of BBB was determined by considering brain parenchymal abundance of IgG within the hippocampal formation and cortex at 6 h and 24 h intervals following final exposure treatment. Neurovascular inflammation was expressed as the abundance of glial fibrillar acidic protein. Two doses of DE were studied and compared to air-only treated mice. Mice exposed to DE had substantially greater abundance of parenchymal IgG compared to control mice not exposed to DE. Increased parenchymal glial fibrillar acidic protein at 24 h post-DE exposure suggested heightened neurovascular inflammation. Our findings are proof-of-concept that inhalation of DE can compromise BBB function and support the broader contention that DE exposure may contribute to neurovascular disease risk. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: Diesel exhaust; Diesel exhaust particle; Blood-brain barrier; Neuroinflammation

Introduction

Air pollutants are associated with a range of chronic disorders including respiratory, cardiovascular and neurodegenerative conditions (Hoek *et al.*, 2001; Katsouyanni *et al.*, 2001; Pope *et al.*, 1992). Diesel exhaust (DE) is an increasingly abundant air pollutant in urbanized communities and considered one of the most serious air pollutants. DE is a heterogeneous mixture of particulate matter, gases and volatile chemicals. Gaseous components of DE include carbon monoxide, carbon dioxide, oxygen, nitrogen, water vapor, nitrogen compounds, sulfur compounds and low-molecular weight hydrocarbons. DE particulate comprises elemental carbon, organic materials, including polyaromatic hydrocarbons and some trace inorganics such as sulfur (Nemmar *et al.*, 2009; Schuetzle, 1983; Strandell *et al.*, 1994). Fine (diameter 2.5 μm) and ultrafine (< 0.1 μm) DE particulate may confer greater toxic effects than larger particulate because of a greater propensity for lung penetration and deposition. Moreover, the greater surface-to-volume ratio associated with smaller DE particulate increases the possibility for absorption of associated cytotoxic compounds via lung epithelial cells (Nemmar *et al.*, 1999; Oberdörster *et al.*, 1996).

Epidemiological and clinical studies have equivocally demonstrated that DE is associated with adverse cardiopulmonary effects (Hoek *et al.*, 2001; Katsouyanni *et al.*, 2001; Pope *et al.*, 1992). Several lines of evidence also suggest that DE and, in particular, the particulate matter may contribute to the risk for onset and progression of neurodegenerative conditions such as Alzheimer's disease, vascular dementia and Parkinson's disease (Block and Calderon-Garciduenas, 2009). The putative mechanisms for a diesel particulate/neurodegenerative risk are presently unclear but are suggested to involve cerebrovascular disturbances (Doty, 2008; Lockman *et al.*, 2004).

Cerebral capillary dysfunction, characterized by brain parenchymal extravasation of plasma proteins, macromolecules and neurovascular inflammation is a feature of many neurodegenerative disorders that commonly precedes frank pathological disturbances (Zlokovic, 2008). Described as a functional blood–brain barrier (BBB), cerebral capillaries feature tightly apposed endothelial cells with highly selective bidirectional transport properties, anchored to extracellular matrices (Zlokovic, 2008). Astrocytes regulate endothelial function and in concert with resident glial cells serve as inflammatory phagocytes when breach of the capillary endothelium occurs (Seifert *et al.*, 2006).

In vitro studies have demonstrated that DE particulate can enhance the non-specific permeability of cultured brain vascular endothelial cells because of reduced expression of tight junction proteins (Hartz *et al.*, 2008). Increased synthesis of systemic and

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local proinflammatory cytokines (Block and Calderon-Garciduenas, 2009), heightened inflammation (Damiani and O'Callaghan, 2007; Muhlfeld *et al.*, 2008) and compromised redox state are suggested to be central for altered capillary function. Synergistic effects of diesel particulate with volatile organic compounds or DE gases might also enhance cerebrovascular disturbances (Block and Calderon-Garciduenas, 2009).

This study reports the effects of transient DE exposure in an *in vivo* murine model. Like other diesel particulate dosimetry studies in animal models demonstrating physiological effects, exposure rates in this study were greater than would occur in most urbanized areas but comparable to some heavy industry settings with less stringent occupational exposure policies (Birch and Cary, 1996; Heinrich *et al.*, 1986; Saverin *et al.*, 1999; Van Niekerk *et al.*, 2002; Wolff *et al.*, 1987). Furthermore, physiological differences in breathing mode for humans (nasal and oronasal) versus mice (obligatory nasal breathers), different airway geometries, alveolar retention of particulate matter, kinetics and clearance are all factors that need to be considered in the context of the total and regional doses that humans may receive (Schlesinger, 1985; Yu and Xu, 1987). Nonetheless, animal dosimetry studies provide insight into possible detrimental effects that may occur because of chronic DE exposure.

Materials and Methods

Animals

A National Health and Medical Research Council of Australia-accredited Animal Ethics Committee approved the experimental animal procedures described (Telethon Institute for Child Health Research animal ethics approval no. 221).

Eight-week-old, male and female BALB/c mice were purchased from the Animal Resources Centre (Murdoch, WA, Australia). Animals were acclimatized for a week with controlled air pressure and temperature on a 12 h light/dark cycle and maintained on standard "Rat and Mouse Cubes" (Specialty Feeds, Glen Forrest, WA, Australia) provided ad libitum with water.

Groups of 12 mice were randomized for treatment in environmental chambers. The treatment regimen was exposure to HEPA-filtered air, or DE regulated to deliver as a mixture with air either 20 mg diesel particulate m^{-3}, or 30 mg diesel particulate m^{-3}. The exposure pattern was for four consecutive days, followed by 3 days with no treatment then another 4 days of treatment as previously described (Mullins *et al.*, 2013). The duration of each treatment was for two consecutive hours. Mice were killed 6 and 24 h after the final exposure to diesel particulate. Throughout the treatment phase, mice exposed to DE showed no clinical signs or distress. The latter is based on an accredited Animal Ethics Committee scoring system of animal activity, grooming, eating behavior and "fur placement." There was no difference in body weight between treatment groups over the duration of DE exposure.

The 8 h time-weighted averages (TWA8h) on days when exposures occurred were 5 mg m^{-3} and 7.7 mg m^{-3} (for the 20 and 30 mg m^{-3} groups respectively). Over the duration of the study, the TWA concentrations were 1.2 and 1.8 mg m^{-3} for the two groups. It should be noted that exposures were to whole exhaust particulate, which in bulk form is approximately 73% of elemental carbon (Lippmann, 2009). The TWA were within the ranges reported from some heavy industry settings (Yu and Xu, 1987; Van Niekerk *et al.*, 2002), but significantly greater than most international exposure guidelines. The American Conference of Governmental Industrial Hygienists and the US National Institute for Occupational Safety and Health recommend concentrations less than 0.02 mg m^{-3} and 0.16 mg m^{-3} respectively (as mean elemental carbon). The experimental design is essentially proof-of-concept and hypothesis generating.

Diesel Exhaust Particle Genesis and Standardization

DE particulate was generated by a light duty indirect injection diesel engine operated on Australian standard diesel fuel (Office of Legislative Drafting and Publishing, Attorney-General's Department, Commonwealth of Australia, Canberra) based on the methods described by Ichinose *et al.* (1998). Each treatment group was placed into new 9 L Perpex exposure chambers maintained and plastic dividers separated mice. The exhaust entering the exposure chamber was continually monitored using a TSI scanning mobility particle sizer, which consists of a differential mobility analyzer (Model 3081, TSI, Shoreview, MN, USA) and a condensation particle counter (Model 3775, TSI, Shoreview, MN, USA). Exhaust carbon oxide, sulfur dioxide, nitric dioxide and nitric monoxide were continually monitored (ENERAC, model 3000E, Holbrook, NY, USA). The temperature of the exposure chamber was maintained between 22 and 25°C (Digitron 2006T Thermocouple; Sifam Instruments, Torquay, UK). Before each exposure, the engine was started and allowed to reach operating temperature. A constant load of ~20% was provided by a large engine driven compressor. Once the operating temperature was reached, the exhaust was connected to the exposure chamber intake, via a balance chamber to provide uniform flow and cool the air. Mass flow controllers were used to meter, precisely, the exhaust and HEPA filtered fresh air injected into each chamber.

Polyaromatic hydrocarbons were determined on diesel particulate samples collected from filters immediately after each 2 h DE exposure. Briefly, samples were extracted in a 4: 1 mixture of hexane and ethylacetate. Extracts were analyzed by an Agilent 6890 Gas Chromatograph (Santa Clara, CA, USA) coupled to a 5973 Mass Spectrometer (Santa Clara, CA, USA) (Bezemer *et al.*, 2011) operating in the selected ion-monitoring mode (Van Niekerk *et al.*, 2002) as previously described (Mullins *et al.*, 2013). The analysis for diesel particulate elements, including nitrogen, carbon, hydrogen, sulfur and oxygen was determined using a Thermo Finnigan EA 1112 Series Flash Elemental Analyser (Thermo Fischer Scientific, Waltham, MA, USA) as previously described (Bredin *et al.*, 2011).

Brain Tissue Collection and Immunofluorescent Quantitative Analysis of IgG and Glial Fibrillar Acidic Protein

Mice were killed with xylazine (0.2 mg 10 g^{-1} body weight) and ketamine (4 mg 10 g^{-1} body weight). Surgical anesthesia was identified by a lack of response to external stimuli, including no blink or pedal reflex. Respiratory depression occurs within minutes of administration. Mice were ventilated until exsanguination by cardiac puncture. Brain tissues were immersion-fixed in 4% paraformaldehyde for 24 h, cryoprotected in 20% sucrose for 3 days, frozen in isopentane/dry ice and stored at − 80 °C for immunofluorescence microscopy. Immunofluorescent micrographs were quantitatively analyzed in 3-D as we have previously detailed (Takechi *et al.*, 2013). Briefly, immunofluorescent micrographs were captured with an mRM digital camera (Zeiss, Oberkochen, Germany)

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attached to AxioVert 200 M (Zeiss, Oberkochen, Germany). At a magnification of 200 × (20 × Zeiss Plan-Neofluar objective with 10 × mRM camera), a minimum of five images per section were captured from randomly selected areas of the cortex and hippocampal formation respectively in the approximate stereotaxic areas of 1.7 mm interaural and – 2.1 mm Bregma. Each 3-D image consisted of 12 Z-stack 2-D images, and the distance between the Z-stack images were 1.225 μm optimized based on Nyquist overlap theory. The voxel intensity of fluorescent signal was then analyzed in 3-D with Volocity 6.2 image analysis software (PerkinElmer, Buckinghamshire, UK). The means of total fluorescent intensities of all the images in cortex or hippocampal formation regions were calculated within each animal.

The integrity of BBB was determined by the presence of IgG (Kittelson *et al.*, 2006) within brain parenchyma. IgG is a high molecular weight peripheral circulation protein widely utilized as a surrogate marker for BBB permeability (Beard *et al.*, 2011; ElAli *et al.*, 2011; Takechi *et al.*, 2008, 2010). Immunofluorescent labeling of IgG was done based on adjusted methods previously reported (Takechi *et al.*, 2013). In brief, brain cryosections of 20 μm were prepared on polysine-coated microscope slides and nonspecific binding sites were blocked with 10% goat serum in phosphate-buffered saline for 30 min. Goat antimouse IgG conjugated with Alexa 488 fluorochrome (Invitrogen, Carlsbad, CA, USA) was applied to the sections at a concentration of 1 : 50 in phosphate-buffered saline and incubated at 4 °C for 20 h. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted with antifade mounting medium.

The cortex and hippocampal formation expression of glial fibrillar acidic protein was determined based on methods we have previously described (Takechi *et al.*, 2013). The brain cryosections were blocked with 10% goat serum and incubated with rabbit antimouse GFAP (1 : 500; Abcam, Cambridge, UK) for 20 h at 4 °C. Goat antirabbit IgG with Alexa 546 was then added to the section for 1 h, and the sections were counterstained with DAPI.

Statistical Analysis

Each treatment group contained 12 mice, which was predicted to provide sufficient power based on the coefficient of variation of the key measures previously published (Pallebage-Gamarallage *et al.*, 2012). A minimum of 60 2-D images were taken from each mouse brain region and in total more than 1440 images per group for the quantitative immunomicroscopy. All values are expressed as mean ± SEM. The animal mean densitometric sum of immunofluorescent data were analyzed using the Kruskal–Wallis test. The Mann–Whitney *U* test was used, following a significant Kruskal–Wallis test, to compare groups based on DE treatment and time of killing after final exposure. Statistical analysis was done using the statistical package SPSS (version 18). $P < 0.05$ was considered as statistically significant.

Results

Diesel Exhaust Analysis

Figure 1 depicts the DE particulate number concentration relative to particle size at the two concentrations of diesel particulate exposure selected. Approximately 80% of particulate matter generated had a diameter less than 100 nm and classified as ultrafine or nanoparticles.

The DE particulates were comprised principally of carbon (73% weight), oxygen (20% weight), hydrogen (5.5% weight), nitrogen (1% weight) and sulfur (0.5% weight) and contained measurable quantities of the volatile organic compounds benzanthracene, benzopyrene, benzofluoranthene and chrysene (Table 1).

In addition to particulate matter, DE included gases of CO, NO_2, NO and SO_2 (Table 2). Exposure to gases was essentially quadrupled in the particulate treatment of TWA 1.8 mg m^{-3} compared to TWA 1.2 mg m^{-3}.

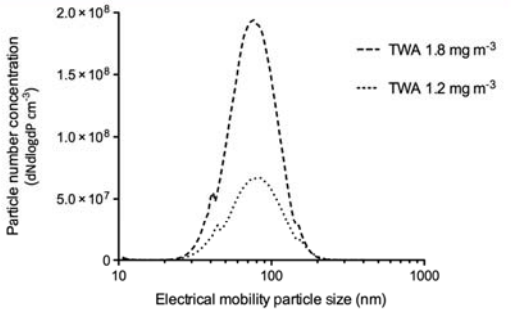

Figure 1. Diesel exhaust particle size and number determined for the two 8 h time-weighted average (TWA) intervention settings were determined using a TSI scanning mobility particle sizer, which consists of a differential mobility analyzer and a condensation particle counter. (dNdlogdP = Normalized concentration as a function of particle diameter.)

Immunofluorescent Quantitative Analysis

Brain parenchymal abundance of plasma-derived IgG was used as a marker of cerebral capillary integrity. Representative images of IgG extravasation in hippocampal formation for the three treatment groups are provided (Fig. 2). At 6 h following final treatment, mice exposed to DE had a significantly greater abundance of IgG within the cortex and hippocampal formation compared to control mice exposed to air alone (Fig. 3A).

Table 1. The concentration of selected polyaromatic hydrocarbons associated with diesel soot analyzed by an Agilent 6890 Gas Chromatograph coupled to a 5973 Mass Spectrometer operating in the selected ion-monitoring mode

Substance	Concentration (mg kg^{-1})[a]
Acenaphthylene	< 1.00
Anthracene	< 1.00
Benz[*a*]anthracene	801
Benzo[*a*]pyrene	483
Benzo[*b*]fluoranthene	< 1.00
Benzo[*g,h,i*]perylene	< 1.00
Benzo[*k*]fluoranthene	343
Chrysene	891
Dibenzo[*a,h*]anthracene	< 1.00
Fluoranthene	< 1.00
Fluorene	< 1.00
Indeno(1,2,3-cd)pyrene	< 1.00
Naphthalene	< 1.00
Phenanthrene	< 1.00
Pyrene	< 1.00

[a]Values are given in mg kg^{-1} of diesel soot.

Table 2. Exposure to carbon monoxide, sulfur dioxide, nitric dioxide and nitric monoxide were continually monitored (ENERAC, model 3000E) during intervention and are indicated for the two TWA8h exposure settings used in this study

Gas	TWA 1.2 mg m^{-3} DE concentration chamber (ppm ± SD)[a]	TWA 1.8 mg m^{-3} DE concentration chamber (ppm ± SD)[a]
CO	110 ± 13	422 ± 85.0
SO_2	2.06 ± 0.58	8.17 ± 2.28
NO	1.49 ± 0.48	5.89 ± 1.9
NO_2	5.18 ± 1.30	20.52 ± 5.13

[a]Values are given in ppm ± SD.
DE, diesel exhaust; PPM, part per meter; TWA8h, 8 h time-weighted averages.

Persistent exaggerated residency of brain parenchymal IgG was found within both cortex and hippocampal formation 24 h after the final exposure to DE (Fig. 3B). The later time of killing also provided clear evidence of a dose effect with mice exposed to TWA 1.8 mg m^{-3} DE concentration having greater abundance of cortex and hippocampal IgG compared to mice exposed to TWA 1.2 mg m^{-3} DE concentration.

Capillary vessel dysfunction and inappropriate blood-to-brain kinetics of plasma proteins and macromolecules is purported to be proinflammatory. In this study, the expression of glial fibrillar acidic protein was used as a marker of neurovascular inflammation (Fig. 4). At 24 h after DE exposure, there was evidence of increased glial fibrillar acidic protein within the cortex and hippocampal formation at the higher rates of exposure (Fig 3D). At 6 h after DE exposure, the effects of DE exposure on glial fibrillar acidic protein expression were not significant (Fig. 3C).

Discussion

This study supports an accumulating body of evidence that chronic exposure to DE can compromise the functional integrity of cerebral capillary vessels. A murine model with subchronic, transient but significant exposure to diesel particulate indicated that repeated exposure episodes over a 2-week period results in exaggerated abundance of plasma-derived IgG in brain parenchyma and this appears to be associated with the level of exposure. The findings also partially support the contention that repeated DE exposure may induce neurovascular inflammation, based on the finding of increased glial fibrillar acidic protein abundance. The upper exposure of TWA 1.8 mg m^{-3} (TWA8h 7.5 mg m^{-3}) investigated in this study is significantly greater than most international exposures guidelines. Some heavy industry settings (example mining) may exceed the exposure levels indicated in this study; however, the findings may not necessarily represent physiological responses in industry and urban settings that are compliant with contemporary health and safety recommendations. Further dose/duration studies are indicated to explore the hypothesis of DE-induced cerebral capillary dysfunction. The model utilized in this study also considered a "total" DE exposure, which includes diesel particulate concentration, complex aromatic hydrocarbons and "gases." The model therefore provides a broad interpretation of the possible effects of DE exposure to BBB function and is hypothesis generating. The study did not permit delineation of what elements are likely to contribute to cerebral capillary dysfunction. Nor does the model explore potential mechanisms for said effects. Collectively, however, the study represents an *in vivo* model showing toxic effects of DE on BBB function.

DE had an equivocal effect on brain parenchymal IgG abundance with almost a twofold and fourfold relative increase at lower and higher concentrations of diesel particulate matter, compared to control mice respectively. The hippocampal formation is principally involved in memory, attention and special awareness and in addition to these functions, cerebral cortex functions contributes to thought, language and consciousness. Hence, the effects of DE exposure on BBB function may be ubiquitous and longer-term pathophysiological effects could be broad. In this study, evidence is provided that DE can increase glial fibrillar acidic protein abundance, a marker of glial activation. In the context of DE as a persistent inflammatory trigger, it is interesting to note the sustained effects of DE on parenchymal IgG abundance (24 h post-DE exposure versus 6 h post-DE exposure). A prolonged effect to transient but repetitive breaches of BBB function may be relevant to disease risk or progression. However, further studies are required to explore the hypothesis of a DE/inflammation and disease–risk axis.

Several components of DE may have compromised BBB integrity and/or promoted inflammation. Exposure to gaseous components of air pollution, including carbon monoxide (Oberdörster *et al.*, 2004), nitrogen oxides, sulfur dioxide and ozone have been

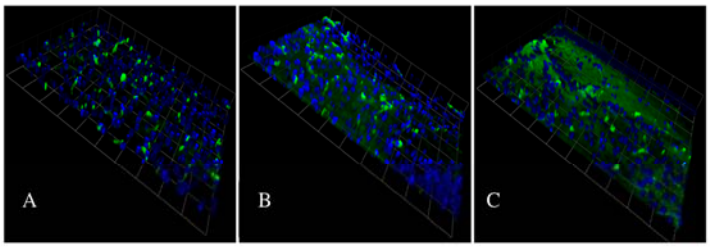

Figure 2. Immunofluorescent microscopy of hippocampal IgG extravasation killed 24 h after the last exposure. The immunofluorescent images show the distribution of IgG (green) in the brain cortex of mice in the control group and the groups exposed to the 8 h time-weighted average (TWA) of 1.2 and TWA 1.8 mg m^{-3} of diesel exhaust particulate. DAPI counterstaining was used to stain the nuclei (blue). (1 gridline = 43 μm.) (A) Hippocampus image of control group of mice killed 24 h after exposure. (B) Hippocampus image of mice inhaled diesel exhaust particulate concentration of TWA 1.2 mg m^{-3} killed 24 h after last exposure. (C) Hippocampus image of mice inhaled diesel exhaust particulate concentration of TWA 1.8 mg m^{-3} killed 24 h after last exposure.

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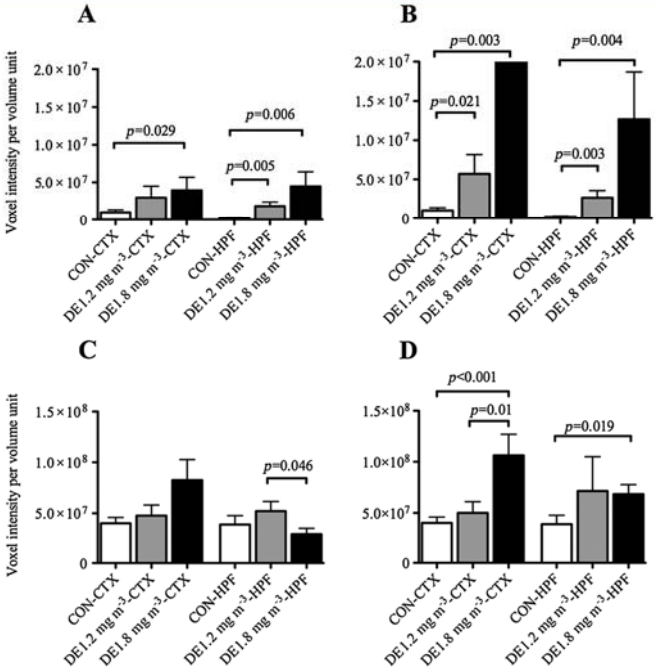

Figure 3. Three-dimensional (3-D) semiquantitative analysis of cerebral IgG extravasation (A,B) and glial fibrillar acidic protein (GFAP) expression (D,C). Pixel intensity of IgG surrounding the cerebrovasculature, and GFAP expression was quantified in two major regions of brain, cortex (CTX) and hippocampal formation (HPF), in mice exposed to fresh air as the control group (CON); the 8 h time-weighted average (TWA) 1.2 mg m^{-3} of diesel exhaust particulate and TWA 1.8 mg m^{-3}. The upper bar charts display IgG extravasation in mice randomized in to control and intervention groups killed 6 h after the final exposure (A) and 24 h after the final exposure (B). The lower bar charts display GFAP expression in mice randomized in to control and intervention groups killed 6 h after the final exposure (C) and 24 h after the final exposure (D). Data are shown as means ± SEM.

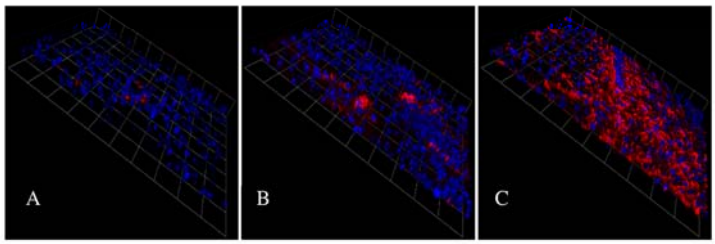

Figure 4. Immunofluorescent microscopy of cortical glial fibrillar acidic protein (GFAP) abundance of mice killed 24 h after final exposure. The immunofluorescent images show GFAP abundance (red) in the cortex of mice in control group and the groups exposed to an 8 h time-weighted average (TWA) of 1.2 mg m^{-3} of diesel exhaust particulate and TWA 1.8 mg m^{-3}. DAPI counterstaining was used to stain the nuclei (blue). (1 gridline = 43 μm.) (A) Cortical image of control group of mice killed 24 h after last exposure. (B) Cortical image of mice exposed at a TWA of 1.2 mg m^{-3} and killed 24 h after the last exposure. (C) Cortical image of mice exposed at a particulate TWA of 1.8 mg m^{-3} killed 24 h after the last exposure.

associated with mortality and hospitalization for ischemic stroke via vascular-mediated effects (Zhu *et al.*, 2012). Carbon monoxide poisoning among other hypoxic exposures disrupts cerebral capillary function (Bezemer *et al.*, 2011). Nitric oxide synthesized by nitric oxide synthase (Ichinose *et al.*, 1998) has been well documented as a vasodilator, but at higher concentrations that might occur with environmental exposure, nitric oxide may become cytotoxic (Zhu *et al.*, 2012). Sulfur dioxide appears to amplify the effects of nitric oxide (Sang *et al.*, 2010). Environmental nitrogen dioxide exposure is also suggested to increase the risk for development and progression of ischemic stroke via vascular-mediated effects (Zhu *et al.*, 2012). However, the effects of the principal DE gases (CO, NO, NO_2 and SO_2) on BBB function and neurovascular inflammation have not been reported *per se*. The TWA 1.8 mg m^{-3} diesel particulate treated group had a fourfold greater amount of CO, NO_2, NO and SO_2 compared to the TWA

1.2 mg m^{-3} diesel particulate treatment arm. Interestingly, the effects of DE on glial fibrillar acidic protein were particularly indicated in the TWA 1.8 mg m^{-3} diesel particulate group but not in the TWA 1.2 mg m^{-3} treatment arm, raising the possibility that gaseous exposure may be particularly relevant in this context (Zhu *et al.*, 2012). Oxidative stress, induced by inflammation and endothelial dysfunction of cerebral vasculature caused by altered expression of some vasoactive factors, is the main potential mechanism leads to ischemic stroke via exposure to DE gases (Sang *et al.*, 2010; Zhu *et al.*, 2012). Oxidative stress also has been well documented to contribute to BBB disruption (Sang *et al.*, 2010); however, the effects of NO_2 and SO_2 on BBB function and neurovascular inflammation have not been reported *per se*.

Polyaromatic hydrocarbons are implicated in several disorders and particularly blood-based cancers (Zhu *et al.*, 2012). The diesel particulate in this study contained measurable quantities of benz[*a*]anthracene, benzo[*a*]pyrene and benzo[*b*]fluoranthene and chrysene. There is presently a paucity of studies investigating the effect of polyaromatic hydrocarbons on BBB integrity and function. In a comprehensive review provided by the Agency for Toxic Substances and Diseases registry, there is little evidence presently supporting polyaromatic hydrocarbons and neurological disorders. Relevant to the polyaromatic hydrocarbons associated with diesel particulate in this study, male and female mice exposed to 0, 175, 350 or 700 mg kg^{-1} day^{-1} acenaphthene by gavage for 13 weeks showed no effect of treatment on behavior, or histopathological effects on nerve or brain samples (US EPA, 1989). Similar findings were reported after 13-week administration of 1000 mg kg^{-1} day^{-1} anthracene, and 500 mg kg^{-1} day^{-1} fluoranthene (US EPA, 1988, 1989). However, no inhalation studies, or pre-pathological studies have been reported, therefore cerebral capillary effects of polyaromatic hydrocarbons cannot presently be excluded.

DE particulate independent of associated polyaromatic hydrocarbons could potentially cause vascular disturbances. An indirect pathway for a cerebral capillary effect is a consequence of diesel particulate deposition within airways triggering alveolar macrophage release of cytokines that have broad systemic effects. Alternatively, some evidence suggests that ultrafine diesel particulate may enter the circulation directly via the nasal olfactory pathway (Block and Calderon-Garciduenas, 2009). Synergistic effects of diesel particulate and polyaromatic hydrocarbons may also be relevant to capillary sensitivity to DE exposure. (Bezemer *et al.*, 2011) reported that acute single phase exposure to respiratory particulate matter activates lung dendritic cells, induces pulmonary inflammation and a T-helper 2-type cytokine response from naïve CD4+ T cells *ex vivo*. Respiratory inhalation or nasal instillation studies with DEP free of PAHs are necessary to consider the specific effects of DEP on cerebral capillary integrity and function.

Conclusions

The overall findings of this study suggest that components of DE have the potential to disrupt BBB function. Cumulative effects from transient but repeated exposure to DE pollutants may induce a state of heightened neurovascular inflammation. The latter may contribute to the onset or progression of some neurological disorders.

Acknowledgments

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Conflict of Interest

The Authors did not report any conflict of interest.

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CHAPTER 4

CHAPTER 4

Discussion

1. Principal Conclusion

Among diverse air pollutants, ambient fine and ultrafine particulate matter are considered a significant and increasing health risk in urbanized communities. Diesel particulate matter may persist in the atmosphere for extended periods and subjects may be exposed for chronic durations. Long-term health risks associated with DEP may be exacerbated by concomitant inhalation of DEP associated volatile organic compounds and diesel exhaust gases (Nemmar et al., 2009b; Schuetzle, 1983a; Strandell et al., 1994a). Health risks associated with exposure to the array of diesel exhaust pollutants have primarily focused on respiratory and cardiac functional effects, with fewer studies investigating CNS sequelae (Hesterberg et al., 2009; Hoek et al., 2001 May; Katsouyanni et al., 2001). At the commencement of my candidacy, several lines of evidence suggested that diesel exhaust pollutants could influence risk for some neurodegenerative disorders via a compromised vascular axis, heightened inflammation and oxidative stress (Calderón-Garcidueñas et al., 2002; Calderón-Garcidueñas, Mora-Tiscareño, et al., 2008; Calderón-Garcidueñas et al., 2004). The primary objective of this study was to explore the indicated hypothesis *in vivo*. The findings provided proof-of-concept data that exposure to diesel exhaust-pollutants compromise cerebral-capillary function, resulting in disturbed kinetics of plasma proteins and heightened GFAP expression by resident inflammatory cells. These observations were made in wild-type adult aged mice free of pathological neurological conditions, supporting the concept that diesel exhaust pollutants could synergistically contribute to risk for such disorders.

2. Principal molecular mechanisms for diesel exhaust/particle-induced brain capillary dysfunction

2.1 Molecular mechanisms underlying diesel exhaust/particle-induced inflammation

The recent discovery of molecular components, underlying the adverse influence of diesel exhaust/particle on the neurovascular unit has developed a better understanding of the BBB reaction in response to systemic immune challenges. Interaction between inhaled diesel exhaust components; alveolar macrophages and airway epithelial cells generate inflammatory biomarkers including IL-1β, TNF-α and granulocyte-macrophage colony-stimulating factor (Laskin, Heck, & Laskin, 1998; Van Eeden et al., 2001; Van Eeden, Yeung, Quinlam, & Hogg, 2005). Cerebral blood vessels express receptors for some of the inflammatory ligands such as IL-1β and TNF-α (Ericsson, Liu, Hart, & Sawchenko, 1995; Nadeau & Rivest, 1999), which are well known for causing neurodegeneration (Ling et al., 2006; Manousakis, Jensen, Chacon, Sattin, & Levine, 2009; Perry, Cunningham, & Holmes, 2007; Qin et al., 2007; Rivest et al., 2000). After binding to their receptors, IL-1β and TNF-α trigger a cascade of molecular events resulting in the activation of the protein complex of nuclear factor-κB (NF-κB) within the BBB endothelial cells and then in brain parenchymal microglial cells resulting in subsequent regulation of various inflammatory mediator genes encoding cyclooxygenase-2 and nitric oxide synthase (Borgerding & Murphy, 1995; Shafer & Murphy, 1997). The overexpression of stated cyclooxygenase-2 and nitric oxide synthase genes prompt the production of the inflammatory mediators prostaglandins and nitrogen monoxide, respectively within brain vascular endothelial cells (Bryan, Bian, & Murad, 2009; Furchgott & Vanhoutte, 1989; Furchgott & Zawadzki, 1980), which can open the BBB (Thiel & Audus, 2001). The high levels of inflammatory molecules induced by IL-1β and TNF-α impair the BBB by regulating P-glycoprotein activity (B. Bauer, Hartz, & Miller, 2007; A. M. S. Hartz, Bauer, Fricker, & Miller, 2004, 2006), a key efflux transporter protein in the BBB that limits transfer of a large spectrum of compounds through the BBB and is considered the main obstacle for entrance of CNS-acting drugs to brain (B. Bauer, Hartz, Fricker, & Miller, 2005; Jette &

Beliveau, 1993). Blood brain barrier disturbances in several conditions such as neurodegenerative diseases and stroke are also indicated by alterations in P-glycoprotein expression and function (Hartz et al., 2008). In addition, these two cytokines were demonstrated to shift the tight junctions from between the endothelial cells of BBB towards the other cell surfaces, which may explain macromolecule extravasation across the BBB during inflammation (Rivest et al., 2000). Along with IL-1β and TNF-α, IFN-γ has also been consistently reported to decrease expression of tight junction protein components by relocalization of zonula occludens protein-1 and junctional adhesion molecule-A in a time and dose-dependent manner (Amasheh et al., 2009; Capaldo & Nusrat, 2009; Ewert et al., 2010). Finally, the inflammatory cytokines induce oxidative stress at the BBB level via generation of reactive oxygen species as well as reactive nitrogen species (Tyml, 2011; Wolin, 2009; Wolin, Gupte, & Oeckler, 2002).

2.2 Molecular mechanisms underlying diesel exhaust/particle-induced oxidative stress

Oxidative stress inflicts damage to all biological components of cells by mechanisms such as DNA damage, lipid and protein peroxidation and fragmentation or cell membrane damage (Sies, 1993). A wide range of studies generally propose that the augmented level of reactive oxygen species and diminished bioavailability of nitrogen monoxide that are induced by the inflammatory state mediate the microvascular dysfunction significantly (Abbott et al., 2010; Liebner et al., 2011; Nakagawa et al., 2009). Diesel exhaust/particle has been shown to stimulate the redox-signalling systems directly as well (Hartz et al., 2008). A number of enzymes are considered to mediate the mechanisms triggering reactive oxygen species generation such as nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) or uncoupled endothelial nitric oxide synthase (C. A. Chen et al., 2011). Diesel exhaust has been previously reported to increase reactive oxygen species through the activation of NADPH oxidase in BBB endothelial cells and CNS microglial cells, which are the main sources of reactive oxygen species in the brain

(Block et al., 2004). Hartz et al. (2008) have demonstrated that reactive oxygen species were found to play a pivotal role in diesel exhaust/particle-induced P-glycoprotein up-regulation in BBB endothelial cells that result in the opening of the cerebrovascular structure. They also demonstrated that exposure to diesel exhaust increases levels of inducible nitric oxide synthase in brain capillaries, however, the role of uncoupled endothelial nitric oxide synthase-induced nitrogen monoxide on BBB permeability and function has not been established. Several *in vivo* studies have reported an increase in protein vascular leakage after blocking nitrogen monoxide production (Harris, 1997; Kurose et al., 1993; Rumbaut, Wang, & Huxley, 2000). In contrast, genetic blockade of uncoupled endothelial nitric oxide synthase in one other study resulted in increased albumin extravasation across BBB in vascular beds, which was also confirmed in wild-type mice after long-term treatment with nitric oxide synthase inhibitor (Predescu, Predescu, Shimizu, Miyawaki-Shimizu, & Malik, 2005).

3. Limitations to Study

The murine model applied in this study is one of the most commonly used strains in animal toxicological studies, however, it might not represent human models. For example, the physiological and anatomical differences between humans and mice in the pulmonary immune system and airway geometries which play the principal roles in the clearance of diesel exhaust components from the lungs and retention of particles in alveoli as well as passage of DEP into the systemic circulation. The physiological differences between modes of inhalation of diesel exhaust for humans (nasal and oronasal) versus mice (obligatory nasal breathers) should also be considered (Schlesinger, 1985; Yu & Xu, 1987).

It is important to emphasize that diesel exhaust possesses a highly complex mixture of two other elements disregarding ultrafine particles: non-ultrafine particles and a variety of gaseous components of toxicological properties. Thus, diesel exhaust

in the model we designed or similar toxicological studies cannot address ultrafine DEPs in isolation. However, it can provide a useful source of data for determining the nature of health hazards imposed by DEPs.

The diesel exhaust mass concentration in the upper exposure group investigated in the study exceeded most international occupational exposure threshold limit values (Oberdörster et al., 2005; Nel et al., 2006), although, the exposure levels may exceed the indicated doses in our study in some heavy industry settings such as mining (Mauderly & Garshick, 2008). Our use of elevated concentrations of DEP, which increases the possibility of observing ultrafine particle induced toxicity, may aggravate the potential interfering effects of other diesel exhaust components such as gases or polycyclic aromatic hydrocarbon.

This study did not explore the potential underlying mechanisms responsible for diesel exhaust/particle-imposed CNS effects. In addition, the effect of dose and duration of exposure to diesel exhaust on different regions of brain was not really explored in this study.

4. Future studies

Following the provided evidence of BBB disruption and neuroinflammation through this study, more *in vivo* dose-duration studies of DEP free of polycyclic aromatic hydrocarbons and gases are necessary to further investigate the short-term and long-term specific effects of DEP on cerebral capillary function, the brain neuronal and non-neuronal cells as well as identifying the specific mechanisms underlying such alterations which can provide us with a better interpretation of the pathophysiological aspects of CNS damage due to air pollution.

In addition, studies are warranted to investigate the extravasation of some other plasma macromolecules through the BBB, indicators of oxidative stress, as one of the hypothesized underlying mechanisms of damage of BBB and brain by air

pollutant nanoparticles, and also specific inflammatory cytokines TNF-α and IL-1β, as some of the known inflammatory biomarkers with harmful effects on BBB, in brain tissue to confirm our findings and broaden our understanding of different harmful properties of DEPs on the cerebrovascular system and their potential role in generating some prevalent neurodegenerative diseases.

5. Final conclusion

The murine model presents an *in vivo* study demonstrating BBB disruption after transient but repeated episodes of diesel exhaust exposure over a short period of time. As accumulating evidence supports the hypothesis that BBB dysfunction precedes diagnostic pathologies of some neurodegenerative diseases, this study explores the association of diesel exhaust exposure in initiation and progression of vascular-based neurodegenerative disorders.

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