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Health effects of subchronic exposure to diesel–water–methanol emulsion emission

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The US Environmental Protection Agency's National Ambient Air Quality Standards for ozone and particulate matter (PM) require urban non-attainment areas to implement pollution-reduction strategies for anthropogenic source emissions. The type of fuel shown to decrease combustion emissions components versus traditional diesel fuel, is the diesel emulsion. The Lubrizol Corporation, in conjunction with Lovelace Respiratory Research Institute and several subcontracting laboratories, recently conducted a health assessment of the combustion emissions of PuriNO$_x$™ diesel fuel emulsion (diesel–water–methanol) in rodents. Combustion emissions from either of two, 2002 model Cummins 5.9L ISB engines, were diluted with charcoal-filtered air to exposure concentrations of 125, 250 and 500 μg total PM/m^3. The engines were operated on a continuous, repeating, heavy-duty certification cycle (US Code of Federal Regulations, Title 40, Chapter I) using Rotella-T® 15W-40 engine oil. Nitrogen oxide (NO) and PM were reduced when engines were operated on PuriNO$_x$™ versus California Air Resources Board diesel fuel under these conditions. Male and female F344 rats were housed in Hazleton H2000 exposure chambers and exposed to exhaust atmospheres 6 h/day, five days/week for the first 11 weeks and seven days/week thereafter. Exposures ranged from 61 to 73 days depending on the treatment group. Indicators of general toxicity (body weight, organ weight, clinical pathology and histopathology), neurotoxicity (glial fibrillary acidic protein assay), genotoxicity (Ames assay, micronucleus, sister chromatid exchange), and reproduction and development were measured. Overall, effects observed were mild. Emulsion combustion emissions were not associated with neurotoxicity, reproductive/developmental toxicity, or *in vivo* genotoxicity. Small decreases in serum cholesterol in the 500-μg/m^3 exposure group were observed. PM accumulation within alveolar macrophages was evident in all exposure groups. The latter findings are consistent with normal physiological responses to particle inhalation. Other statistically significant effects were present in some measured parameters of other exposed groups, but were not clearly attributed to emissions exposure. Positive mutagenic responses in several strains of *Salmonella typhimurium* were observed subsequent to treatment with emulsion emissions subfractions. Based on the cholesterol results, it can be concluded that the 250-μg/m^3 exposure level

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was the no observed effect level. In general, biological findings in exposed rats and bacteria were consistent with exposure to petroleum diesel exhaust in the F344 rat and Ames assays. *Toxicology and Industrial Health* 2006; **22:** 65–85.

Key words: *diesel; emulsion; health assessment; Tier II*

Introduction

Variations in ambient concentrations of ozone and particulate matter (PM) in urban areas have been associated statistically with concurrent variations in adverse respiratory and cardiovascular health outcomes in populations (Dockery *et al*., 1993; Pope *et al*., 1995; 2004; US EPA, 1996; 2004a; Samet *et al*., 2000; Dominici *et al*., 2002). To address this problem, the US Environmental Protection Agency (EPA) has tightened the National Ambient Air Quality Standards for ozone and PM (US EPA, 1996; 2004a). Although many sources of air contaminants contribute to ozone and PM levels, mobile source emissions are significant contributors in most areas. As part of a strategy to reduce all on- and off-road mobile source emissions, the EPA and California have set increasingly stringent standards for diesel emissions (CA EPA, 1998; US EPA, 2002; 2004b,c,d). In response, diesel engine manufacturers have implemented numerous technological advances in engine design and exhaust after-treatment, and sulfur has been reduced in diesel fuel. These changes have led to an 80% reduction in emissions of PM and an 80–90% reduction in emissions of nitrogen oxides (NO_x) (a key precursor for ozone formation) from new diesel vehicles during the past decade (Langer *et al*., 2000; Liedtke, 2004).

Many older diesel-powered vehicles are still in use, due to the long service life of diesel engines. Therefore, reducing emissions from the entire diesel fleet requires technologies that can be applied readily to older vehicles. One solution is for national, state, and local governments to require mobile on- and off-road vehicles to use retrofit emission reduction technologies (US EPA, 2004b,c,d). Most of these technologies are designed to treat emissions downstream of the combustion chamber. Although after-treatment devices, such as PM traps, have been shown to decrease adverse health indices in animal studies, these and other mechanical technologies require the user to modify the existing vehicle/exhaust system (Langer *et al*., 2000; McDonald *et al*., 2004). An alternative approach is to burn alternative diesel fuels that, by design, reduce emissions compared to petroleum diesel (Barnes *et al*., 2000; Langer *et al*., 2000; Park *et al*., 2001). One type of fuel shown to reduce NO_x and PM emissions compared to traditional diesel fuels is the diesel emulsion (Barnes *et al*., 2000; Langer *et al*., 2000; Park *et al*., 2001). The presence of emulsion oxygenates (eg, water and ethanol) in these fuel blends (1) decreases PM precursors in the pre-mix burn phase and (2) lowers the peak combustion temperature in the engine to decrease the formation of NO_x (Langer *et al*., 2000).

PM and NO_x are clearly reduced when vehicles are operated on these fuels (Barnes *et al*., 2000). However, given that the emulsion formulation alters combustion and requires the presence of emulsifiers and other chemical components, there is a need to confirm that the new fuel does not present new health hazards (US EPA, 1999).

Currently, there is a void of toxicity data on emissions from combustion of diesel–water emulsion in comparison to 100% petroleum diesel fuels. As interest in the use of diesel emulsions builds within the US and abroad, there is a need for a broader health assessment of the emission hazards of this technology. This study was undertaken to fulfill the EPA Tier II requirements for registration of alternative diesel fuels under Section 211 (b) of the Clean Air Act as amended in 1999. The study was conducted in concordance with the guidelines outlined in the US Code of Federal Regulations (CFR) 79 F. The emissions tested were from a commercially available diesel-emulsion fuel blend (PuriNOx™ All Weather Generation 2 Fuel) developed by The Lubrizol Corporation. This study was

conducted as a requirement for registration of the fuel in the US.

Materials and methods

Good laboratory practice (GLP) guidelines

All work was conducted and completed under the EPA GLP regulations consistent with those described in EPA 79.60, Good Laboratory Practices Standards for Inhalation Exposure Health Effects Testing.

Experimental subgroups

Rats were assigned to each of four blocks that corresponded to specific health assays (Table 1), special histology and neurotoxicity (SH/NTX); micronucleus, sister chromatid exchange, and glial fibrillary acidic protein (MN/SCE/GFAP) assays; general subchronic toxicity and general subchronic toxicity and recovery (GST/GSTR) assessments; and development and reproductive toxicity (DART) assays. Blocks started exposure on a staggered schedule over approximately three weeks to facilitate health assessment. Specific health assays measured within each block are presented in Table 2.

Animals and housing

A total of 412, 6- to 8-week-old CDF® (F344)/CrlBR rats (Charles River Laboratories, Inc., Raleigh, NC) were received, quarantined, and conditioned prior to study start. Rats were conditioned while quarantined in wire cages in stainless steel inhalation chamber basket units (Hazleton H2000, Lab Products, Maywood, NJ). Rats were housed in chambers throughout the study except those rats assigned to the GSTR subgroup (during recovery), DART breeding pairs (daily after the 6-h exposure during mating), and DART females during gestation days 15–20. The latter subgroups of rats were housed in polycarbonate shoebox cages during the specified times.

Chambers were serviced daily and washed weekly, and chamber baskets were rotated one position weekly to facilitate equivalent exposure

Table 2. Health evaluations

- GST/GSTR (general subchronic toxicity/general subchronic toxicity recovery)
  - Ophthalmology
  - Hematology and clinical chemistry
    - Pre-exposure
    - After 30 days of exposure
    - Study termination
  - Gross necropsy
  - General histopathology
  - Post-exposure recovery
- DART (development and reproductive toxicity)
  - Fertility, reproductive toxicity, teratology
- MN/SCE/GFAP
  - Micronucleus (MN)
  - Glial fibrillary protein (GFAP)
  - Sister chromatid exchange (SCE)
- SH/NTX (special histology/neurotoxicity)
  - Histology of brain and peripheral nerve
  - Histology of reproductive organs
  - Histology of lungs and lesions
  - Particle distribution in lungs

Table 1. Experimental design

Treatment group[a]	Males (subgroup)*						Females (subgroup)*					
	GST	GSTR	SH/NTX	DART	MN/SCE/GFAP	Total	GST	GSTR	SH/NTX	DART	MN/SCE/GFAP	Total
Control	10	10	5	10	5	40	10	10	5	30	5	60
Low	10	–	5	10	5	30	10	–	5	30	5	50
Mid	10	–	5	10	5	30	10	–	5	30	5	50
High	10	10	5	10	5	40	10	10	5	30	5	60
CP[b]	–	–	–	–	10	10	–	–	–	–	10	10
GFAP PC[c]	–	–	–	–	5	5	–	–	–	–	5	5
Total	40	20	20	40	35	155	40	20	20	120	35	235

[a]Treatment group denotes clean-air control, low-, mid- or high-concentration level, or positive control treatments.
[b]Cyclophosphamide treatment by IP injection to provide positive controls for MN and SCE assays.
[c]Positive control trimethyltin hudroxide-10.
*An additional 22 rats (11/sex) served as sentinel animals for microbiological screening.
GST, general subchronic toxicity subgroup; GSTR, general subchronic toxicity recovery subgroup (30 days post-exposure); SH/NTX, special histopathology/neurotoxicity subgroup; DART, developmental and reproductive toxicity subgroup; MN/SCE/GFAP, micronucleus/sister chromatid exchange/glial fibrillary acid protein subgroup.

among all groups of rats. Chamber temperature, humidity, flow, pressure, and oxygen were monitored throughout exposure. Room lighting was on a 12-h per day cycle from approximately 06:00 to 18:00 h. Unlimited tap water was available *ad libitum*. Rats were fed (Teklad Certified Rodent Diet (W), Harlan Teklad, Madison, WI) *ad libitum*, except during the daily 6-h exposure period and during the periods of fasting prior to necropsy (GST/GSTR).

Prior to exposure, rats were weighed and randomly assigned to a group by a computerized data acquisition system (Path/Tox; Xybion, Cedar Knolls, NJ) and identified by tail tattoo. Blood from sentinel rats was drawn for serological analysis prior to study start, at the end of the exposure period, and at the end of the recovery period. The serum was analysed for the presence of antigens to common rodent pathogens. These included cilia associated respiratory Bacillus, Kilham rat virus (KRV), H-1 virus (H-1), lymphocytic choriomeningitis virus (LCM), *Mycoplasm pulmonis* (*M. pul.*), parvovirus (KRV, RPV), pneumonia virus of mice (PVM), rat coronavirus/sialodacryoadenitis virus (RCV/SDA), Reovirus (Reo 1, 2, 3), and Sendai virus (Sendai). Tested rats were negative for all pathogens.

Exposures

Rats were exposed to diesel emulsion emissions (DEME) 6 h/day, five days/week except during the mating and gestation period, when all rats were exposed seven days/week. No more than two sequential non-exposure days (eg, weekends) occurred during the study, and no exposure week consisted of fewer than four exposure days. Due to the staggered-start study, combined with the in-study implementation of seven day/week exposures, groups were exposed for variable times. Study day '1' was considered to be the start of DEME exposure for each subgroup. Total exposure days for each subgroup were as follows:

The exposures included four treatment groups: three dilutions of engine exhaust and clean air (control). Target DEME exposures were set based on PM at 125 (low), 250 (mid), and 500 (high) μg PM/m^3. These PM concentrations were spaced evenly to facilitate exposure response assessment. The high exposure level was chosen as the maximal achievable concentration based on the maximal allowable chamber temperature for rats.

Generation of emissions

DEME were generated alternately from two, 2002 model 5.9-L Cummins ISB turbo diesel engines, using diesel–water–methanol emulsion fuel (PuriNO_x™ All Weather Generation 2 Fuel, The Lubrizol Corporation, Wickliffe, OH) and 15W-40 lubrication oil (Rotella T®, Shell, Houston, TX). Crankcase oil and filters (Fleetguard LF3349, Cummins, Columbus, IN) were changed after every 200 h of engine operation. The intake was from the clean air source used for control exposure chambers. The engines were mated to eddy current dynamometers (Alpha-240, Kiel, FRG) and operated on repeated, slightly modified, transient engine cycles based on the EPA Engine Dynamometer Schedule for Heavy-Duty Diesel Engines (40 CFR, Chapter I). The two engines/dynamometers were rotated on an approximately monthly basis so that the total exposure hours were roughly equivalent for both engines. Further details on the exposure system design and the duty cycle are described elsewhere (McDonald *et al*., 2001; 2004; Reed *et al*., 2004).

PuriNO_x™ fuel was supplied in six 550-gallon stainless steel totes as a water–methanol blend diesel fuel composed of 74% wt. California Air Resources Board (CARB) diesel fuel (CARB, 2003; US DOE, 2004)+5.7% methanol+3.5% wt. proprietary emulsifying package (PNOX 1121[A], The Lubrizol Corporation)+16.8% wt. deionized water. Specifications for the CARB diesel fuel used in the blend were consistent with standard specifications (API gravity (35), sulfur percent mass (0.006),

	SH/NTX	MN/SCE/GFAP	GST/GSTR	DART
Total No. exposure days	69/Gender	71/Gender	71/Gender	Males 73; Females 61–73

cetane number (53.8), aromatic hydrocarbon percent mass (19), distillation temperature amount recovered, 50% (564°F), and 90% (627°F)). The fuel was characterized in detail for emulsion characteristics and found to be acceptable for product specification for specific gravity (15.6°C American Society for Testing and Materials (ASTM) D4052 method), kinematic viscosity (40°C ASTM D445 method), and percent water (ASTM D6304 method).

Characterization of exposure

PM mass was the key concentration parameter by which exposure chamber DEME concentrations were monitored and controlled. PM was measured gravimetrically each day by filter measurement and reported as the aerosol concentrations. A real-time PM monitor (DUSTTRAK, TSI, St. Paul, MN) was used to assist operational adjustments of exposure atmospheres to target values.

Table 3 summarizes the daily and periodic measurements that were conducted, including the frequencies of measurements and calibrations, measurement techniques, methods of calibration, and sampling intervals. Daily measurements were collected from each chamber for PM, NO_x, sulfur dioxide (SO_2), total hydrocarbon (THC), carbon monoxide (CO), and carbon dioxide (CO_2). Specific methods of analyses have been described elsewhere (McDonald *et al*., 2004; Reed *et al*., 2004). PM was measured once in the study during non-exposure hours to ensure soot was not re-entrained and passed to exposure chambers. The homogeneity of test atmosphere distribution was determined during the pre-study test and early in the exposure period. The size distribution of the particles was measured in the high-level exposure chamber in the pre-study phase and three times during the study, at approximately the beginning, middle, and end of the exposure period to establish the stability and consistency of particle size distribution. Size was measured by two methods: a Micro Orifice Uniform Deposit Impactor (MOUDI™; MSP Corp., Minneapolis, MN) and a Scanning Mobility Particle Sizer (SMPS™; TSI, Inc., Minneapolis, MN). This combination was necessary because the MOUDI was used to measure the material from 50 nm to 10 μm in diameter, and the SMPS was used to measure the material in the smaller size range from ~15–500 nm.

Following the completion of rat exposures, exhaust PM and semivolatile organic compounds (SVOCs) were collected on 8×10-inch Teflon™-impregnated glass fiber filters (Pall-Gelman, East Hills, NY) followed by two (in series) polyurethane foam/crosslinked divinyl benzene (PUF/XAD-4 PUF) cartridges using a custom-built, in-line aluminum filter holder (Tisch Environmental, Inc., Village of Cleves, OH). Material was collected at the same dilution as the high exposure level with no exposure chamber in place (filter holder replaced the chamber). The flow through the samples was

Table 3. Exposure atmosphere characterization measurements, techniques, and sample frequency

Measurement	Technique	Measurement frequency	Sampling interval
Filter particle mass	Filter (Pallflex, 47 mm diameter)	Daily	Duration of exposure
Real-time particle mass	Dust-Trak nephelometer	Daily	Duration of exposure
Oxides of nitrogen	Chemiluminescence	Daily	20 min alternating[a]
Carbon monoxide	Photoacoustic gas analyser	Daily	1, 20-min sample/level
Carbon dioxide	Photoacoustic gas analyser	Daily	1, 20-min sample/level
Total hydrocarbon	Flame ionization detector/ photoacoustic gas analyser[b]	Daily	1, 20-min sample/level
Sulfur dioxide	Photoacoustic gas analyser[c]	Daily	1, 20-min sample/level
Particle size	Impactor[d], SMPS[e]	Four times during study[f]	1 pre-study, three during study

[a]Samples alternated every 20 min between chambers.
[b]The photoacoustic gas analyser was used as a backup in the case of flame ionization detector failure.
[c]The photoacoustic gas analyser was subject to interferences from other analytes when measuring sulfur dioxide. Samples were collected twice per month at each exposure level to measure sulfur dioxide directly by wet chemical techniques.
[d]Cascade impactor.
[e]SMPS, scanning mobility particle sizer.
[f]Measurements were made once prior to study start and three times during study duration (beginning, middle, and end of study).

approximately 300 L/m for a 6- to 8-h duration. The organic fraction of the PM and SVOC samples was extracted separately with dichloromethane for 24 h in a Soxhlet extractor. Extracts were concentrated to approximately 4 mL by rotary evaporation (vacuum distillation) followed by a gentle stream of nitrogen. Each extract concentration was determined by spiking three separate 10-μL aliquots onto a microbalance and allowing the dichloromethane to evaporate at room temperature for several minutes. After the mass of the aliquot stabilized, the residual mass was weighed, giving the concentration of the extract. Equivalent mass concentrations of PM and SVOC collected from each engine were combined. Extracts were then evaporated under nitrogen and exchanged into dimethyl sulfoxide for mutagenicity tests. The PM+SVOC extract and a filter (PM), and sorbent (SVOC) blank extract (processed in the same way as the sample) were analysed at BioReliance (Rockville, MD) for mutagenicity.

PM and SVOC samples were subjected to a bacterial reverse mutation assay (Ames assay), as described in 40 CFR 79.68. Briefly, the samples were tested individually using standard procedures both with and without S9 metabolic activation in five tester strains: TA1535, TA1537, TA98, TA100, and TA98NR. TA98NR is an acetylase-deficient strain, thus rendering the strain less susceptible to nitroaromatics in the assay (gift of Dr H Rosenkranz, University of Pittsburgh; Dr L Claxton, EPA). Thus, when used in parallel with TA98, the presence of nitroaromatics in the exhaust emissions could be identified by a profile of positive mutagenicity results in TA98 and attenuated results in TA98NR. Negative (solvent) and strain-specific positive controls were included for PM and SVOC. The potential of these subfraction extracts to cause point mutations in these tester strains in a dose-related manner was measured relative to the solvent groups. Appropriate positive-control groups served as intra-assay controls; their sole purpose was to demonstrate the reliability of the test procedures employed.

Dose levels tested for PM were 100, 333, 1000, 2000, 3333, and 5000 μg/plate for TA1535, TA1537, TA98, TA100, and TA98NR. For SVOC, dose levels tested were 6.7, 10, 67, 100, 333, 667, 1000, 3333 and 5000 μg/plate for all test strains.

Health evaluations

Body weight, clinical examinations, and feed consumption

All rats were weighed individually using the Path/Tox data acquisition system on study day −7 (to randomly assign rats to groups by weight), at day −1 or −2, then twice weekly thereafter throughout the course of the study, except for gestating rats. Body weights for gestating female rats were recorded on gestational days 0, 3, 6, 9, 12, 15, 18, and 20 to track gestation. Body weights were also recorded at the time of necropsy.

A detailed clinical examination was performed when body weights were measured, and any clinical abnormalities were recorded using Path/Tox software. These observations included, but were not limited to, the following: reactivity to general stimuli; description and severity of any convulsions, tremors or abnormal motor movements (including posture or gait abnormalities); and description of any abnormal behaviors, emaciation, dehydration, masses, lesions, etc.

Food consumption data were obtained daily for each chamber basket unit holding rats at each exposure level. The food consumed per basket unit (housing up to 24 rats) was obtained by subtracting the morning feeder weight (feeder plus feed) from the full feeder weight (feeder plus feed) recorded the prior night. Food consumption data were obtained from exposure days 21 through 73. This period consisted of the only consecutive study days where food consumption data could be effectively compared between control and exposure levels. Prior to exposure days 21 through 73 and subsequent to this period, the nominal rat count per basket unit changed due to staggered starts for different study subgroups, breeding (no food consumption data were obtained during this period), and staggered sacrifices for study groups. For each basket unit, the amount of food consumed was converted to grams of food consumed/gram of rat in each basket (total weight). These numbers were based on body weight obtained at the weekly weigh sessions (eg, data were converted at only those dates corresponding to weigh sessions). Food consumption data were statistically analysed for only a select group of chamber basket units among all exposure levels. For males, basket 1 (total of six) at each exposure level was analysed. This basket contained male rats

from each representative subgroup (same number of rats from each group at each exposure level). For females, basket 4 (total of six) in each chamber was analysed comparatively. All baskets analysed contained 24 rats per basket with a full complement of body weights.

Ophthalmology

All rats assigned to the GST/GSTR subgroups were examined within one week prior to the initiation of exposures. The consulting board-certified veterinary ophthalmologist, based on the pre-study examination, recommended rats to exclude from placement on the study. Only rats designated for the terminal sacrifice subsequent to exposure (GST) were examined again within five days of the conclusion of the exposures.

Examinations were performed following pupillary dilation with tropicamide eye drops. Examinations were performed using an indirect ophthalmoscope and included observation of the external globe and adnexa, as well as the optical media and retina.

DART

The DART subgroup involved an additional 10 male and 30 female rats per treatment group, as shown in Tables 1 and 2. Beginning at eight weeks of age (ie, two weeks before exposures started), approximately 144 female rats received daily vaginal cytology smear examinations. A saline vaginal lavage was performed on each rat and examined for cell types present. Acyclic rats were culled and not placed in the study. Rats having a normal estrus cycle were randomly assigned to exposure groups.

After seven weeks of exposure, daily vaginal cytology examinations of the 30 females per exposure group in the DART subgroup resumed. These evaluations were conducted immediately following daily exposure, or at approximately that same time on non-exposure weekend days. At nine weeks of exposure, each of the 30 females was mated to a male rat from the same treatment group. Males from the DART, GST/GSTR, SH/NTX, and MN/SCE/GFAP subgroups were used for mating ($N=30$ males per exposure group). The mating pairs were removed from the chambers after each day's exposure, or at approximately that same time of day on non-exposure weekend days. The mating pairs were housed in shoebox-type cages under similar environmental conditions as the exposure chambers. Three days prior to the start of the mating period, males were removed from the chambers after the exposure period and placed in shoebox cages designated with their rat number. During this period, no bedding was changed. These males were returned to their normal chamber spaces prior to exposure on each of these three days. Throughout the mating period, the males were placed into their designated shoebox cages, and the appropriate females were introduced into the cage several minutes later. During the mating period, the shoebox cages were not washed, and the frequency of bedding changes was minimized (one change per week). In the morning, before exposures began, each of the paired rats was returned to the normal individual housing location within the exposure chambers. Daily overnight mating continued for two weeks or until daily vaginal cytological examinations of the mated females revealed a plug and the presence of sperm in the vaginal lavage cell smear. This day was defined as gestation day 0. Vaginal smears for the detection of mating evidence were made prior to daily exposures during the mating periods. Any female that did not become pregnant after mating with the initial male was placed with a proven male from the same treatment group. Females with evidence of mating were exposed every day from gestation day 0 through gestation day 15 (inclusive). Any female that aborted was sacrificed at the first evidence of abortion.

On gestation day 20, all surviving rats were euthanized by CO_2 inhalation. The abdominal, thoracic, and pelvic cavities were opened by a ventral mid-line incision, and the ovaries and uterus were removed from the body. Pregnancy status (gravid or non-gravid) and gross necropsy finding were recorded. The uterus was weighed and examined. For gravid females, the number of *corpora lutea* on each ovary was recorded. The uterus was opened and the development of the fetuses was classified using the following criteria: (a) live fetus: a fetus that responded to touch; (b) dead fetus: a fetus that did not respond to touch and was not breathing; (c) early resorption: an implantation for which it was not grossly evident that organogenesis

had occurred; (d) late resorption: an implantation for which it was grossly evident that organogenesis had occurred; and (e) a fetus with autolysis was considered a late resorption.

The number and location of implantation sites, live and dead fetuses, and early and late resorption(s) were recorded. Uteri with no macroscopic evidence of implantations were opened and placed in 10% aqueous ammonium sulfide solution for detection of implantation sites.

External examinations of fetuses included the eyes, palate, head shape, trunk, and extremities. Each live fetus was then weighed, externally sexed, and euthanized by an intrathoracic injection of sodium pentobarbital. Dead fetuses were examined, but not weighed or sexed. One half of the fetuses of each litter had visceral contents removed and fixed in Bouin's solution for visceral evaluation using the Wilson free-hand slicing technique (Wilson, 1965). The remaining one-half of the live fetuses were fixed in 95% isopropyl alcohol for skeletal examination. These fetuses were subsequently macerated in 2% potassium hydroxide, stained with Alizarin Red S, and cleared with increasing concentrations of 25 and 100% glycerin. The fetuses were then examined for skeletal formation and ossification.

The incidences or the means and standard deviations of maternal and fetal observations were calculated. Calculations and statistical analysis of Cesarean-section and fetal parameters were performed using the litter as the unit of analysis. The number of *corpora lutea*, implantations, live and dead fetuses, early and late resorptions, and gravid uterine weights were calculated as the total number for each exposure group divided by the number of litters evaluated. The following parameters were calculated by determining the percent loss for each litter followed by group mean calculations.

$$\text{Pre-implantation loss } \% = \frac{\textit{Corpora lutea} - \text{No. implants} \times 100}{\text{No. } \textit{corpora lutea}}$$

$$\text{Post-implantation loss } \% = \frac{\text{No. implants} - \text{No. viable fetuses} \times 100}{\text{No. implants}}$$

General histopathology

Rats in the GST and GSTR subgroups were sacrificed by overdose of barbiturate and received a full necropsy. Fresh weights were obtained for the liver, kidneys, lungs, adrenal glands, brain, vagina, uterus, ovaries, testes, epididymides, seminal vesicles with coagulating gland, and prostate. Pituitary weights were obtained after fixation. All organs were preserved in 10% neutral buffered formalin (NBF), except for the testes, epididymides, and eyes, which were preserved in Bouin's solution. In addition to the listed organs, the nasopharynx, pituitary, thyroid/parathyroid, thymus, trachea, heart, sternum with marrow, salivary gland, spleen, pancreas, aorta, skin, gastrointestinal tract, urinary bladder, lymph node, mammary gland, thigh muscle, femur, spinal cord, exorbital lachrymal gland, and Zymbal gland were examined microscopically. Microscopic evaluations were limited to the control- and high-exposure level groups with the exception of the lungs, which were examined in all exposure groups. Tissues were trimmed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

Histopathologic evaluations included determining the presence, nature, and severity of morphologic abnormalities. A scale from 0 to 4 was used to score the severity of morphologic abnormalities.

Special histopathology

Five males and five females per exposure groups were designated for special neuropathology, reproductive system pathology, and pulmonary distribution of particles. Rats were weighed, heparinized, euthanized by overdose of barbiturate, the hearts were cannulated, and tissues fixed by whole-body perfusion of buffered 4% paraformaldehyde. The lungs were fixed *in situ* by intratracheal instillation of 4% paraformaldehyde via gravity feed at a pressure of approximately 25 mmHg.

After perfusion fixation, the pelts were removed from the dorsal head, neck, shoulders and both hind legs. The bones of the cranium and cervical vertebral column were exposed. The nasal cavity was gently flushed with 4% buffered paraformaldehyde. The lungs were carefully removed and tied-off. Other organs and tissues (see General histopathology section above), including reproductive organs, but not the brain, spinal cord, peripheral

nerve, or nasal tissue, were removed and placed in 10% NBF, except for the testes, epididymides, and eyes, which were preserved in Bouin's solution. The carcasses were placed in a tissue bag with 4% buffered paraformaldehyde and held at 4°C overnight. The following day, the cranium and cervical vertebral column were removed. The brain (cerebrum, cerebellum with pons, and medulla) was removed, weighed, and the length and width of the brain was recorded. The brain, spinal cord at the cervical swelling, left and right sciatic nerves at the mid-thigh and sciatic notch, the left tibial nerve at the knee, and the nasal cavity were removed and placed in 10% NBF. All tissues were held in 10% NBF for at least 48 h before further processing.

After at least 48 h of formalin fixation, the sciatic or tibial nerve(s) and cross-sections of the forebrain, center of the cerebrum, midbrain cerebellum, and medulla oblongata were processed for embedding and histopathology. The spinal cord at the cervical swelling (C3–C6) and proximal right sciatic nerve (mid-thigh and sciatic notch) were placed in labeled cassettes. A longitudinal section of the left sciatic nerve was also placed in a cassette with the cross-sections of the right sciatic nerve. The lungs and other organs were trimmed, processed and stained with hematoxylin and eosin. Neural tissues from only the clean-air control and high-level treatment groups were processed and examined. The testes, epididymides, seminal vesicles, and ovaries were examined microscopically from all SH/NTX subgroup rats (all exposure groups). The lungs and trachea of all whole-body perfusion-fixed test rats were examined for inhaled particle distribution. These assessments were qualitative, noting regional distribution of particles or particle-containing macrophages and intralumenal versus interstitial particle location.

Clinical chemistry and hematology

Hematology and clinical chemistry were evaluated on all rats assigned to the GST/GSTR subgroup after one month of exposure, at the end of exposure and at the end of the recovery period (GSTR only). In addition, at the one-month bleed, the SH/NTX rats group served as clinical chemistry and hematology backup rats for those GST/GSTR rats where adequate samples were not obtained. Prior to blood collection, rats were fasted overnight. At the end of exposure and recovery, the rats were anesthetized with halothane (one-month) or overdose of barbiturate for necropsy. Blood was collected via the retro-orbital sinus at the one-month bleed (prior to exposure) and by cardiac puncture during necropsy at the end of exposure and after the recovery period.

For hematology analyses, approximately 0.5–1.0 mL of whole blood was collected from each rat into tubes containing EDTA as an anti-coagulant. For clinical pathology analyses, approximately 1 mL of whole blood was collected from each rat into microtube serum separator tubes for centrifugation and separation into cellular and serum fractions. Clinical chemistry parameters included the following (Hitachi 911, Roche Diagnostics., Indianapolis, IN): alanine aminotransferase (ALT), albumin, albumin/globulin ratio, alkaline phosphatase (ALP), aspartate aminotransferase (AST), bile acids, blood urea nitrogen (BUN), BUN/creatinine (CRE) ratio, serum calcium, serum chloride, cholesterol, creatinine, gamma glutamyl transpeptidase (GGT), glucose, inorganic phosphorous, potassium, sodium, sorbitol dehydrogenase (SDH), total bilirubin, total globulin, and total protein. Hematology parameters were assayed by automated analyser (Advia 120, Bayer Corp., Tarrytown, NY) and included the following: differential white blood cell count (lymphocytes, neutrophils, eosinophils, monocytes), hematocrit, hemoglobin, platelet count, red blood cell count, white blood cell count, and methemoglobin (IL 682 CO-oximeter, Instrumentation Lab., Lexington, MA).

Glial fibrillary acidic protein

Subsequent to blood collection for the SCE assay, the skull was opened and the brain removed for the GFAP assay from all MN/SCE/GFAP rats plus rats dosed with 7.0 mg/kg, intraperitoneal (i.p.) of the known hippocampal neurotoxicant, trimethyltin (TMT). Brains were dissected into the following six regions: cerebellum, cerebral cortex, hippocampus, striatum, thalamus/hypothalamus, and remaining brain as specified in the EPA Guidelines (40 CFR 79.67). Each brain region was placed into individually labeled tubes, frozen over dry ice, and stored at −70°C until processing for protein analysis. GFAP analyses were performed on each brain

region by sandwiched enzyme-linked immunosorbent assay (ELISA) versus known GFAP standards as described previously (O'Callaghan, 2002).

Sister chromatid exchange

On the day following the last day of exposure, MN/SCE/GFAP rats were sacrificed by overdose of barbiturate. The chest was opened and heparinized peripheral blood samples (2–4 mL) were obtained from the abdominal aorta of all exposed rats in this subgroup plus the appropriate cyclophosphamide (CP)-treated, positive-control rats (five males and five females exposed to clean air throughout the exposure period and dosed with 5 mg/kg CP i.p. 24 h prior to sacrifice). Blood samples were analysed by BioReliance Corporation. The frequency of SCEs was determined by culturing whole-blood lymphocytes in the presence of 5-bromo-2′-deoxyuridine (BrdU) for two cell cycles. Briefly, cultures were initiated by adding 0.5 mL of whole blood per 5.0 mL of complete culture medium (RPMI 1640 culture medium) supplemented with 10% fetal bovine serum, antibiotics (penicillin G, 100 μg/mL and streptomycin sulfate, 0.1 mg/mL, 25 mM HEPES buffer, 20 μg/mL phytohemagglutinin and an additional 2 mM l-glutamine) in T-25 sterile disposable tissue culture flasks and incubated at 37°C. Approximately 21 h after initiation of the cultures, the cells were exposed to 5.0 μg/mL of BrdU. Dividing cells were arrested in metaphase and harvested for microscopic evaluation of SCEs. At approximately 68 h, colcemid at 0.2 μg/mL was added to each flask and incubated for approximately 3 h. The cells were swollen by hypotonic treatment (0.075 molar potassium chloride (KCl)), fixed, and washed with methanol:acetic acid (3:1), capped, and stored overnight or longer at 2–8°C. To prepare slides, the cells were collected by centrifugation and resuspended in fresh fixative. One to two drops of fixed cells were dropped onto a glass microscope slide and air-dried. The slides were stained using a modified fluorescence plus Giemsa technique (Perry and Wolff, 1974). Slides were stained in Hoechst 33258, 5 μg/mL for 10 min; mounted in phosphate buffer, pH 6.8; exposed to an ultraviolet lamp at approximately 60°C for 4–6 min; and then stained in 5% Giemsa for 6–10 min. The potential of the test substance to increase SCEs in an exposure–responsive manner was measured relative to the clean-air control group. The CP-treated group served as a positive control to demonstrate the reliability of the test system.

Slides were coded. Metaphase cells were examined under oil immersion without prior knowledge of treatment groups. Whenever possible, a minimum of 25-sec division metaphases per rat was scored for SCEs. At least 100 consecutive metaphase cells were scored for the number of cells in first-, second- or third-division metaphase for each rat as an indicator of toxicity (cell cycle delay). At least 1000 cells were scored for mitotic index per rat.

Micronucleus

Subsequent to blood removal for the SCE assay, MN/SCE/GFAP rats and CP-treated, positive-control rats (five males and five females exposed to clean air throughout the exposure period and dosed with 40 mg/kg CP i.p. 24 h prior to sacrifice), both femurs were removed to provide marrow cells for the MN assay. Bone marrow smears for this endpoint were prepared and scored for micronuclei. Briefly, femur proximal heads were removed and the contents of each femur were eluted in fetal bovine serum by aspiration through a needle fitted to a plastic syringe. The resulting cell suspension was centrifuged to isolate cells. The final cell pellets were resuspended in a small volume of fetal calf serum to facilitate smearing on glass microscope slides. Two smears were prepared from each rat. The slides were air-dried and shipped to BioReliance for analyses. Slides were coded, fixed by dipping in methanol, stained with the nucleic acid-specific stain, Acridine orange, and scored. Using medium magnification, an area of acceptable quality was selected so that the cells were well spread and stained. Using oil immersion, 2000 polychromatic erythrocytes were scored per rat for the presence of micronuclei. The number of micronucleated normocytes in the field of 2000 polychromatic erythrocytes was also enumerated. The proportion of polychromatic erythrocytes to total erythrocytes was recorded per 1000 erythrocytes. The proportion of polychromatic erythrocytes to total erythrocytes in test substance-treated rats was never $<20\%$ of the control value. The potential of the test substance to increase MNs in an exposure–responsive manner was measured relative to the

clean-air control group. The CP-treated group served as a positive control to demonstrate the reliability of the test system.

Statistical analyses

For all analyses, multivariate and univariate, the criterion for statistical significance was set at $P \leq 0.05$.

Body weight and organ weight data were tested for homogeneity with Bartlett's test. For homogenous data, Dunnett's t-test was performed, and for non-homogenous data, the modified t-test was performed to determine statistical difference from control values. For food consumption, least squares regression lines with a common slope and different y intercepts were fit to the data from each exposure group for both males and females for gram of food consumed per gram of body weight values over the period for which data was analysed. A P-value was assigned to deviation from the control group line.

For DART data, the number of *corpora lutea*, implantation sites, live fetuses, gravid uterus weights, and fetal body weights by litter and by gender were analysed by one-way analysis of variance (ANOVA). If a significant F ratio was obtained ($P<0.05$), Dunnett's test was used for pair-wise comparisons to the control group. The percent pre-implantation loss, percent post-implantation loss, number of dead fetuses, early and late resorptions, and the percent of male and female fetuses were compared using the Kruskal–Wallis test. If a significant effect occurred ($P<0.05$), the Mann–Whitney U-test was used for pair-wise comparisons of each treated group to the control group. The incidences of malformation and variations were compared using the χ^2-test with the litter as the experimental unit. The total number of litters with external, visceral, and skeletal malformations as well as the total number of litters with malformations and variations were also statistically compared. If a significant effect occurred ($P<0.05$), the Fisher's Exact test was used for pair-wise comparisons to the control group.

Hematology and clinical chemistry were evaluated using the Kruskal–Wallis test (Hollander and Wolfe, 1973). If significant evidence of a treatment effect ($P<0.05$) was found, individual comparisons of exposed groups against controls were performed using the multiple comparison procedure of Dunn (1964).

For the SCE assay, a Dunnett's t-test was used to test statistical significance from control and treated groups. For the MN assay, micronucleated polychromatic erythrocytes were tested in control versus treated groups by the Kastenbaum–Bowman's test. GFAP data were subjected to analysis of variance followed by *post hoc* comparison of treatment means (JMP®, SAS).

Results

Exposures and characterization

Daily average chamber temperatures (~23°C), chamber relative humidity (~40–60%), oxygen concentrations (~20%), and chamber exhaust flows (~600 L/min) were acceptable throughout the study at all exposure levels.

Summary exposure concentrations of PM, NO_x, nitrogen oxide (NO), NO_2, CO, CO_2, THC, and SO_2 are shown in Table 4. PM concentrations were within 5% of targets for the study. Only five

Table 4. Summary of particle mass and gas analyte concentration data[a]

	Treatment group			
Atmospheric component	Control	Low	Mid	High
CO (ppm)	0.4±0.2	11.3±2.7	18.1±3.6	30.5±6.6
CO_2 (ppm)	2286.4±566.6	3637.1±728.5	4912.5±911.7	6966.2±1519.0
SO_2 (ppb)	4.7±2.0	12.2±5.3	28.0±9.5	64.6±23.7
Total hydrocarbon (ppm)	0.3±0.3	1.2±0.3	1.8±0.6	2.8±0.8
NO_x (ppm)	ND[b]	12.5±1.5	20.6±5.7	33.9±8.9
NO (ppm)	ND[b]	1.5±0.4	1.3±1.1	2.6±1.7
NO_2 (ppm)	ND[b]	14.2±4.2	22.1±6.1	36.7±9.9
Particle mass (μg/m^3)	6.4±3.3	131.0±32.7	253.9±40.5	503.8±86.2

[a]Values are mean plus or minus one standard deviation.
[b]ND, not detected.

Table 5. Monthly summary of particle mass and number size distribution (high-exposure level)

	Particle mass distribution		
	Mass median diameter (μ)	% <0.56 (μ)	% 0.56–10.8 (μ)
Month 1	0.081	84	16
Month 2	0.087	85	15
Month 3	0.096	93	7

	Particle number distribution	
	Median size (nm)	GSD[a]
Month 1	33.6	1.6
Month 2	34.4	1.6
Month 3	34	1.6

[a]GSD, geometric standard deviation.

exposure days in the low-exposure level, two exposure days in the mid-exposure level, and seven exposure days in the high-exposure level were outside of 15% of target values. Control background PM averaged 6.4 μg/m^3. NO_x, CO, CO_2, SO_2, and THC scaled with dilution at all exposure levels. NO_x was composed of approximately 90% NO and 10% NO_2, and the concentration of NO_x at the highest exposure level was approximately 34 parts per million. SO_2 values averaged 64.6 parts per billion at the highest exposure level. Small but measurable background amounts of CO, CO_2, SO_2, and THC were present in the control chamber. The median diameter of PM was between 0.088 μm and the median number distribution was ~34.2 nanometers (Table 5).

DEME PM and SVOC tested positive in the bacterial reverse mutation assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537, and/or TA98NR. For PM specifically, positive responses were observed with tester strains TA98, TA100, TA1535, TA1537, and TA98NR in the presence of S9 activation and with tester strains TA98, TA100, TA1537, and TA98NR in the absence of S9.

For SVOC, positive responses were observed with tester strains TA98, TA100, and TA98NR in the presence and absence of S9 activation.

Health responses

Survival, body weight, clinical signs, feed consumption

There were no exposure-related effects on mortality, morbidity, or clinical signs. No consistent exposure-related body weight effects were noted in the GST/GSTR, SH/NTX, DART, and MN/SCE/GFAP subgroups (data not shown). Some statistically significant differences from control were seen at some measurement intervals in both genders in all subgroups. No difference in body weight was apparent in the GSTR subgroup subsequent to exposure.

Food consumption per gram of body weight for both males and females in all exposure groups decreased over time as the study progressed (data not shown). However, the amount of food consumed (grams) per gram of rat weight for exposed groups was greater than controls, as shown in Table 6. No exposure–response relationship was observed.

Ophthalmologic examinations

All rats assigned to the study had congenital corneal dystrophy (CDD) at study onset. Examination of all 80 rats assigned to the GST group post-exposure showed no CDD progression during the study or other signs of ocular pathology.

Table 6. Summary food consumption analyses

Gender	Chamber	Mean ± SEM of deviations from fitted regression line (mg food/g rat)	*P*-value against control
Male	Control[a]	0.0 ± 1.4	–
	Low	4.1 ± 1.7	0.073
	Mid	6.1 ± 1.0	0.008
	High	11.3 ± 1.9	<0.001
Female	Control[a]	0.0 ± 2.6	–
	Low	11.8 ± 2.6	0.003
	Mid	5.0 ± 1.9	0.107
	High	6.3 ± 1.3	0.048

[a]Control value set at 0 for the purpose of reporting regression analyses.

Clinical chemistry and hematology

Statistically significant exposure effects on clinical chemistry values of rats are summarized in Table 7. The magnitude of most effects was modest, and there was little consistent evidence for effects across genders and exposure times. Most statistically significant effects were observed after three months of exposure (terminal sacrifice). Most effects disappeared after 30 days of recovery in clean air exposure. Cholesterol was slightly increased in females at the interim sacrifice (mid; maximum 11%), but decreased in both females (high; maximum 14%) and males (high; maximum 16%) at the terminal sacrifice. Effects on liver parameters (serum enzymes: ALT, ALP, AST, GGT, and bile), although in some cases large in the relative magnitude of change from control (eg, up to 167% of control in the low exposure GGT males at the terminal sacrifice), were variable among genders and exposure times. Electrolyte values (chloride (Cl), sodium (Na), calcium (Ca), and phosphorus (phos)) were variable in direction of change, slight in magnitude (eg, max 21% phos in mid exposure females at interim sacrifice), variable among genders, and lacked exposure–response relationships. In general, slight decreases were observed in the absolute and calculated parameters within the protein panel of exposed rats. Albumin was slightly decreased in females at the interim (low; maximum 6%) and recovery sacrifices (high; maximum 6%), respectively. Total protein was decreased slightly in both males and females at the terminal sacrifice (high; maximum 7%). Concomitant decreases in calculated male globulin levels were observed (high; maximum 15%) at the interim sacrifice. Albumin/globulin ratios increased (low; maximum 8%) slightly in females at the interim sacrifice, but increased (mid; maximum 15%) slightly in males at the terminal sacrifice. Increases in BUN and BUN/CRE were observed in females at the interim sacrifice (low, mid; maximum 40%). In males at the terminal sacrifice, CRE was slightly increased as well (mid; maximum 19%). However, decreases in female BUN (mid, high; maximum 30%) and in male and female BUN/CRE (mid, high; maximum 34%) were present at the terminal sacrifice. Glucose was also slightly elevated in females (low, mid; maximum 45%) at the terminal sacrifice.

Statistically significant exposure effects on hematology values of rats are summarized in Table 8. The magnitude of most effects was modest, there was little coherent evidence for effects across exposure times, and many effects lacked an exposure–response relationship. Platelet values were slightly decreased in females (low, mid; maximum 17%) at the interim sacrifice. Statistical changes in white blood cell (WBC) counts (decrease in female – low, mid; maximum 43% (interim sacrifice); and male – low; maximum 40% (terminal sacrifice); increases in female – mid; maximum 32%) were variable among genders and exposure times and lacked an exposure response. These changes were

Table 7. Significant effects of exposure on clinical chemistry parameters of rats[a]

Parameter	Interim	Terminal	Recovery
Alanine aminotransferase		↓Female, H (26%)	
Gamma glutamyl transpeptidase	↑Male, L (70%)	↑Male, L (167%)	↓Male, H (30%)
Sorbitol dehydrogenase	↑Female, L (54%)	↓Male, H (41%)	
Bile	↓Female, M (43%)		
Cholesterol	↑Female, M (11%)	↓Female, H (14%); ↓Male, H (16%)	
Alkaline phosphatase	↑Female, L (133%), M	↓Female, H (35%); ↑Male, M (35%)	
Albumin	↓Female, L (6%)	↓Female, H (6%)	
Total protein		↓Female, H (6%); ↓Male, H (7%)	
Globulin		↓Male, H (15%)	
Albumin/globulin ratio	↓Female, L (8%)	↑Male, H (15%)	
Blood urea nitrogen (BUN)	↑Female, L, M (34%)	↓Female, M, H (30%)	
Creatinine		↑Male, M (19%)	
BUN/creatinine ratio	↑Female, L (40%), M	↓Female, M, H (34%); ↓Male, M (19%)	
Calcium	↑Female, L, M (6%); ↓Male, H (6%)		
Phosphorus	↑Female, M (21%)	↑Female, M (12%)	
Chloride	↓Female, M (2%)		

[a]Arrows indicate increases (↑) or decreases (↓) from control ($P<0.05$). Letters indicate the exposure groups differing significantly from controls (L = low, M = mid, H = high). Values in parentheses are the largest percent differences between exposed and control group means and immediately follow the indicator for the group having the greatest difference.

Table 8. Significant effects of exposure on hematology parameters of rats[a]

Parameter	Interim	Terminal	Recovery
Hemoglobin	↑Male, M, H (4%)		
Hematocrit	↑Female, H (6%)	↑Male, M (6%)	
Red blood cell count		↑Male, M (4%)	
Platelets	↓Female, L, M (17%)		
White blood cell count	↑Female, L, M (43%)	↑Female, M (32%); ↓Male, L (29%)	
Lymphocytes	↑Female, L, M (61%)	↑Female, M (30%); ↓Male, L (28%)	
Neutrophils		↑Female, M (47%)	
Monocytes	↑Female, M (57%)		

[a]Arrows indicate increases (↑) or decreases (↓) from control ($P < 0.05$). Letters indicate the exposure groups differing significantly from controls (L = low, M = mid, H = high). Values in parentheses are the largest percent differences between exposed and control group means and immediately follow the indicator for the group having the greatest difference from control.

reflected in statistically observed changes in lymphocyte values. In addition, female monocyte (mid; maximum 57% (interim sacrifice)) and neutrophils (mid; maximum 47% (terminal sacrifice)) differential values were increased. Changes in red blood cell parameters (hematocrit, red blood cell (RBC) count, hemoglobin) were generally slight and lacked an exposure–response relationship in males and females at the interim and terminal sacrifices (maximum increase 6% for all parameters). However, net-hemoglobin values in males (low, mid, high; maximum 144%) at the interim sacrifice were increased, but corresponding values were decreased in females (low, mid, high; maximum 56%) at the terminal sacrifice.

Necropsy and pathology gross observations

Gross observations were generally unremarkable in all study groups at all sacrifice times. Discolored lungs, consistent with inhalation of PM, were fairly prevalent in the high-level exposure group males and females.

Organ weights

Organ weight changes were minimal. Absolute organ weights and organs as a percentage of brain weight were unchanged compared to control in all exposed groups at the terminal sacrifice. As a percentage of body weight, female low-level kidney weights (5%) and mid-level liver weights (19%) were decreased from control values. Relative male (7%) and female (5%) brain weights were slightly decreased. No changes in organ weight parameters were observed at the recovery sacrifice.

Microscopic pathology

Exposure-related findings were limited to the lungs and due to DEME PM in GST/GSTR rats (Figure 1). Observations included accumulation of black/brown PM within the cytoplasm of alveolar macrophages and minimal alveolar macrophage hyperplasia. PM accumulation was exposure related and correlated with the presence of gross necropsy observations of discolored lungs. Essentially, no particles were evident in the cytoplasm of

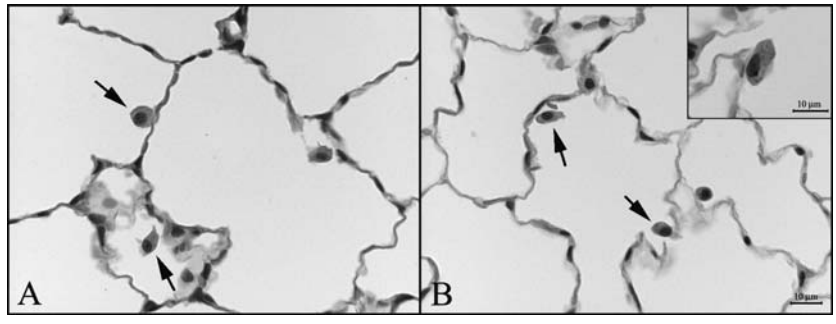

Figure 1. Photomicrographs illustrating pulmonary histology. (A) Lung from a control male rat demonstrating normal alveolar macrophages with lightly eosinophilic, vacuolated cytoplasm (arrows). (B) Lung from high-exposure level group (400 µg PM/m^3) male rat demonstrating macrophages with more dense, faintly brown-tinged cytoplasm with indistinct particulate material (arrowheads). Higher magnification (inset) demonstrates an alveolar macrophage with more discrete particles.

any alveolar macrophages of control rats. Some resolution of PM accumulation and macrophage hyperplasia was evident after the 30-day recovery period for the GSTR rats.

Microscopic findings in other organs were minimal and similar between controls and high-exposure level rats and incidental to DEME exposure. Findings included the following: minor foci of chronic inflammation within the liver and/or myocardium; alveolar histiocytosis; minimal renal tubular concretions/mineralization; scattered foci of inflammation or mineralization in other organs/tissues; testicular atrophy; sperm granulomas of the epididymides; foci of hemorrhage or fibrosis; and hepatodiaphragmatic nodules.

SH/NTX subgroup

Special histopathology neuropathology indicators were normal. No lesions were detected in either paraffin-embedded nervous tissue or plastic-mounted, teased preparations of tibial nerves from high-exposure level or control rats.

For particle distribution, PM was contained within the cytoplasm of macrophages. Observations were essentially as described above for the GST/GSTR subgroup. PM was only rarely seen in tracheal sections of exposed rats. Particles were not found within epithelia or free within interstitial tissue.

No exposure-related lesions were observed in reproductive tissues from either males or females. No aberrancy in spermatogenesis or oogenesis was detected histologically.

DART

Rats placed on study cycled normally through the course of exposure. The pregnancy rate was 75% in the clean-air control exposure group, 67% in the low-exposure level group, and 63% in the mid- and high-exposure level groups. A minimum of 19 pregnant females with viable fetuses per study group were available for examination.

No statistically significant difference from control was observed for the following parameters: number of *corpora lutea*, implantations, live and dead fetuses, early and late resorptions, percent pre- and post-implantation loss, and the mean gravid uterus and mean fetal body weights by litter and by gender. No exposure-related differences from control malformation or variation incidence were observed. The pattern and distribution of the malformations did not indicate a teratogenic response in any exposure group. Various common developmental variations were observed at each exposure level at a similar frequency.

A statistically significant increase in the percentage of male versus female fetuses (64% males versus 36% females) was observed in the high-exposure group, compared to the control (46% males versus 54% females). A similar ratio (59% male fetuses versus 41% female fetuses) was observed in the 125 $\mu g/m^3$ exposure group, whereas the fetal gender ratio in the 250 $\mu g/m^3$ group (48% males versus 52% females) was comparable to the control group.

GFAP

The validity of the GFAP assay as an indicator of neurotoxicant-induced gliosis was verified with a positive control, TMT (Tables 9 and 10). Large increases in hippocampal GFAP were observed in both male and female positive control rats, even after a relatively short post-dosing interval (five to seven days). A slight decrease in GFAP was noted in the hypothalamus/thalamus and rest of brain of males at the low- and mid-exposure levels (Table 9). A slight decrease in GFAP was observed in the

Table 9. Mean GFAP levels in specific regions of male rat brains

Brain area	TMT	Control	Low	Mid	High
Striatum		1.09 ± 0.04	1.07 ± 0.06	1.14 ± 0.05	0.98 ± 0.03
Hippocampus	6.87 ± 1.32	3.11 ± 0.08	3.10 ± 0.05	3.19 ± 0.09	2.84 ± 0.07
Cortex		1.35 ± 0.06	1.33 ± 0.10	1.38 ± 0.09	1.19 ± 0.09
Hypo/thalamus		3.02 ± 0.10	2.61 ± 0.09 +	2.74 ± 0.21	2.21 ± 0.12 +
Cerebellum		3.78 ± 0.15	3.66 ± 0.13	3.40 ± 0.22	3.49 ± 0.13
Rest of brain		3.10 ± 0.19	2.64 ± 0.10 +	2.69 ± 0.08	2.20 ± 0.12 +

+, Statistically different from control, $P \leq 0.05$.
Each value represents the mean ± SEM for the concentration of GFAP (μg/mg total protein).
TMT, trimethyltin hydroxide, 7.0 mg/kg, i.p., positive control.

Table 10. Mean GFAP levels in specific regions of female rat brains

Brain area	TMT	Control	Low	Mid	High
Striatum		0.94±0.04	0.96±0.03	0.96±0.06	0.88±0.04
Hippocampus	7.95±0.42	3.38±0.23	2.89±0.06+	2.84±0.05+	2.92±0.17+
Cortex		1.27±0.04	1.27±0.03	1.17±0.03	1.25±0.06
Hypo/thalamus		2.47±0.14	2.44±0.08	2.36±0.08	2.35±0.07
Cerebellum		3.72±0.13	3.52±0.15	3.26±0.09+	3.56±0.13
Rest of brain		2.43±0.22	2.65±0.12	2.37±0.12	2.58±0.17

+, Statistically different from control, $P \leq 0.05$.
Each value represents the mean±SEM for the concentration of GFAP (μg/mg total protein).
TMT, trimethyltin hydroxide, 7.0 mg/kg, i.p., positive control.

hippocampus (low, mid, high) and cerebellum of the female brains (mid), as shown in Table 10. Levels of GFAP were not affected in any brain region of the females. Thus, gliosis, which is reflected by an increase in GFAP, did not occur in the brain regions examined in either gender in this study.

SCE

As shown in Table 10, no statistically significant increases in average SCE frequencies were observed in exposed rats. The positive control group (5 mg/kg CP) exhibited statistically significant increases in SCE frequencies in both genders. No appreciable difference was observed in average generation time or in mitotic index in exposed versus control rats.

Micronucleus

Slight reductions (up to 8%) in the ratio of polychromatic erythrocytes to total erythrocytes were observed in exposed groups relative to the negative controls (data not shown). There was no exposure response. The number of micronucleated polychromatic erythrocytes was not statistically increased relative to controls at any exposure level (see Table 11). As shown in Table 12, CP induced a significant increase in micronucleated reticulocytes in both male and female rats.

Discussion

DEME caused only slight to modest health outcomes in this study of subchronically exposed F344 rats. The most noteworthy effects of DEME were observed in the several statistical changes in clinical chemistry and hematology. However, most statistical hits were slight to modest in magnitude and inconsistent among exposure times and genders. Based on the most consistent observations at three months of exposure and recovery (eg, cholesterol), a NOEL could be determined as the low-exposure level (250 $\mu g/m^3$).

The PM exposure concentrations were within the range of very high environmental to occupational levels of petroleum diesel emissions (PDE) (Lloyd and Cackette, 2001; US EPA, 2002). The DEME exposure concentrations employed in this study were also comparable to the latest subchronic

Table 11. Sister chromatid exchange (SCE) in exposed rats

Exposure	Female			Male		
	SCE mean±SE*	AGT	MI	SCE mean±SE	AGT	MI
0	6.8±2.2	25	4.9	7.6±1.8	25	4.1
Low	6.4±1.9	26	4.6	7.2±1.9	26	4.6
Mid	6.6±2.2	25	3.3	6.6±1.9	25	3.0
High	6.4±1.3	26	4.1	6.5±1.7	25	4.3
CP	21.6±4.4+	27	3.2	20.3±2.1+	27	3.9

*The standard error was calculated using the data of all 125 metaphases scored.
+, Statistically different from control, $p \leq 0.05$.
MI, Mitotic index: (Number of cells in mitosis out of 1000 cells).
AGT, Average generation time: Number of hours in BrdU × 100/(number M_1 cells × 1)+(number M_2 cells × 2)+(number M_3 cells × 3).
CP, cyclophosphomide, 5.0 mg/kg, i.p., positive control.

Table 12. Percent micronucleated reticulocytes in exposed rats

Exposure	Female Number/1000 PCEs (mean ± SD)	Male Number/1000 PCEs (mean ± SD)
0	0.9 ± 0.55	1.5 ± 0.35
Low	0.7 ± 0.45	0.9 ± 0.42
Mid	1.6 ± 1.08	1.1 ± 0.55
High	1.4 ± 0.22	1.3 ± 0.76
CP	9.2 ± 2.71+	13.9 ± 4.95+

+, Statistically different from control, $p \leq 0.05$.
CP, cyclophosphomide, 40.0 mg/kg, i.p., positive control.

health study conducted with PDE in this institute (whole exhaust diluted based on PM to 30, 100, 300 and 1000 μg PM/m^3) under very similar engine operation (2000 versus 2002 Cummins) and atmospheric sampling conditions (Reed *et al*., 2004). In addition, the DEME exposures were roughly comparable to human equivalent concentrations of similar levels of PDE based solely on PM size and deposition fractions (Reed *et al*., 2004). However, one caveat of the Tier II program and the EPA-required testing of PuriNO$_x$™ and other alternative diesel fuels is that no baseline Tier II study exists for any blend of petroleum diesel. Although the study conducted by Reed *et al.* (2004) was performed under similar environmental and exposure conditions, in most cases the fuel, the exposure times, and health assessments varied from those specified in 40 CFR 79. This makes direct comparison of the possible health effects elicited in studies with PDE and DEME difficult at best.

However, the DEME generated in this study was similar in nature to PDE. Particle size and number counts were consistent with typical PDE and with PDE generated in other studies at this institute and elsewhere (Kittelson, 1998; McDonald *et al*., 2004; Reed *et al*., 2004). At a similar PM level (125 versus 100 μg/m^3) however, PDE and DEME differed in concentration from component to component. For example, SO_2, CO and NO_x differed from those measurements reported by Reed *et al.* (2004) and McDonald *et al.* (2004) when similar engines (circa 2000) were operated on national certification diesel fuel. DEME contained roughly 2.25 times more NO_x, three times more CO, and 12 times less SO_2/m^3 than the PDE used in the 2004 Reed study. The contributions of slightly different magnitudes of components between DEME and PDE to health outcome are unknown. As might be expected, both SVOC and PM components of DEME were mutagenic in several strains of bacteria. These results are similar to those of PDE and reflect the majority petroleum diesel component of the PuriNO$_x$™ fuel (US EPA, 2002).

The generally mild effects observed in survival, clinical signs, body weight, ophthalmology, and food consumption were consistent with the relatively low concentrations of DEME used in this study and other studies of rodents exposed to PDE (Mauderly, 1999; US EPA, 2002). Overt toxicity was not a necessary goal of this study. Similar gross indicators of toxicity were unaffected by up to six months of PDE at similar levels (Reed *et al*., 2004) and in an earlier Tier II study of biodiesel emissions (Finch *et al*., 2002). Ophthalmology and food consumption endpoints have not been addressed thoroughly in these or other PDE exposure studies. The corneal dystrophy observed in all exposure groups, including control, is widely recognized as a congenital condition in the F344 rat strain (Losco and Troup, 1988; Bruner *et al*., 1992). The lack of progression and the lack of other ophthalmology indicators of toxicity indicated no effect of DEME on this parameter. Interestingly, the DEME-associated slight increase in food consumption has not been observed in other studies with PDE and biodiesel emissions (Mauderly, 1999; Finch *et al*., 2002). In fact, PDE exposure has been shown to decrease food consumption in cases where the food was present in the exposure chamber during exposures (Maejima and Nagase, 1989). The modest nature, the lack of a clear exposure–response, and the lack of a consistent body weight exposure effect suggests that these observations were not clinically significant and not clearly associated with DEME exposure.

The effect of PDE and other combustion emissions on developmental and reproductive parameters have not been assessed thoroughly. In the current study, there were no exposure effects on pregnancy rates or developmental and reproductive indices. The statistical increase in male fetus numbers, while valid, was not supported by a clear exposure–response relationship in the other exposure groups and, thus, was likely of little toxicological consequence. These observations reflect the results observed in mouse fertility/reproduction after exposure to 6 g PM/m^3 PDE for 5–10 weeks

(Pepelko and Peirano, 1983) and those of rats exposed to biodiesel emissions (Finch *et al*., 2002). Interestingly, fetal masculinization (as measured by anogenital distance and sex hormone disruption) has been reported when pregnant female rats have been exposed to high levels of PDE (5.63 mg PM/m^3); however, the ratio of male and female fetuses in this study was near 50% in all exposed and control groups (Watanabe and Kurita, 2001). Collectively, these studies suggest that the results observed in the current study are likely to be unrelated to exposure.

The slight to modest nature of several observations of organ weight changes were not associated with clear histopathology nor clearly attributed to exposure to DEME. In general, slight and variable effects on organ weights were observed in F344 rats exposed to similar levels of PDE for up to six months (Reed *et al*., 2004). Although measured by absolute, percent body weight and percent brain weight indices, no consistent or coherent pattern of organ alterations exists in PDE/DEME exposure studies that span the environmental range of PDE or DEME (US EPA, 2002; Reed *et al*., 2004). Outside of the lung and beyond occasional and sporadic observations unrelated to exposure, there was no histopathology present in any of the organs or tissues examined in this study.

One of the most pronounced but expected results of DEME exposures was accumulation of PM within macrophages and associated macrophage hyperplasia. These observations are comparable with PDE and other PM sources and constitute a normal response of the lung to PM (Mauderly, 1999; US EPA, 2002). Consistent with the observations in this study, no overt pathology resulted from PM accumulation in macrophages from rats exposed to low-level PDE for up to six months (Reed *et al*., 2004). In general, except in conditions of severe particle overload where normal clearance mechanisms are overcome, PM accumulation and PM-associated macrophages have little pathological effect (Mauderly, 1999; Sydbom *et al*., 2001).

Of the numerous statistically significant observations within clinical chemistry and hematology parameters measured or calculated in this study, the one most clearly related to exposure was serum cholesterol after three months of exposure. The cholesterol findings have precedents in the PDE literature. Reed *et al.* (2004) observed decreases in cholesterol in male and female F344 rats exposed to PDE for one week and six months. Similar changes have been observed in rats exposed to levels of PDE ranging from 0.5–6.6 mg PM/m^3 PDE (Brightwell *et al*., 1986; Maejima and Nagase, 1989). This observation is certainly consistent among PDE exposures, but the mechanism of effect remains uncertain. Other effects likely associated with exposure after three months were serum total protein, gender-specific changes in other protein parameters, and possibly female BUN and BUN/CRE ratios. Differential effects on total protein and albumin were observed in F344 rats exposed to PDE for six months (Reed *et al*., 2004) as well as those exposed to PDE for up to 30 months at 0.5–3.7 mg PM/m^3 (Maejima and Nagase, 1989), but all were slight and of undetermined clinical significance. BUN measurements subsequent to PDE were variable. Maejima and Nagase (1989) observed slight increases in BUN in some groups of rats exposed to PDE while Reed *et al.* (2004) observed slight decreases in females exposed to PDE for six months. In the current study, the observed slight decreases subsequent to DEME exposure at various time points, predominantly in females, is certainly consistent with PDE exposure studies, but are difficult to interpret and likely of little clinical significance.

The minimal statistical changes observed after exposure in hematology parameters were not clearly attributable to treatment with DEME. The lack of gender concurrence among effects and the lack of exposure–response relationships support this scenario. These observations were similar in scope (eg, lack of clear attributed effect) observed in the latest PDE study conducted at this institute (Reed *et al*., 2004). Other high-exposure PDE studies observed alterations in many hematology parameters, but the clinical significance was not determined (Brightwell *et al*., 1986).

In summary, consistent with the good general health of all exposed rats, the statistically significant changes in clinical chemistry and hematology were not supported by any gross/microscopic pathology or damage to any major organ system.

The current study was not designed to evaluate the carcinogenic effects of DEME. The carcinogenicity of chronic PDE exposures has been assessed

in several studies of rats (Pepelko and Peirano, 1983; Heinrich *et al*., 1986; 1995; Mauderly *et al*., 1987; 1996; Ishinishi *et al*., 1988; Brightwell *et al*., 1989) and reviewed extensively (HEI, 1995; CA EPA, 1998; Mauderly, 1999; US EPA, 2002). In short, rats exposed chronically to extremely high concentrations of PDE develop lung tumors, which appear to be a somewhat non-specific response accompanying the overloading effect of the lung with poorly soluble PM (Heinrich *et al*., 1995; Nikula *et al*., 1995; Mauderly, 1997). Lifetime exposure studies of tumorigenesis are not called for in the EPA Tier II design and were not possible in the 90-day format in the current study. However, indicators of clastogenesis and chromosomal alterations (eg, carcinogenic potential), bone marrow micronuclei, and SCE were unaffected by DEME in the current study. The absence of exposure effects was similar to those observed by Reed *et al.* (2004) in strain A/J mice exposed to PDE for six months at similar exposure levels. In rats, no changes in micronucleus counts or SCE have been observed subsequent to exposure to PDE (Pepelko and Peirano, 1983; Morimoto *et al*., 1986; Ishinishi *et al*., 1988) or biodiesel emissions (Finch *et al*., 2002). In fact, the only rodent model showing PDE-induced, exposure-related increases in MN or SCE were Syrian hamsters exposed to PDE at 6 mg PM/m^3 for six months (Pepelko and Peirano, 1983).

A universal cellular reaction to damage of the central nervous system is hypertrophy of astrocytes. The defining characteristic of this response, often termed reactive gliosis, is the enhanced expression of the major intermediate filament protein of astrocytes, GFAP. Thus, an increase in the brain concentration of GFAP serves as a biochemical indicator of neurotoxicity. Central nervous system levels of GFAP have not been assessed previously in rats exposed to PDE or DEME but, nevertheless, are a requirement of the Tier II testing procedures. The decreases observed in GFAP levels in some brain regions of some exposure groups in the current study, although relatively small (none $>29\%$), were statistically significant. However, these observations cannot be interpreted as adverse given that prior studies (and the positive controls in this study) indicate that GFAP levels increase rather than decrease in response to known neurotoxicants (eg, TMT) (O'Callaghan, 1991; Norton *et al*., 1992). One speculative possibility for these effects may be associated with male corticosterone levels. Small, neuroendocrine-regulated decreases in GFAP have been reported in association with high physiological levels of corticosterone (O'Callaghan *et al*., 1991). Thus, it is possible that the exposed males had elevated levels of corticosterone sufficient to result in small decreases in GFAP. While enhanced expression of GFAP serves as a signature of brain injury-induced glial activation (gliosis), small decreases in GFAP, such as those observed in the present study, are likely to reflect exaggerated physiological effects with no known adverse consequences (O'Callaghan *et al*., 1991). Overall, the data suggest that under the exposure conditions used, damage-induced gliosis did not occur in the brain regions examined.

Using these cumulative data to survey the comparative risks of PDE, DEME, and other alternative fuels (eg, biodiesel emissions) is difficult given that no baseline PDE exposure study has been conducted in the Tier II program. However, the results of this, the third of three Tier II studies conducted at this institute on a novel alternative diesel fuel, were consistent with the mild biological responses observed after exposure to reasonable concentrations of biodiesel emissions (Finch *et al*., 2002) and another study of DEME (a Lubrizol emulsion fuel consisting of a diesel–water blend, Reed *et al*., 2005). This body of evidence and that of other PDE exposure studies suggests that the risks of DEME are no greater than that of PDE and other alternative diesel fuels (Reed *et al*., 2004).

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