Title
Diminished Defenses In Children May Lead To Increased Susceptibility To Inflammatory Effects of Air Pollutants

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Diminished Defenses In Children May Lead To Increased Susceptibility
To Inflammatory Effects Of Air Pollutants

A thesis submitted in partial satisfaction
of the requirements of the degree Master of Science
in Clinical Research

by

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2012
Children are considered to be highly susceptible to the adverse effects of pollutants such as diesel exhaust particles (DEP). Phase II enzymes are a key defense against the oxidant effects of pollutants. Twenty adults and fifteen children (11-16 years of age) underwent a single-blind placebo controlled randomized exposure study to differing DEP nasal challenges. Gene expression levels of 3 sentinel Phase II genes (GSTP1, NQO1, HO-1) and markers of inflammation were measured before and 24 hours after DEP challenge. We found that nasal lavage cell count increased with increasing DEP challenge doses in adults and children. However, at the highest doses children had greater cellular inflammation and decreased Phase II enzyme expression as compared to adults. Children appear to have a diminished ability to generate protective enzymes in response to higher DEP exposures as compared to adults. Impaired antioxidant defenses in children may lead to increased vulnerability to pollution-induced airway diseases.
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Chapter 1 – Background

Oxidative Effects of Diesel Exhaust Particles on Highly Susceptible Children

Diesel Exhaust Particles

Exposure to air pollutants is believed to be a significant factor contributing to the increased incidence of allergic diseases in industrialized countries. Traffic-related air pollution and its potential adverse health effects are a concerning issue. Diesel exhaust emissions are an important contributor to airborne pollution. Diesel fuel is widely used throughout the world as a source of power to fuel vehicles and generators including trucks, buses, agricultural equipment, and electric generators. Diesel engines are widely viewed as being more efficient and versatile than gasoline engines or alternative vehicle fuels; however the exhaust from diesel engines contain harmful substances that have been shown to be hazardous to human health.

According to the World Health Organization, particulate matter adversely affects more people in the world than any other air pollutant. Diesel exhaust is a major source of particulate matter (PM). Diesel exhaust is a heterogeneous mixture of thousands of gases and fine particulate matter.\(^1\) Diesel exhaust particles (DEP) are a major component of diesel combustion products and are present in high concentrations in urban air from mobile sources. DEP consist of a carbon core surrounded by trace metals and adsorbed organic polycyclic aromatic hydrocarbons. Electron microscopy studies have shown that DEP have a mass median diameter of 0.2 µm, a size that makes them easily respirable and capable of...
depositing in the airways leading to potential airway inflammation. DEP consist of a mixture of fine and ultrafine particles. Particles smaller than 2 µm in diameter have a greater ability to deposit in the peripheral airways and alveoli. In addition to the ultrafine size of DEP, toxicity caused by DEP may also be due to its metal content which is adsorbed on the DEP surface. These trace metals are able to generate reactive oxygen intermediate species from airway cells leading to inflammatory effects in the airways and alveoli.\textsuperscript{[3-6]}

**Air Pollution and Oxidative Stress**

A proposed mechanism by which particulate matter in diesel exhaust exerts its adverse effects in the lungs of healthy and asthmatic individuals is via induction of oxidative stress. As reactive oxygen species come into contact with proteins, lipids, and DNA they lead to cellular damage. When there is an overproduction of reactive oxygen species such as upon exposure to PM, one’s natural antioxidant defenses may be overwhelmed leading to cellular oxidative stress and inflammation of the airways.\textsuperscript{[7]}

There are 2 metabolic pathways for the detoxification of DEP compounds: Phase I and Phase II metabolism pathways (Figure 1a). In phase I metabolism, expression of the cytochrome P450 genes is induced. Polycyclic aromatic hydrocarbons (PAHs) contained in DEP can cause cellular damage due to their tendency to accumulate in cell membranes. PAHs are lipophilic, thus can directly cross the cell membrane and bind to the cytosolic arylhydrocarbon receptor (AHR). The PAH-AHR complex translocates to the nucleus where it is able to activate transcription from xenobiotic response element (XRE) thus activating Phase I drug metabolizing enzymes such as cytochrome P450 enzymes. Ultimately, this metabolic pathway can activate transcription of genes containing the antioxidant response element (ARE) which promotes Phase II drug metabolizing enzyme activity. Phase II enzymes protect cells from oxidative stress by
conjugating oxidatively labile compounds so that they become hydrophilic moieties which are soluble and excretable. Phase II genes contain both a XRE and an ARE in their 5’ flanking regions. Thus, DEP metabolism occurs via direct induction of Phase II enzymes or via indirect induction through Phase I enzymes. It may be that the extent to which Phase II enzymes are produced affect the degree to which DEP is able to exert its inflammatory effects. [8]

**Phase II Enzymes**

NADPH:Quinone oxidoreductase (NQO1) and Glutathione S-transferases (GSTs) are important Phase II enzymes expressed in the respiratory tract. GSTs represent a group of multifunctional proteins which protect against the toxic effects of xenobiotics. GSTs catalyze the conjugation of glutathione (GSH) to products of oxidative stress, a variety of electrophils and reactive intermediates rendering them soluble and excretable. Many GST isoenzyme families have been identified including the Mu family (GSTM1), the Theta family (GSTT1), and the Pi family (GSTM1). [9] GST genes are expressed in a variety of tissues as well as the respiratory tract. Generation of reactive oxygen species induces a decrease in GST concentrations, which in turn leads to the increase in transcription of GST genes. [10]

NQO1 is a flavoenzyme that can detoxify quinones to hydroquinones which are less toxic. Previous studies have shown that NQO1 expression can be induced by DEP in human nasal cells. [11] Heme oxygenase 1 (HO-1) is a phase II detoxifying enzyme that has been found to be induced via the ARE in the promoter region of the gene. HO-1 is induced by DEP as well, and appears to play an important role in cytoprotection against DEP chemicals including quinones. [12]

Kinetics of GST production in vivo in humans has been described in several different studies. Gupta et al. reported a doubling in the specific activity of GST in peripheral lymphocytes
obtained from human participants 10 h after administration of oltipraz, a compound used in chemoprevention via a proposed mechanism of activation of phase II enzymes. Elevations in levels of glutathione were also observed. The mRNA of phase II enzymes increased after dosing to reach a peak on day 2 and declined to baseline levels over the subsequent week. Riedl et al. studied the effect of the antioxidant sulforaphane on phase II enzyme expression. The phase II enzyme gene expression was measured following 3 days of daily ingestion of sulforaphane. The phase II gene expression was specifically measured 2 hours after the 3rd oral sulforaphane dose. Oral sulforaphane was found to effectively induce Phase II enzyme expression in the upper airway of human subjects in a dose-dependent manner.

Evidence from in vitro studies suggest that inflammation associated with DEP exposure may be mediated by oxidative stress. Sagai et al found that DEP could produce oxygen radicals such as superoxide and hydroxyl radicals in vitro in lung endothelial cells without any biological activating systems. In a study of the effects of DEP on alveolar macrophages and lymphocytes in a mouse model, it was found that DEP exposure greatly increased inflammatory markers in pulmonary lavage fluid. In response to the DEP exposure, alveolar macrophages showed a time-dependent increase in antioxidant intracellular cysteine and glutathione levels suggesting a protective mechanism of the lung in response to oxidative stress. In a murine ovalbumin (OVA) sensitization model, Whitekus et al. demonstrated that thiol antioxidants N-acetylcysteine (NAC) and bucillamine (BUC) were able to prevent a decrease in intracellular reduced glutathione: glutathione disulfide ratios after exposure to organic DEP extracts. In addition, NAC and BUC were also able to prevent the generation of lipid and protein peroxidation in the lungs of mice exposed to OVA and DEP suggesting that oxidative stress plays an important role in the mediation of adverse effects by DEP. Indeed, it appears that DEP
may increase oxidative stress in 2 ways -- directly by the chemicals they contain, and indirectly by increasing activation of intracellular signaling and transcriptional activation of inflammatory cytokine and cytokine genes.

**DEP and Allergy**

Exposure to diesel exhaust and diesel exhaust particulates has been shown to induce a marked inflammatory response in the human airways. Salvi et al. performed a controlled diesel exhaust exposure study in which healthy adults were exposed to either air or diluted diesel exhaust for 1 hour. Bronchial biopsies revealed that diesel exhaust exposure resulted in a marked cellular inflammatory response in the airways with increased neutrophils, mast cells, lymphocytes, and endothelial adhesion molecules. The mechanism of this cellular response appears to be mediated by the enhanced gene transcription of interleukin-8, a trend toward an increase in IL-5 in the bronchial tissue, and increased expression of growth-related oncogene-alpha in the bronchial epithelium. In another study, inhalation of diesel exhaust particulates was shown to result in an increase in sputum neutrophils and myeloperoxidase 4 hours after exposure as well as increased exhaled carbon monoxide levels. Exposure to diesel exhaust and DEP appear to evoke an airway inflammatory response in healthy human airways.

Human exposure studies have shown that particulate pollutants such as DEP can augment inflammation and allergic responses in both the upper and lower airways by increasing IgE production and skewing immune reactions toward a Th2 response. In a nasal challenge study with 0.3 mg of DEP, Diaz-Sanchez et al. demonstrated that DEP could enhance local IgE production with a concomitant increase in epsilon mRNA production in the upper human airways. In subsequent studies, DEP have been shown to synergize with allergen at the human upper respiratory mucosa to enhance allergen-specific IgE production, initiate a TH2
cytokine environment, and even promote primary allergic sensitization. In a study in which DEP were administered with ragweed in ragweed sensitized individuals, it was shown that DEP augmented a ragweed-driven allergen-specific IgE response. Challenge with DEP plus ragweed also resulted in an increase in the mRNA expression of Th2-type cytokines and a decrease in mRNA expression of Th1-type cytokines. \[^{[21]}\] DEP have been demonstrated to sensitize atopic individuals to a neoantigen, keyhole limpet hemocyanin (KLH). After serial challenges with intranasal KLH followed by DEP and repeat KLH challenges, KLH sensitization was assessed. While challenge with KLH alone resulted in an IgA and IgG anti-KLH response, challenge with DEP plus KLH elicited an IgE anti-KLH response as well demonstrating DEP ability to sensitize to a neoantigen. \[^{[22]}\]

Air pollution in the form of particulate matter in diesel exhaust has been associated with a number of adverse health consequences. Chronic exposure to particulate matter has been shown to increase ones risk of cardiovascular disease, lung disease, and lung cancer. \[^{[23]}\] Respiratory diseases including asthma in particular have been shown to be closely correlated with PM levels. Short-term exposure to diesel exhaust has been shown to be associated with exacerbation of characteristic features of asthma including inflammation and hyperresponsiveness. In a study investigating acute effects of exposure to diesel exhaust, asthmatics were found to have increased airway hyperresponsiveness to methacholine challenge, increased airway resistance, and elevated levels of the inflammatory marker IL-6 in the sputum. \[^{[24]}\] McCreanor et al. examined the effects of roadside diesel traffic on individuals with asthma. Although changes in respiratory symptoms before and after exposure were small, there were significant reductions in forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) when exposed to higher levels of fine particles. This finding was more prominent in those with moderate asthma as compared to
mild asthma.\textsuperscript{[25]} Epidemiological studies conducted have also supported a relationship between PM levels and asthma exacerbations. A study of asthmatic individuals living in the San Fernando Valley which ranks as one of the regions in the U.S. with the highest levels of air pollution revealed that both increased ozone and particulate matter levels were associated with adverse health outcomes. There were significant increases in daily or weekly asthma symptoms, increased asthma-related emergency department visits, and hospitalizations as ozone, PM2.5 and PM10 were increased.\textsuperscript{[26]}

**Air Pollution and Children**

Epidemiological studies have highlighted the association between air pollutant levels and adverse health effects. Children appear to be especially vulnerable given their developing immune and respiratory systems. At birth, the lung is not fully developed. During early childhood, the number of alveoli in the lungs increases from 24 million at birth to 257 million at age 4. Development of the lung continues until adolescence. The lung epithelium is immature as well, resulting in increased permeability.\textsuperscript{[27]} The more narrow airways of children make them more susceptible to inflammation caused by air pollution.\textsuperscript{[28]} Infants and children generally breathe more rapidly than adults, which increase their exposure to pollutants in the air. Additionally, young children often breathe through their mouths, bypassing the filtering effect of the nose and allowing more pollutants to be inhaled. Children and adults have unequal exposure to ambient air pollution. Children generally spend significantly more time outdoors than adults, thereby differentially taking more air pollutants into their lungs. Children also tend to participate in more outdoor physical activities than adults.\textsuperscript{[29]} Also, children may be differentially more susceptible to the adverse effects of air pollutants given the relative immaturity of their immune system and their suboptimal ability to combat and detoxify harmful xenobiotics.\textsuperscript{[30]}
The Southern California Children’s Health Study is a population-based, long-term evaluation of air pollution and respiratory health of 6,000 children in the 4th, 7th, and 10th grade classrooms in twelve Southern California communities. Numerous analyses and findings have been derived from this comprehensive evaluation which began in 1993. Major conclusions from the study include findings that chronic exposure to air pollution including particulate matter can lead to increased risk of asthma and asthma symptoms which may ultimately result in diminished lung function at the age of 18 years when reversibility of lung function deficits are unlikely. Children living in more polluted communities were also observed to have reduced growth of lung function and more school absences. Living within 75 meters of a major road was associated with an increased risk of asthma and wheezing symptoms. This relationship was greatest among children without a parental history of asthma and who had lived in the home before 2 years of age. This may indicate a greater vulnerability in younger infants and toddlers to the adverse effects of air pollution. However, if living greater than 150-200 meters from a major road, no increased risk of asthma was observed. Interestingly, the increased risk with proximity to major roads was found to be greater in girls than in boys.\textsuperscript{31} In the 4th grade cohort over a 4 year period, children who were exposed to the higher levels of ambient air pollutants (PM10, PM2.5, PM2.5-10, nitrogen dioxide (NO2), inorganic acid vapor) had slower annual rates of lung function growth with diminished forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC), forced expiratory flow at 75% of expired FVC (FEF 75), and maximal midexpiratory flow (MMEF).\textsuperscript{32} When following the cohort of children from 10 years to 18 years of age, Gauderman et al. found that a low FEV1 was associated with higher exposure to NO2, acid vapor, PM10, PM2.5, and elemental carbon. The findings from this study showed that lung development was adversely affected in children who were exposed to high levels of air pollution.
In a study investigating potential consequences of changes in air quality when a child moved to areas with different levels of air pollution, Avol et al. discovered that annual lung function growth rates as measured by FEV1, MMEF, and peak expiratory flow rate (PEFR) increased in children when moving from areas with higher PM10 levels to lower levels. These finding were most pronounced when analyzing changes in PM10 levels. Thus, it appears that decreased levels of air pollution exposure during adolescence may have a significant positive impact on lung function growth. [34]

Studies on the adverse effects of air pollution on childhood asthma have taken into consideration where a child’s home is located, as well as how much traffic-related exposure there is for children at their schools and in their commuting times to school. In a prospective study examining new diagnoses of asthma, the relationship between new-onset childhood asthma and traffic-related pollution near homes and schools was studied. It was found that children exposed to higher levels of traffic-related pollution at home and at school had a higher incidence of being diagnosed with asthma. [35] When investigating the frequency of severe wheeze in asthmatics, commuting time to school was measured as a proxy for on-road air pollution exposure. The on-road commuting time likely represents increased exposure to ultrafine particles and emitted from vehicles and buses on the road. Although there was no statistically significant relationship between commute time and prevalence of asthma, there was a correlation between commuting time among those with commuting times 5 minutes or longer and incidence of severe wheeze in children with asthma. Interestingly, similar findings were found for asthmatic children who walked to school with longer walking commute times being associated with increased incidence of severe wheeze. [36]
The importance of exposure of children to diesel exhaust particles while riding in a school bus cannot be underestimated. Behrentz et al. studied air pollutant concentration levels of black carbon, particle-bound polycyclic aromatic hydrocarbons, nitrogen dioxide, fine particulate counts, and fine particulate matter (PM$_{2.5}$) during long commutes on school buses on the Los Angeles Unified School District bus routes and bus stops. Mean exposures for these pollutants during the bus commute were between 20-40 times higher than that found at bus stops. The increased exposures that occurred inside the school buses resulted mainly from exposure during the commute itself, and not from idle time waiting at bus stops or during times of loading and unloading.\(^{[37]}\) It appears that children riding in school buses may be exposed to up to 4 times the level of diesel exhaust as compared to others riding in cars. Diesel exhaust levels in school buses are higher in the back of the bus as compared to the front of the bus.\(^{[38]}\) The PM$_{2.5}$ particulate concentrations inside school buses were up to 5-10 times the background levels of PM$_{2.5}$.\(^{[39]}\)

**Antioxidants**

Previous studies have shown that children may be more vulnerable than adults to the inflammatory effects of oxidant pollutants. The oxidant effects of environmental tobacco smoke (ETS) and DEP are likely to be quite similar. Gong et al. studied the effects of ETS on non-smoking adults and children who had no recent exposure to ETS. In this study, the participants underwent a controlled chamber exposure to 2 hours of ETS or clean air. Twenty-four hours after ETS exposure, nasal lavages performed revealed significantly higher increases in pro-inflammatory cytokines including TNF-$\alpha$, GM-CSF, and IL-$1\beta$ in children as compared to adults. The children also exhibited a more robust cellular response as measured in the nasal lavage fluid. These findings suggest that ETS had a significantly greater effect in children as compared to adults. In a murine model examining the effects of ETS in young (less than 2 weeks old) and old
mice (8 weeks old)), it was shown that age played a significant factor in allergic sensitization by ETS. The mice were exposed for 10 consecutive days to 1% nebulized ovalbumin protein and 20 min/day either in the presence or absence of 1 hour of daily smoke from 5 cigarettes. The ability of the mice to mount total and allergen-specific IgE was determined. After exposure to ETS, the IgE, cellular and cytokine responses to ETS were more exaggerated in the younger mice.\textsuperscript{[40]} These studies illustrate that the young may be more susceptible to the adverse effects of oxidant particulate pollutants, but the mechanism for these findings has not yet been elucidated. A potential hypothesis is that increased susceptibility is due to under-developed Phase II enzyme responses in children leading to a diminished ability to combat oxidative stress.

Given that children may be more vulnerable to oxidative stress as compared to adults, antioxidant therapy may be a valuable intervention modality. Antioxidant defenses play an important role in protecting the lung from insult. Epidemiologic studies have suggested that dietary intake of antioxidant foods and vitamins may have a positive effect on respiratory function.\textsuperscript{[41, 42]} In a cross-sectional analysis of the Children’s Health Study, the association between pulmonary function and intake of fruits, vegetables, juices, vitamin A, C, and E of children 11-19 years of age was studied. Gilliland et al. found that low intake of fruit juices was associated with statistically significant decreases in FVC and FEV1 in boys. This trend was similar in girls, but results did not achieve statistical significance. There were no significant associations between intake of whole fruits and vegetables and lung function. Low vitamin A, C, and E intake was associated with decreased lung function.\textsuperscript{[43]} However, in a randomized, placebo-controlled trial examining the effects of 16 weeks of daily oral supplementation with Vitamin C and magnesium in adults with asthma, there was no evidence of a beneficial effect on asthma control. Outcome measures included change in FEV1, FVC, peak flow, change in
symptoms, and bronchodilator use. Similarly, a parallel group randomized placebo-controlled trial examining potential effects of Vitamin E on asthma control did not show any significant benefit.

Since children appear to be especially vulnerable to DEP-induced oxidative stress, future study of antioxidant supplements in this age group may be beneficial. It is important to further investigate whether dietary intake of antioxidants in children could contribute to antioxidant defenses directly or indirectly by increasing expression of antioxidant genes to ultimately improve clinical disease states such as asthma.
Chapter 2 - Thesis

Diminished Defenses In Children May Lead To Increased Susceptibility To Inflammatory Effects Of Air Pollutants

Introduction

Accumulating evidence regarding the adverse effects of air pollution in children is of growing concern. Adverse health effects in children have been demonstrated in those living in more polluted communities. Many of the detrimental effects have been linked to pollution from mobile sources, including cars, trucks, planes, and diesel-powered equipment. Long-term exposure to outdoor air pollution has been shown to affect the respiratory health of children. Children living in more polluted communities have been shown to suffer reduced growth of lung function, increased asthma exacerbations, and increased incidence of newly diagnosed asthma. [32]

Diesel exhaust emissions are an important contributor to airborne pollution. Diesel exhaust particles (DEP) are a major component of diesel combustion products and are present in high concentrations in urban air from mobile sources. They consist of a carbon core surrounded by trace metals and adsorbed organic polycyclic aromatic hydrocarbons which have been shown to have inflammatory effects in the airways and alveoli. [4, 5] DEP have a mass median diameter of 0.2 µm, a size that makes them easily respirable and capable of depositing in the airways leading to potential airway inflammation. Human and animal exposure studies have shown that particulate pollutants such as DEP can augment inflammation and allergic responses in both the upper and lower airways.

Exposure to air pollutants such as diesel exhaust particles is believed to be a factor contributing to the increased incidence of allergic diseases in industrialized countries.
DEPs have been shown to participate in the development of allergic airway inflammation. [8] DEPs can synergize with allergen at the human upper respiratory mucosa to enhance allergen-specific IgE production, initiate a TH2 cytokine environment, and even promote primary allergic sensitization. [21] Short-term exposure to diesel exhaust is associated with exacerbation of asthma, increasing airway hyperresponsiveness and bronchoconstriction. [24, 25]

Particulate air pollutants are thought to mediate many of their toxic effects via generation of reactive oxygen species and oxidative stress. [7, 45] Generation of free radicals subsequently leads to upregulation of proinflammatory cytokines. The pathways involving redox-sensitive transcription factors and cell-signaling pathways have recently been elucidated in bronchial epithelial cells both in vitro and in vivo. [46] Phase II enzymes such as glutathione-S-transferases (GSTs) are thought to be a key defense against the oxidant effects of pollutants such as DEP. GSTs participate in antioxidant defenses by catalyzing the conjugation of products of oxidative stress to glutathione making them more hydrophilic and excretable. [47] GST genotypes are expressed in a variety of tissues including the respiratory tract. Generation of reactive oxygen species induces a decrease in glutathione-S-transferase concentrations, which in turn leads to the increase in transcription of GST genes. [10] Variants in the genes of phase II enzymes, particularly GSTM1 and GSTP1, have been shown to modify the inflammatory and adjuvant effects of DEP, as well as ozone, secondhand smoke and other air pollutants. [48-50]

We postulated that phase II gene expression in the upper airways is induced by oxidant pollutants such as DEP and determines inflammatory outcomes. In support of this, previous in vitro studies have shown that over-expression of these enzymes will decrease DEP-induced induction of pro-inflammatory mediators or IgE production from bronchial epithelial and B cells respectively. We compared inflammatory responses in response to nasal DEP challenge in
children and adults and examined differences in phase II antioxidant defenses. We tested the hypothesis that the enhanced vulnerability of children to pollution-induced airway disease is due to their diminished Phase II antioxidant defenses.

**Methods**

**Participants**

We enrolled twenty adults aged 23-45 years and fifteen children aged 11-16 years living in Los Angeles, California (Table 1). Individuals recruited through advertising and referrals completed the informed consent process and subsequently underwent study screening procedures. This included a brief medical history to confirm 1) no history of oral antihistamine or nasal steroid spray use in the 3 months prior to the study, 2) no history of smoking in the two years prior to the study, 3) no history of having lived with a smoker in the two years prior to the study, 4) no history of perennial allergic rhinitis or asthma, 5) no history of an upper respiratory tract infection for at least 8 weeks before the study. Use of antihistamine, intranasal, or immunosuppressive medication including any form of corticosteroids was prohibited during the study period. There were no other inclusion or exclusion criteria. Based on interview responses, none of the volunteers had any atypical exposure to pollutants. Verbal and written consent were obtained from all adult participants. Assent was obtained from all pediatric participants as well as written consent from a parent. All study procedures were approved by the Human Subject Protection Committee of the University of California at Los Angeles. Each visit was monetarily compensated.

**Sample Size**

We estimated the necessary sample size based on power for testing the central hypotheses that changes in phase II enzyme expression occur in response to DEP and differs between adults and
children. We used the preliminary data in which GSTM1 expression was measured in 4 adults after exposure to two doses of DEP: 0 (saline) and 300µg. Comparing the 0 to 300 DEP exposures in adults, we calculated that we would need only 5 subjects to get 80% power at a 5% level of significance for a 2-fold difference. However, to compare adults with children, we assumed greater variability in children so that we would need twice as many children as adults. We calculated that we would need 18 adults and 36 children to get 80% power at a 5% level of significance for a 1.5 fold difference. We assumed a 10% drop-out rate, so would need to recruit 20 adults and 40 children. [11] Unfortunately, due to the logistical difficulty in recruiting children, only 15 children were able to be recruited. We did not have any subjects drop out. Thus, the study was not able to meet the original planned criteria. However, the sample size used in our study was comparable to previous studies which were performed analyzing effects of oxidant pollutants on children and may be representative of ‘typical’ sample sizes. [11, 40]

Procedures

The study was conducted in a single-blind randomized exposure design with each subject acting as his or her own control. All subjects were exposed to 4 differing diesel exhaust particle nasal challenge doses – 0 (control), 30, 100, 300 µg on 4 separate occasions, at least 4 weeks apart, and in a randomized sequence. Each exposure to a DEP challenge dose required 2 separate visits scheduled 24 hours apart. The first of each pair of visits consisted of a nasal lavage to establish pre-challenge baseline levels. Unfortunately, as noted below these data are no longer available. This was followed 10 minutes later by a DEP challenge dose. Twenty-four hours later, a post-challenge nasal lavage was performed and collected (Figure 1b).

Nasal washes and DEP challenges were performed as previously described. [20] RNA was extracted from nasal lavage cell pellets. RNA purification performed by RNeasy Mini Kit and
RNase-Free DNase Set (QIAGEN Valencia, CA), followed by cDNA synthesis using Sensiscript RT Kit (QIAGEN). All qRT-PCR was performed by using TaqMan predesigned probes and primers using the TaqMan Universal PCR Master Mix (Applied Biosystems Carlsbad, CA) and TaqMan PRISM 7700 Sequence Detection System. The primers and probes were supplied by PE Applied Biosystems, using their Assays-on-Demand Gene Expression Products system. 

Assay and public identification numbers are listed below.

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DNA for genotyping was isolated from buccal cells. DNA genotyping was performed for GSTM1 as previously described. \[51\]

Pro-inflammatory cytokines IL-8, GM-CSF, and TNF-α were measured from nasal lavage supernatants obtained before and 24 hours after DEP exposure by commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits following the manufacturer’s instruction. Total cell count and differential counts were measured as previously described. \[52\]

**Data analysis/Statistical analyses**

Descriptive statistics (mean and standard deviation) were generated to characterize the study population and summarize major outcome variables. The major outcome variables included the total cell count, cytokine levels, and phase II enzyme expression 24 hours post challenge for each differing DEP challenge condition. Phase II enzyme expression was summarized as relative to
the saline control. The primary comparisons were between responses by adults vs. children for each outcome variable and challenge dose. Each individual acted as his/her own control. All tests were two-sided and a p-value < 0.05 was considered statistically significant. Comparisons between groups for total cell count and Phase II enzymes were done by rank sum tests. Pearson correlations were also calculated between two outcomes across and within challenge conditions. The p-values were calculated using the null hypothesis of zero correlation.

Role of the funding source

The National Institutes of Health: National Institute of Environmental Health Sciences and Environmental Protection Agency played no role in study design, subject recruitment, data collection, data analysis, or the compilation of this report.

Results

Children showed a greater susceptibility to the pro-inflammatory effects of DEP. In both adults and children, the degree of cellular infiltration strongly increased with the dose of the nasally administered DEP challenge (Figure 2). Higher concentrations of DEP elicited a larger number of total cells recovered from nasal lavage fluid obtained 24 hours after DEP exposure. However, at the highest dose (300ug), cell influx was significantly greater in children than in adults (p=0.025). At baseline (saline) and the lowest DEP challenge dose of DEP30, children had a statistically significant lower cell counts than adults. The lower initial cell count in children augments the reversal at the highest DEP300 challenge dose with children showing greater cell counts (Table 2a).

Expression of phase II enzymes GSTP1, HO-1, and NQO1 measured in cell pellets derived from nasal washes after challenge with DEP was significantly higher than saline in both adults and children (Figures 3-5). Higher concentrations of DEP challenge corresponded with higher
levels of phase II enzyme relative gene expression in a dose-dependent fashion. At the highest DEP challenge dose of 300µg, GSTP1 expression differed significantly between adults and children with adults exhibiting a more robust response (p=0.005) (Figure 3). HO-1 expression in response to DEP100 (p=0.005) and DEP300 (p=0.012) was higher in adults as compared to children (Figure 4). There were no statistically significant differences in NQO1 expression between adults and children for differing DEP challenge doses (Figure 5).

Differing DEP challenge doses caused a change in differential cell count numbers. In both adults and children, the proportion of macrophages and lymphocytes were significantly increased following challenge with DEP, and correspondingly the proportion of neutrophils was decreased. At the two highest doses, children also had proportionally more macrophages than adults and fewer neutrophils (Table 2b).

In adults, inflammatory cytokine levels of IL-8, TNF-α, and GM-CSF found in nasal washes after challenge with DEP were significantly higher than prechallenge levels. Higher DEP challenge dose concentrations were associated with increased levels of these proinflammatory cytokines in a dose-dependent fashion in adults (Figure 6).

Scatter plot data for adults and children showed that the degree of cellular infiltration was inversely correlated with Phase II enzyme gene expression in both adults and children at the higher DEP challenge doses (Figure 7). In adults, the negative correlation between cell count and GSTP1 expression was statistically significant at the DEP challenge doses of DEP100 (r= -0.44; p=0.045) and DEP300 (r= -0.70; p=0.001). Likewise, the negative correlation between cell count and HO-1 expression was statistically significant at the DEP challenge doses of DEP100 (r= -0.54; p=0.013) and DEP300 (r= -0.65; p=0.002). However, there was no statistically significant negative correlation between cell count and NQO1 expression. In adults,
GSTP1 and HO-1 phase II enzyme expression at the highest DEP challenge dose was positively correlated (r=0.84; p=<0.0001), but no such correlation was found with NQO1. In children, there was a statistically significant negative correlation between cell count and GSTP1 expression at the DEP100 challenge dose (r= -0.73; p=0.002) and the DEP300 challenge dose (r= -0.77; p=0.002). Similar results were found for NQO1 at DEP100 (r= -0.63; p=0.013) and DEP300 (r= -0.79; p=0.001). The negative correlation was also found for cell count and HO-1 expression at DEP100 (r= -0.54; p=0.038), and a negative correlation trend without statistical significance at DEP300 (r= -0.55; p=0.052).

Discussion

Children comprise a subpopulation that is considered to be at increased risk of adverse health outcomes from air pollution. Not only do children have smaller airway diameters and faster minute ventilation rates as compared to adults, they also tend to spend more time outdoors engaged in physical activity ultimately leading to overall increased exposure to air pollutants. Our results suggest that children may have further reason for their vulnerability to ambient air pollutants, as they appear to have diminished antioxidant defenses.

In our study, adults demonstrated a dose-dependent relationship of increased antioxidant enzyme expression in response to increasing DEP nasal challenge doses. Correspondingly, with increased antioxidant enzyme expression, there were decreased levels of inflammatory markers. These findings were consistent with previous studies showing that antioxidant defenses protect against the proinflammatory and oxidizing effects of DEPs. Children demonstrated a similar pattern as the adults at lower levels of DEP exposure. However, at the highest DEP challenge dose of 300 µg, antioxidant expression in children did not increase accordingly as in the adults. The relatively lower antioxidant enzyme activity in children at the highest DEP challenge dose
suggests that there may be a plateau of antioxidant enzyme expression in children as opposed to a more robust anti-inflammatory response elicited by adults. This likely accounts for the findings of increased inflammatory markers in children when challenged with the highest DEP dose as their limited antioxidant enzyme activity cannot effectively combat the DEP-induced inflammation. Ultimately, the increased inflammation associated with impaired antioxidant defenses may lead to increased adverse health effects in children. It is of interest to note that the negative correlations between total cell count and most of the Phase II enzymes suggest that the higher the Phase II enzyme response, the lower the inflammatory response. At baseline (saline) and the lowest DEP challenge dose of DEP30, children had a statistically significant lower cell counts than adults. The lower initial cell count in children augments the reversal at the highest DEP300 challenge dose with children showing greater cell counts.

The highest DEP challenge dose used in our study was 300 µg. In everyday settings, comparable doses could be encountered when residing in Los Angeles for 40 hours, standing near a bus when its engine is being started up, or being in a freeway tunnel for 10 minutes. Our findings in children exposed to diesel exhaust particles are particularly important given the ubiquitous nature of diesel-powered buses and cars worldwide as well as the use of diesel-powered school buses for children. Children who ride in diesel-powered school buses have even greater DEP exposure given that in-vehicle pollution was shown to be much higher than those found in the ambient air due to the tendency of school buses tend to drive through polluted areas and behind high emitting vehicles as well as from the bus exhaust itself which leaks into the cabin. Children in school buses may be exposed to 4 times the level of diesel exhaust as compared to a child in an adjacent car and up to 8 times the average statewide level. School bus idling where children congregate is an important additional source of diesel exhaust
exposure. Appropriate measures have already begun to take place in order to decrease exposure of children to diesel emitted by school buses. Findings from our study emphasize the urgency in which these protective measures should be implemented.

Exposure to elevated levels of air pollutants such as DEP may result in adverse clinical consequences that affect the upper and lower airways. Findings from the Children’s Health Study revealed that long-term exposure to air pollution is associated with reduced growth of lung function in children as demonstrated by substantial deficits in attained FEV1 measured at 18 years of age. Indeed, there were 3 to 5 times more children living in high outdoor pollution communities that exhibited diminished FEV1 as compared to those living in low outdoor pollution communities. While we have focused on the effects of DEP on healthy adults and children, it seems that the inflammatory sequelae may be magnified in those with pre-existing lower airway disease. Previous studies have demonstrated adverse clinical consequences in asthmatic subjects following DEP exposure. Short-term exposure to DEP has been shown to exacerbate airway hyperresponsiveness and airway resistance in asthmatics. Asthmatic children have demonstrated increased symptoms of cough and sputum production after exposure to increased air pollution levels. Given that children appear to be more vulnerable to adverse effects of DEP than adults, it may be that asthmatic children in particular are especially sensitive.

Further evidence for the key role of oxidative stress in the DEP model is the ability of antioxidants to block the untoward effects of oxidant pollutants. In a murine ovalbumin sensitization model, the adjuvant effects of DEP were greatly diminished following administration of antioxidant agents. Ritz et al. conducted a study of airway epithelial cells examining the use of sulforaphane, an isothiocyanate derived from cruciferous vegetables and a potent inducer of antioxidant phase II enzymes. After supplementation with sulforaphane,
production of proinflammatory cytokines was greatly diminished following DEP exposure. \cite{57}

Riedl et al. studied the effect of the antioxidant sulforaphane on phase II enzyme expression in a human model. Oral sulforaphane was found to effectively induce Phase II enzyme expression in the upper airway of human subjects in a dose-dependent manner. \cite{14} Clinically, previous studies involving asthmatics have revealed mixed results regarding efficacy of antioxidant therapy. \cite{42, 44, 58, 59} Since children appear to be especially vulnerable to DEP-induced oxidative stress, future study of antioxidant supplements in this age group may be beneficial. It is important to further investigate whether dietary intake of small molecule antioxidants in children could contribute to antioxidant defenses directly or indirectly by increasing expression of antioxidant genes to ultimately improve clinical disease states.

Individuals appear to vary in their response to oxidative stress. These differences are to some extent genetically determined. Polymorphisms in phase II enzymes such as glutathione-S-transferase P1 (GSTP1) lead to increased susceptibility to reactive oxygen species and may affect level of atopy as well as severity of bronchial hyperresponsiveness and asthma. \cite{50, 60} The efficacy of antioxidants appears to be affected by genetic polymorphisms of GSTM1. In a study examining the harmful effects of ozone on pulmonary function, results showed that asthmatic children with absent GSTM1 activity displayed an enhanced response after antioxidant supplementation as compared to children with protective GSTM1 activity.

In conclusion, we have shown that antioxidant enzyme gene expression may govern the extent of the proinflammatory effects of DEP. Children have a diminished ability to generate protective enzymes with higher levels of DEP exposure as compared to adults. Impaired antioxidant defenses in children may lead to increased vulnerability to pollution-induced airway diseases.
Acknowledgements

DEPs were a generous gift from Dr. M. Sagai (National Institute for Environmental Studies, Tokyo, Japan). These particles were generated by a light duty, four-cylinder diesel engine (4JB1 type; Isuzu Motors) using standard diesel fuel prepared as previously described.
Chapter 3 – Statistical Analysis

Design

In our study, we utilized a crossover design. Each patient was randomly assigned to a sequence of challenge conditions. Each participant underwent all 4 challenge conditions in a random order which was determined by drawing a challenge condition from a bag which contained all challenge conditions that the subject had not undergone already. An advantage of using a repeated measures crossover design includes reduction of confounding covariates because each subject serves as his/her own control. Also, a crossover design allows one to achieve a given power using a smaller sample size.

A potential limitation in using a crossover design is the order effect because theoretically, the order in which the challenge dose was given could potentially affect the outcome measure. Unfortunately, we were unable to assess for the possibility of order effect given that this recorded information was lost. While each individual underwent each of the 4 different challenge doses in a random fashion, the exact order of the DEP challenge doses for each individual was not available. Therefore, we were unable to effectively rule out a potential order effect. A second disadvantage of using a crossover design is the possibility of a carry-over effect between challenge conditions. In our study, we did have a specified washout period of 4 weeks which should have been a sufficient amount of time to minimize a potential carry-over effect.

Alternative Analysis Strategy

A potential way to analyze a repeated measures study would have been to use a linear mixed effects model. Such a model would have allowed us to evaluate the impact of different challenge doses on outcomes of interest while controlling for baseline values and random effects. A linear mixed effects model is able to account for within-individual correlations, which allows the
intercept to vary as a random variable from person to person. The slope would be able to
describe the relation between the outcome variable of interest, y and time as a function of the x
moderators. By calculating changes in slope, it may have been possible to develop a dose-
response model. Calculating a slope would also have allowed us to see the patterns of change.

Limitations

The main statistical concern for our study is that we are missing pieces of data due to loss of
raw data and other key pieces of information. The data was originally collected between 2003-
2004. Over time, the data has been misplaced, misfiled, and otherwise missing. The statistical
analysis of our data is thus limited and suboptimal, but still able to yield some clinically and
statistically significant results. In addition, it is unfortunate that we didn’t have pre-challenge
baseline levels. If we had this information, it would have allowed us to quantitate the pre-
challenge variability and to better assess our outcome variables.

We had no raw data for Phase II enzyme expression of GSTP1, HO-1, and NQO1. The data
available were ratios in which the Phase II enzyme values for each challenge dose at 30, 100, and
300 µg were divided by the baseline expression (control dose of normal saline) for each
individual. With incomplete raw data, there would be an even greater concern if there was a lot
of variability in the baseline values. When working with ratios instead of raw data, if there is a
lot of variability in the baseline values for each individual, the ratios will be greatly skewed. For
example, if by chance, the baseline expression was lower than usual, the ratio would be
improperly inflated. And vice versa, if by chance the baseline expression was higher than usual,
the ratio would be improperly deflated. It may however be a reasonable alternative to use ratios
rather than raw data for analysis. Numerous previous studies from our laboratory examining
Phase II enzyme expression challenge data utilized ratios. [14] Previous challenge data from our
laboratory did not reveal significant variability in individual baseline levels of phase II enzyme expression.

We have cytokine data only for the adults, but not for the children. In our study, we were able to use the cell count as a measure of inflammation in response to a DEP challenge. A stronger argument regarding inflammation could have been made had we had the data on cytokine levels for children for each DEP challenge dose as well. In determining total cell count, we did not attempt to control for the size of the nasal passage of each participant which would have differed between adults and children. If we did adjust the total cell count for nasal surface area, our findings of an increased inflammatory response in children as compared to adults would have been even greater.

We have only summary statistics for our group of adult and children participants. We do not have individual data for the age, weight/height, gender, or ethnicity. This limitation did not allow us to analyze whether any of the specific patient characteristics had a statistically significant effect on the outcome measure. The lack of individual data on the children did not allow us to thoroughly evaluate whether age was significant variable or not. The age range in children of the participants was between 11-16 years which may have potentially yielded statistically significant differences in outcome measures between the youngest and the oldest child participants. While we are comparing responses to diesel exhaust challenge in ‘adults’ and ‘children’, the average age of the children was ‘14’. Ideally, we would have liked to be able to use children that were younger in order to have a more distinct group from the ‘adult’. However, it would have been logistically difficult recruiting and obtaining IRB approval for younger children. It would also have been difficult for younger children to be able to perform the necessary nasal lavage procedures to gather the data.
A minor limitation of our study was that it was single-blinded vs. double-blinded. Originally, it was thought that a single-blind design would be sufficient given that the outcome variables measured were all objective outcomes. However, for the administrator of the DEP challenge doses, knowing the challenge dose may have affected the technique or how effectively the challenge dose was administered. There were practical reasons for utilizing a single-blind instead of double-blind design. A double-blinded design would have required another study administrator to be present to help with administering the challenge doses. A potential disadvantage of involving another administrator is that it may have increased the chance for human error, for example administering the wrong dose of DEP.

When first beginning analysis of our data, relative expression of the phase II enzymes GSTP1, HO-1, and NQO1 were plotted for differing DEP challenge doses in adults and children. (Figures 8-13). These plots were performed to determine if there were any general trends in the data, and to determine whether transformation of the data would be necessary. Performing log of phase II enzymes and log of cell counts did not appear to improve the interpretability of the graphs. When viewing these figures, it seemed that there was a general trend with increased phase II enzyme expression and cell count corresponding with higher DEP challenge doses for adults and children. However, closer analysis showed variability for each individual where some individuals plateaued at a DEP challenge dose of 100, while other individuals actually had decreased phase II enzyme expression and cell count with increasing DEP challenge doses. The fact that we did not have individual patient characteristic data did not allow us to be able to offer potential explanations of why the trajectory curve for each individual behaved as it did. One advantage of our analysis is that we used non-parametric tests allowing us to make fewer assumptions and have greater applicability.
Data for a 4\textsuperscript{th} phase II enzyme, GSTM1 was collected for our study, but was ultimately missing at the time of data analysis. We also do not have the information on which participant had a present vs. null GSTM1 genotype. We know that GSTM1 genotype was present in approximately 50\% of the adults and children, and null in the remainder. It would have been very informative to have this information because it was an important variable that likely would have affected the inflammatory response as measured by cell count and cytokine levels. Having information about each participant’s genotype for GSTM1 could have explained some of the individual variability that we observed when analyzing individual trajectories of phase II enzyme expression and cell count with each DEP challenge dose. Having GSTM1 data could also have increased our power or ability to distinguish the effects of DEP challenge doses on cytokines and cell count.

While performing statistical analysis of our incomplete data set, it became very apparent that we had many statistical limitations. We were unable to perform many tests that may have allowed us to better analyze our data. Nevertheless, we found several interesting statistically significant correlations and trends that supported our initial hypotheses. The process of dealing with missing data and suboptimal data entry and records taught me many important lessons that will help me to be better equipped to be involved in future clinical studies and trials.
Table 1. Participant Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Adults (n=20)</th>
<th>Children (n=15)</th>
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<tbody>
<tr>
<td>age (years)</td>
<td>29 (4.5)</td>
<td>14 (1.6)</td>
</tr>
<tr>
<td>weight (kg)</td>
<td>72 (13.7)</td>
<td>60 (13.5)</td>
</tr>
<tr>
<td>height (cm)</td>
<td>170 (11.4)</td>
<td>165 (13.4)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>women</td>
<td>8 (40%)</td>
<td>6 (40%)</td>
</tr>
<tr>
<td>men</td>
<td>12 (60%)</td>
<td>9 (60%)</td>
</tr>
<tr>
<td>Ethnicity</td>
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</tr>
<tr>
<td>Hispanic</td>
<td>4 (20%)</td>
<td>8 (53%)</td>
</tr>
<tr>
<td>Non-Hispanic</td>
<td>13 (65%)</td>
<td>4 (26%)</td>
</tr>
<tr>
<td>Mixed</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (10%)</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>GSTM1 genotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>present</td>
<td>10 (50%)</td>
<td>7 (46%)</td>
</tr>
<tr>
<td>null</td>
<td>10 (50%)</td>
<td>8 (54%)</td>
</tr>
</tbody>
</table>

Data are mean (SD) or number (%).

Table 2a. Total cell counts for adults and children

<table>
<thead>
<tr>
<th></th>
<th>Total Cell Counts (x 10⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adults</td>
</tr>
<tr>
<td>DEP Challenge Dose</td>
<td></td>
</tr>
<tr>
<td>0 µg</td>
<td>5.61 ± 1.84</td>
</tr>
<tr>
<td>30 µg</td>
<td>7.53 ± 4.26</td>
</tr>
<tr>
<td>100 µg</td>
<td>21.43 ± 12.87</td>
</tr>
<tr>
<td>300 µg</td>
<td>31.08 ± 14.70</td>
</tr>
</tbody>
</table>

Results are the mean cell differential percentage for the 35 subjects ± 1 SD. * p<0.05 for differences between children and adults.
Table 2b. White blood cell differential for adults and children

<table>
<thead>
<tr>
<th>DEP Challenge Dose</th>
<th>White Blood Cell Differential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>eosinophil %</td>
</tr>
<tr>
<td>Adults</td>
<td></td>
</tr>
<tr>
<td>0 µg</td>
<td>0.33 ± 0.52</td>
</tr>
<tr>
<td>30 µg</td>
<td>0.18 ± 0.29</td>
</tr>
<tr>
<td>100 µg</td>
<td>0.15 ± 0.29</td>
</tr>
<tr>
<td>300 µg</td>
<td>0.18 ± 0.34</td>
</tr>
<tr>
<td>Children</td>
<td></td>
</tr>
<tr>
<td>0 µg</td>
<td>0.40 ± 0.54</td>
</tr>
<tr>
<td>30 µg</td>
<td>0.20 ± 0.37</td>
</tr>
<tr>
<td>100 µg</td>
<td>0.20 ± 0.41</td>
</tr>
<tr>
<td>300 µg</td>
<td>0.19 ± 0.33</td>
</tr>
</tbody>
</table>

Results are the mean cell differential percentage for the 35 subjects ± 1 SD. * p<0.05 for differences between children and adults
Figure 1a. Metabolic Pathways for Detoxification of Diesel Exhaust Particles.

ARNT - Aryl hydrocarbon receptor nuclear translocator
**Figure 1b. Study Profile.** A single-blind randomized exposure design was used to test responses to 4 different DEP doses – 0 (control), 30, 100, 300 µg. Subjects had nasal lavage sampling immediately before and 24 hours after each DEP challenge. DEP was administered by nasal spray into one nare via an atomizer. Each subject was randomized to one of 24 possible sequences of 4 DEP exposures. A four week wash-out period was observed between each DEP exposure. Each concentration of DEP was given to each subject only once.
Figure 2. Total Cell Count vs. DEP Challenge Dose in Adults and Children

*\( p=0.014 \), **\( p=0.015 \), ***\( p=0.025 \)

Error bar represents ± 1 SD
Figure 3. GSTP1 Relative Gene Expression in Adults vs. Children
Figure 4. HO-1 Relative Gene Expression in Adults vs. Children

![Bar chart showing HO-1 expression in adults and children across different DEP challenge doses. The chart includes error bars and statistical significance markers (*) and (**).](chart.png)

- Adults
- Children

* p=0.0048   ** p=0.0124
Figure 5. NQO1 Relative Gene Expression in Adults vs. Children
Figure 6. Mean Cytokine Levels vs. DEP Challenge Dose in Adults
Figure 7. Scatterplot of Cells vs. Phase II Enzymes in Adults and Children

ADULTS

CHILDREN
Figure 8. GSTP1 Relative Gene Expression in Adults
Figure 9. GSTP1 Relative Gene Expression in Children
Figure 10. HO-1 Relative Gene Expression in Adults
Figure 11. HO-1 Relative Gene Expression in Children
Figure 12. NQO1 Relative Gene Expression in Adults
Figure 13. NQO1 Relative Gene Expression in Children
References


