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Authors: Hallberg, Lance M, Ward, Jonathan B, Wickliffe, Jeffrey K, and Ameredes, Bill T

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Advanced Collaborative Emissions Study Auxiliary Findings on 2007-Compliant Diesel Engines: A Comparison With Diesel Exhaust Genotoxicity Effects Prior to 2007

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Lance M Hallberg¹, Jonathan B Ward Jr², Jeffrey K Wickliffe³
and Bill T Ameredes^{1,4}

¹Sealy Center for Environmental Health and Medicine, University of Texas Medical Branch, Galveston, TX, USA. ²Department of Preventive Medicine and Community Health, University of Texas Medical Branch, Galveston, TX, USA. ³School of Public Health and Tropical Medicine, Tulane University, New Orleans, LA, USA. ⁴Division of Pulmonary Critical Care & Sleep Medicine, Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX, USA.

ABSTRACT: Since its beginning, more than 117 years ago, the compression-ignition engine, or diesel engine, has grown to become a critically important part of industry and transportation. Public concerns over the health effects from diesel emissions have driven the growth of regulatory development, implementation, and technological advances in emission controls. In 2001, the United States Environmental Protection Agency and California Air Resources Board issued new diesel fuel and emission standards for heavy-duty engines. To meet these stringent standards, manufacturers used new emission after-treatment technology, and modified fuel formulations, to bring about reductions in particulate matter and nitrogen oxides within the exhaust. To illustrate the impact of that technological transition, a brief overview of pre-2007 diesel engine exhaust biomarkers of genotoxicity and health-related concerns is provided, to set the context for the results of our research findings, as part of the Advanced Collaborative Emissions Study (ACES), in which the effects of a 2007-compliant diesel engine were examined. In agreement with ACES findings reported in other tissues, we observed a lack of measurable 2007-compliant diesel treatment-associated DNA damage, in lung tissue (comet assay), blood serum (8-hydroxy-2'-deoxyguanosine [8-OHdG] assay), and hippocampus (lipid peroxidation assay), across diesel exhaust exposure levels. A time-dependent assessment of 8-OHdG and lipid peroxidation also suggested no differences in responses across diesel exhaust exposure levels more than 24 months of exposure. These results indicated that the 2007-compliant diesel engine reduced measurable reactive oxygen species-associated tissue derangements and suggested that the 2007 standards-based mitigation approaches were effective.

KEYWORDS: Diesel, emissions, engine, ACES, comet, 8-hydroxy-2'-deoxyguanine

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CORRESPONDING AUTHOR: Lance M Hallberg, Sealy Center for Environmental Health and Medicine, University of Texas Medical Branch, 301 University Blvd, Galveston, TX 77555-1110, USA. Email: lmhallbe@utmb.edu

Introduction

In 2001, because of diesel exhaust (DE)-related health concerns, the Environmental Protection Agency (EPA) and the California Air Resources Board (CARB) issued new standards for diesel fuels and exhaust emissions. To meet the new exhaust emission standards, manufacturers made improvements to traditional diesel engines, referred to here as pre-2007-compliant diesel engines, using new engineering technologies. Diesel engines with the new technologies are often referred to as new technology diesel engines (NTDEs) and are referred to here as 2007-compliant diesel engines. This distinction is made here to emphasize that the diesel engine used in the Advanced Collaborative Engine Study (ACES) was a 2007-compliant diesel engine. Accordingly, we review some pre-2007-compliant diesel engine studies and findings, to compare with our results, obtained as part of the ACES project. Although some of these data have been presented in prior publications,^{1,2} our emphasis in this discussion is to (1) highlight the contrast in

findings between pre-2007-compliant and 2007-compliant diesel engines, (2) provide in-depth discussion of study characteristics making the ACES unique, and (3) to discuss potential ACES advantages and limitations of study, possibly influencing the results.

Pre-2007 diesel engine biomarker studies

Since the early 1900s, research has been performed to investigate the health effects of exposure to diesel emissions. Here, we highlight some of those early studies for comparison with our research of 2007-compliant diesel engines, to set the context for the results of the ACES project.

Studies of DE particulate extract. From 1954 to 1955, Kotin et al^{3,4} investigated the toxic and carcinogenic potential of DE particulate (DEP) collected under varying engine performance



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Table 1. Aromatic hydrocarbon estimated on 1-minute samples of pre-2007-compliant diesel engine exhaust with varying load and engine revolution speed under conditions of inefficient operation.

QUANTITIES EXPRESSED IN MICROGRAMS PER MINUTE							
RPM	LOAD	CONDITION	PYRENE	COMPOUND X	3,4-BENZOPYRENE	1,12-BENZPERYLENE	ANTHANTHRENE
1000	0	Inefficient operation	137	22	146	22	0
	¼		267	76	465	42	43
	½		586	175	772	124	223
	¾		1800	640	1320	610	472
	1		2500	639	876	1265	469
1200	0		208	0	9	79	4.3
	¼		257	0	47	40	24
	½		448	278	437	171	197
	¾		888	488	432	930	820
	1		1912	614	1706	976	944
1400	0		188	0	80	0	20
	¼		177	56	78	0	16
	½		220	76	1372	368	69
	¾		734	337	982	1071	577
	1		822	346	1687	944	666

Adapted from Kotin et al.⁴

conditions. Table 1 illustrates how diesel emission composition changes, under the varying engine revolutions per minute and loads parameters that were used. C57BL and A-strain mice were skin painted with acetone extracts of DEP 3 times a week (30 µL) up to 24 months. The authors found different toxic effects of DEP extracts that were animal strain dependent and sex dependent. As shown in Table 2, they initially found low tumor induction due to toxicity. However, A-strain females were sufficiently resistant to the toxic effects of the extracts, such that tumors were provided sufficient time for formation to occur. C57BL mice (both male and female) had a 64% survival rate at the time of the appearance of the first tumor and produced only 2 tumors by the end of the experiment, 22 months later. Male and female A-strain mice had a 16% and 80% survival rate, respectively, at the time of first tumor appearance. A-strain males produced a total of 4 tumors, whereas A-strain females produced a total of 17 tumors, by the end of the experiment (23 and 17 months, respectively). Although there were some differences in the number and timing of tumors, the investigators concluded that DEP extracts were both toxic and carcinogenic.^{3,4}

Subsequently, in mutagenicity studies (Ames Assay) of DEP fractionated extracts, Huisinigh et al,⁵ found that the majority of mutations were from the neutral (uncharged) fraction which contained the polynuclear aromatics, phenols,

ethers and ketones. These studies were the first data indicating the potential of DEP extracts to promote DNA mutations.

More recently, in 1999, Hiura et al proposed that DEP acts as an adjuvant, and that it is the organic adherents on the DEP particles that are responsible for its pro-inflammatory and tissue damaging effects. Using an in vitro approach, stock DEP was suspended in phosphate-buffered saline, diluted in cell media (doses: 50, 100, 200, 400 µg/mL), and added to the cell cultures. In both murine and human macrophage cell lines, exposed to increasing levels of DEP, the appearance of blebbing and apoptotic bodies was associated with increased cell death. Those findings were in contrast to a simultaneously studied bronchial epithelial cell line, which lacked this toxic response.⁶ In addition, when the macrophage cell lines were exposed to either washed DEP or carbon black, modeling the carbonaceous center of DEP particles, no toxic effects were detected. Those findings implicated DEP particulate adherents, and not the particle carbonaceous center, in the toxic effects seen. Furthermore, pretreatment with an antioxidant reversed apoptotic effects of DEP; for example, pretreatment with *N*-acetylcysteine, in these experiments, inhibited apoptosis by 80%.⁶ The authors concluded that macrophage cell lines exposed to DEP underwent apoptosis as a result of the induction of reactive oxygen species (ROS).⁶

Table 2. Diesel exhaust experiments.

EXPERIMENT	DATE STARTED	NUMBER OF ANIMALS	STRAIN, SEX	SAMPLE MATERIAL	NUMBER OF DAYS TILL FIRST TUMOR	SURVIVORS AT TIME OF FIRST TUMOR	TOTAL TUMORS	LENGTH OF EXPOSURE, (DAYS)
VIII	March 5, 1952	52	C57BL; 40 F, 12 M	Extract of particulate material obtained during warm-up	392	33	2	670
XII	May 19, 1952	50	A, M	Extract of particulate matter obtained during full load	471	8	4	700
XII (2)	February 13, 1953	25	A, F	Extract of particulate material obtained during full load	400	20	17	891

Adapted from Kotin et al.⁴**Table 3.** Concentrations of key components of exposure atmospheres from pre-2007-compliant diesel engines^a compared with the Advanced Collaborative Emissions Study 2007-compliant engine^b.

COMPONENT	UNITS	CONTROL		LOW		MEDIUM		HIGH	
		TDE	NTDE	TDE	NTDE	TDE	NTDE	TDE	NTDE
DEP	ug/m ³	10 (10)	0.3 (0.2)	350 (70)	0.2 (0.2)	3470 (450)	0.9 (0.6)	7080 (810)	6.3 (4)
CO	ppm	1 (1)	0.9 (0.9)	3 (1)	1.1 (0.5)	17 (7)	1.9 (0.5)	30 (13)	6.4 (2.6)
NO	ppm	0	0.001 (0.002)	0.6 (0.3)	0.3 (0.2)	5.4 (1.5)	1.3 (0.4)	10.0 (2.6)	6.6 (2.4)
NO ₂	ppm	0	0.002 (0.004)	0.1 (0.1)	0.1 (0.01)	0.3 (0.2)	0.9 (0.05)	0.7 (0.5)	4.4 (0.3)
HC vapor	ppm	3 (1)	—	4 (1)	—	9 (5)	—	13 (8)	—
CO ₂	%	0.2 (0.04)	0.1 (0.05)	0.2 (0.03)	0.2 (0.08)	0.4 (0.06)	0.2 (0.03)	0.7 (0.1)	0.5 (0.1)

Abbreviations: DEP, diesel exhaust particulate; HC, hydrocarbon; NTDE, new technology diesel engine; TDE, traditional diesel engine.

^aThe mean (SE) of weekly mean values during 30 months of exposure.Adapted from Mauderly et al.¹³^bThe mean (SD) concentrations for each analyte of all measurements throughout the ACES.Adapted from McDonald et al¹⁷, HEI Research Report 184, Appendix 1, Table 3 (2015).

Although there are many other exemplary studies comprehensively reviewed by Claxton,^{8–12} the above studies of particulate extracts of DEP are examples that demonstrated the mutagenic potential of DEP components, in rodent and bacterial systems. However, the effects of direct inhaled DEP exposure on mutagenesis remained controversial, until the late 1990s, when a number of *in vivo* animal studies were undertaken and demonstrated that inhaled DEP could also promote mutagenic potential, as outlined below.

Studies of DE particulates in vivo. In 1987, Mauderly et al¹³ performed a lifetime inhalation study of pre-2007-compliant diesel engine exhaust emissions (as shown in Table 3) in F344/CR male and female rats. The rats were exposed 7 h/d, 5 d/wk, for up to 30 months to whole exhaust diluted to a nominal soot concentration of 0.35 mg/m³ (low), 3.5 mg/m³ (medium), or 7.0 mg/m³ (high) or to filtered air. At 24 months, analysis of DE soot lung burden averaged 0.6, 11.5, and 20.8 mg in the low-dose, medium-dose, and high-dose groups, respectively.

There was little difference in survival between the control and high-dose groups with respect to DE soot lung burden. Histologically, both active and chronic inflammations were observed at focal accumulations of DEP in lung macrophages, mainly in alveoli adjacent to the bronchial terminals. Epithelial cell-lined air spaces also were altered with fibrosis near sites of DEP accumulation, with sites of epithelial hyperplasia and squamous cell metaplasia, located next to fibrotic loci. Both benign bronchial alveolar adenomas and malignant adenocarcinomas were also observed. Tumor response to those exposures was significantly higher at the high dose, and the response was greater than would be predicted from the tumor response observed at the low dose, possibly indicating that clearance of DEP from the lung at the higher dose was decreased, as compared with the low-dose clearance. In addition, DNA from rats exposed to the high dose also displayed high adduct levels, compared with control.¹³ As a result of those findings, the authors concluded that DE at high concentrations over a lifetime was a carcinogen.

Later, in 1997, Ichinose et al investigated the effects of exposure to DEP on lung tumor development and the induction of the DNA adduct 8-oxodeoxyguanine (8-OHdG) in Institute of Cancer Research (ICR) mice. Diesel exhaust particulate (dose: 0.05, 0.1, 0.2 mg/mouse) suspended in phosphate buffer containing 0.05% Tween 80 was administered with an intratracheal cannula. After 10 weeks of DEP exposure, a dose-dependent increase in 8-OHdG in lung tissue DNA was observed. The investigators also detected a 24% to 31% increase in tumor formation after 12 months. Interestingly, inclusion of β -carotene produced a partial reduction in 8-OHdG adduct formation, indicating a possible contribution by ROS to the DNA damage observed. This study indicated that in vivo DEP exposure in mice leads to an increase in 8-OHdG DNA adduct formation, with a concomitant increase in lung tumor formation.¹⁴

Exposure to DE also has been associated with epigenetic changes that affect transcriptional pathways. Jiang et al, in a double-blind, crossover study design, examined how acute exposure to DE changed genomic methylation patterns in circulating human peripheral blood mononuclear cell (PBMC). Nonsmoking human volunteer subjects who were physician-diagnosed with asthma for at least 1 year or those who had undergone methacholine challenge with a resulting provocation concentration decrease in 20% (PC₂₀) of ≤ 8 mg/mL methacholine, served as both control and treated subjects. The investigators detected changes in methylation patterns in PBMC. The changes were mainly associated with genes involving the protein kinase and nuclear factor κ B pathways. These pathways are associated with oxidative damage responses. In addition, gene polymorphisms along with changes in methylation patterns influence gene functionality as observed in this study with glutathione S-transferase, protein-1 (GSTP1). The GSTP1 showed an increase in methylation; however, only individuals with the A > G polymorphism showed a marked change with exposure, whereas subjects with the G allele (lower functional activity) did not. This study provided evidence that human exposure to DE induced epigenetic changes in methylation patterns in genes that were induced in response to the presence of ROS.¹⁵

In summary, in vitro and in vivo studies, such as those outlined above, indicated that DNA damage was associated with DE/DEP exposure. Those studies contributed to the development of diesel emission controls and the eventual development of the NTDE that were considered necessary to protect the public against the deleterious effects of DEP being dispersed into the atmosphere by diesel engines.^{16–25}

The 2007-Compliant Engine: ACES

Overall study rationale and framework

As indicated above, in 2001, because of DE-related health concerns, the EPA and CARB issued new diesel fuel and emission standards. A tiered adoption of these standards began with the

manufacture of 2007-compliant diesel engines targeting particulate matter (PM) emission reduction by 2007, and reductions in NO_x, due by 2010. To meet these rigid standards, industry redesigned the emission exhaust controls and fuel formulations, resulting in considerably reduced emissions.²⁶ A cooperative multiparty effort was conceived in 2005, with the Health Effects Institute and the Coordinating Research Council developing the ACES. This study was conducted in 3 phases:

- *Phase 1.* Emissions from 4 heavy-duty diesel engines were characterized. These engines were equipped with emission control systems meeting the 2007 PM standards, and characterization was conducted by Southwest Research Institute, in Houston, Texas.²⁷
- *Phase 2.* In this phase, testing was performed on another set of diesel engines meeting the 2010 emission standards to reduce NO_x emissions.²⁸
- *Phase 3.* Lovelace Respiratory Research Institute (LRRI) was selected to perform the rodent exposure studies using 2 diesel engines selected from phase 1.

The study focused on the induction of oxidative stress as a result of DE emission exposure, with specific emphasis on damage of DNA, proteins, and lipids. Thus, the experiments were designed to answer the following questions:

1. Does DE from 2007-compliant diesel engine damage DNA in an exposure-dependent manner?
2. Does DE from 2007-compliant diesel engine damage the hippocampus through lipid peroxidation?

Methods

DE exposures. Exposures to DE were conducted at LRRI in Albuquerque, New Mexico. In total, 2 of 4 engines from phase 1 were randomly selected by the ACES oversight committee for the phase 3 health studies and designated as engine “B” and “B’.” These engines would be switched out periodically for maintenance, similar to what would occur under real-world conditions. These engines were fitted with 2007-required technologies, including a diesel oxidative catalyst, diesel particulate filter (DPF), and active regeneration system with exhaust fuel injection (all active regeneration was triggered by the engine control module and a water-cooled high-pressure loop exhaust gas recirculation system).²⁷ The analysis of the DE indicated the following: (1) the average particle mass across the study was $<12 \mu\text{g}/\text{m}^3$ (Table 3), with most of the mass coming off during DPF regeneration, (2) this mass exchange occurred once or twice per 16-hour duty cycle of engine operation, and (3) the particle size range was between 15 and 20 nm.²⁹

Exhaust dilutions. The animal bioassay doses were DE dilutions described as low, medium, high, and control. These

dilutions of DE were based on predetermined nitrogen dioxide (NO₂) concentrations, rather than particulate concentrations, because PM concentrations in the exhaust were typically beneath reliable detectability levels, although particulates were continuously monitored per protocol. NO₂ exposure has been linked with noncancer effects seen in emissions from pre-2007-compliant diesel engine and has been found to have the highest concentration in NTDE emissions.^{13,30} Thus, for these studies, NO₂ levels were selected as 4.2 ppm (high), 0.8 ppm (medium), and 0.1 ppm (low), based on the following criteria. The highest concentration of NO₂ (4.2 ppm) served as the maximum tolerated dose and was selected based on a chronic study of exposure to NO₂ at 9.5 ppm, for 7 h/d more than 6 months, corresponding to ACES 4.15 ppm for 16 h/d exposure.^{30,31} The low-exposure level (0.1 ppm) represented a potential no-observed-adverse-effect level approaching the national air quality standard for NO₂ (0.053 ppm). The medium-concentration level was a concentration between the low and high levels, based on previous studies performed at LLRI.

Selection of the rat strain. Two strains of rats were originally considered for this study: the F344 and the Wistar WU. Both of these strains had been previously used in chronic studies and in studies of DE (F344 in the United States and Wistar WU in Europe). At the time the ACES oversight committee deliberated the advantages of each strain, the National Toxicology Program (NTP) was considering the Wistar Han strain in lieu of the F344 strain in chronic bioassays³² due to the following criteria: (1) longevity, (2) use in chronic inhalation studies (including studies of DE), (3) availability of an historical database of cancer incidence, and (4) the maximum body weight reached by males. The NTP decision and the previously mentioned factors persuaded the oversight committee to recommend the Wistar Han strain for the ACES. Accordingly, samples from a total of 160 rats (80 males, 80 females) were used in these studies.

Comet assay. An alkaline-modified comet assay was used to measure DNA strand breaks in lung tissue, as a consequence of oxidative damage. During analysis of comet images, the presence of pyknotic cells was noted, indicating cells that are undergoing nuclear condensation as part of the necrotic and apoptotic process. Comet slides were scored blindly, by at least 2 individual slide readers. Both pyknotic and nonpyknotic cells were initially analyzed, as part of the comet assays performed, but after comparing analysis results with and without pyknotic and finding no difference, in further analysis, pyknotic cells were excluded.

Samples from the 1-, 3-, 12-, and 24-month time points were prepared for analysis; however, 9 rat samples demonstrating pyknotic cells were removed from further analysis. Analysis of the following comet parameters was conducted: (1) tail length (TL; comet head diameter–comet length), (2) tail moment (TM; TL × %DNA in the tail), (3) olive moment (OM; tail centroid – head centroid × %DNA in the tail), and (4)

%DNA in the tail. All TM values were expressed as percentages of respective male or female control values at each time of DE exposure.

8-Hydroxydeoxyguanine assay. As a surrogate measurement of DNA damage, the presence of 8-OHdG fragments, the result of excision of adducted guanine nucleotides during DNA repair, was measured in the serum. These fragments are considered an acceptable measure of DNA damage.^{33–35}

Thiobarbituric acid reactive substances assay. Previous studies suggested that DE particles may be able to bypass the blood-brain barrier by impairment of the nasal-respiratory and olfactory barriers.^{36,37} Particles entering the brain in this fashion could result in peroxidation reactions, from ROS generation by particulates, within the brain lipids. To investigate this possibility within our study, the thiobarbituric acid reactive substances (TBARS) assay was used to detect the presence of TBARS formed as a result of lipid peroxidation in the hippocampus.

Statistics. Descriptive statistical analyses were performed on all groups and assays, with tests for normality of the tested data. If the data were not normally distributed, then equivalent non-parametric tests were performed. Comparisons were made to test for sex differences; if no sex differences were noted, then male and female cohorts were combined for further analysis. In addition to sex differences, data were analyzed for both differences between DE exposure groups (low, mid, and high) and DE exposure duration. Data are reported as the mean ± SEM. General linear models were used (analysis of variance) to analyze the data, and when the analysis was found to be significant ($P < .05$), discrete post hoc comparisons between DE groups were performed using Bonferroni correction, to control for multiple comparisons.

Results

DNA damage assessment

Comet assay 1- and 3-month exposures. No significant differences were observed after 1 month of DE exposure between any concentration tested (treated vs control, male vs female, all $P > .05$ (Figure 1A). A similar lack of statistically significant differences was observed with measures of comet TL (Figure 2A) and percentage of DNA in the tail (Figure 3, Table 4).

Likewise, no significant differences were noted 3 months postexposure in TM (Figure 1B), TL (Figure 2B), and %DNA in the tail (Figure 3, Table 4), in comparing controls versus DE-treated animals and within the male versus female comparison. Pyknotic cells were observed in the 1-month group (1 control, 3 high-concentration samples) and the 3-month group (1 low-concentration, 3 medium-concentration, and 1 high-concentration samples), no correlations were observed with respect to exposure group, sex, exposure duration, or batch processing (Table 4).

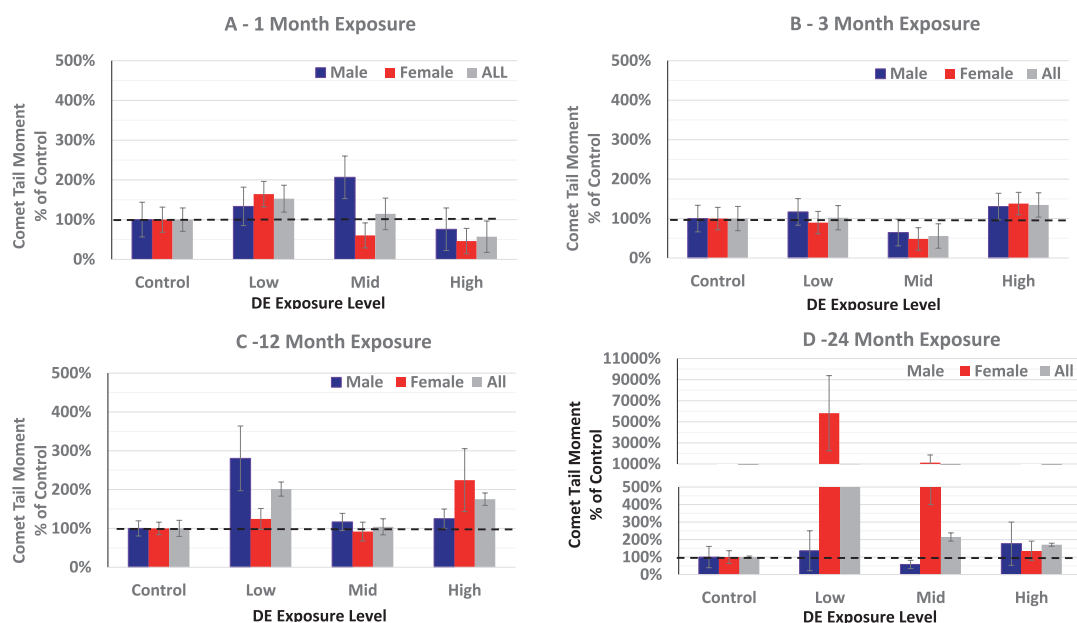


Figure 1. Tail moment data from modified comet assay in rat lung tissue. No significant differences were found for (A) the 1-month exposure group ($P > .05$ for exposure level and for sex), (B) the 3-month exposure group ($P > .05$ for exposure level and for sex), (C) the 12-month exposure group ($P > .05$ for exposure level and for sex), and (D) the 24-month exposure group ($P > .05$ for exposure level and for sex) (please note differences in y-axis scales). Data shown are the mean tail moment $\pm$ SEM, $N = 4$ to 5 animals/bar. For male and female groups at each DE exposure time, data were normalized using control group tail moment averaged in panels (A) at 1 month: 15 ± 7 and 26 ± 8 pixels, (B) at 3 months: 28 ± 9 and 33 ± 9 pixels, (C) at 12 months 0.4 ± 0.1 , and 0.4 ± 0.1 , (D) at 24 months 0.6 ± 0.4 and 0.1 ± 0.04 , respectively. DE exposure levels were based on NO_2 levels where low = 0.1 ppm, mid = 0.8 ppm, and high = 4.2 ppm. DE indicates diesel exhaust.

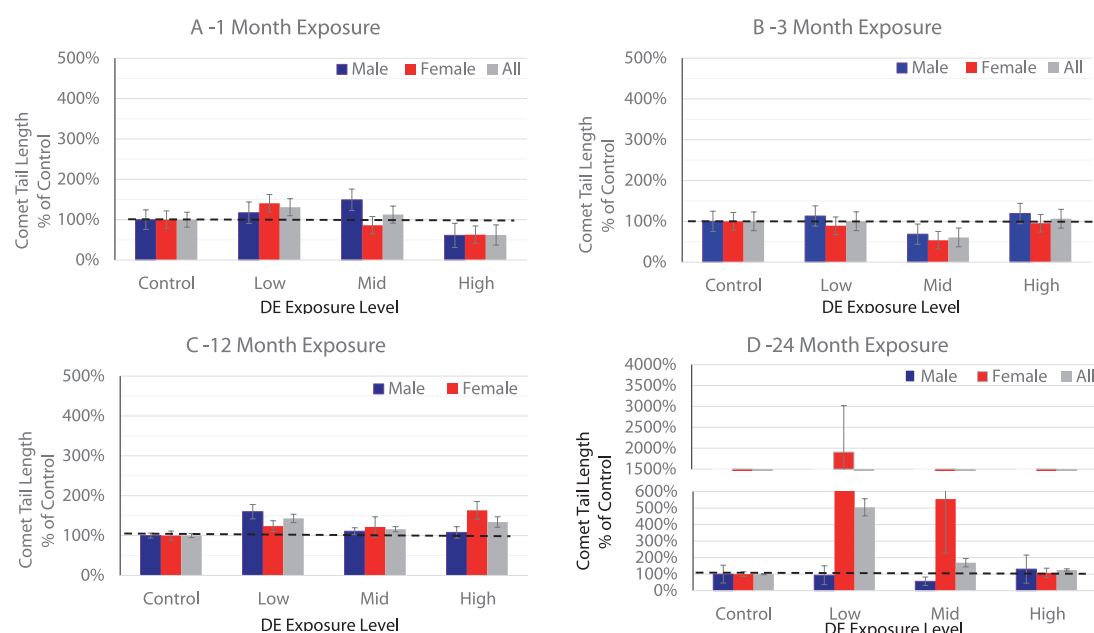


Figure 2. Tail length data from modified comet assay in rat lung tissue. (A) For the 1-month exposure group, a significant difference was found between the low-exposure level and high-exposure level ($P = .04$). (B) For the 3-month exposure group, $P > .05$. (C) For the 12-month exposure group, $P > .05$. (D) For the 24-month exposure group, $P > .05$ (please note differences in y-axis scales). Data shown are the mean tail length $\pm$ SEM; $P > .05$, number of animals, data normalization, and DE exposure levels are as described in Figure 1. DE indicates diesel exhaust.

Comet assay 12- and 24-month exposure results. In the 12- and 24-month groups, no sex differences were observed ($P > .05$ for TM [Figure 1C and D], TL [Figure 2C and D], %DNA in tail [Figure 3A and B], and OM [Figure 4A

and B]). Overall, no interaction between time and sex factors was noted to affect DNA damage, in neither the 12- nor 24-month groups. Because sex differences were absent in all tests up to this point, sexes were merged for all

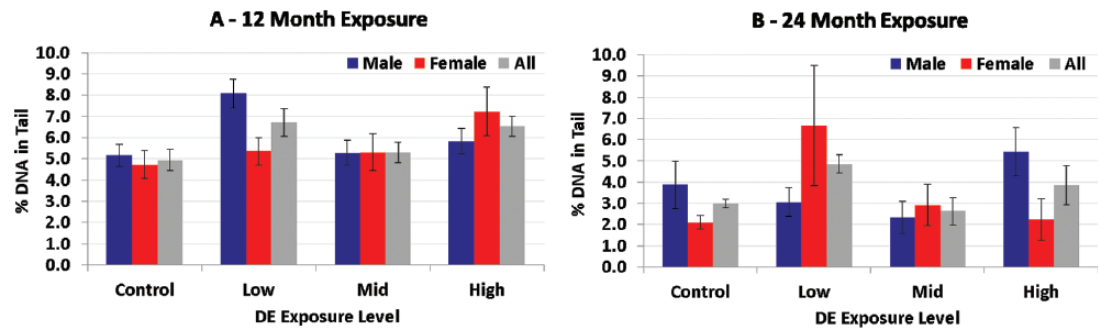


Figure 3. %DNA tail data from modified comet assay in rat lung tissue. (A) For 12-month exposure group. (B) For 24-month-exposure group. Data shown are the mean %DNA±SEM; $P>.05$, number of animals, data normalization, and DE exposure levels are as described in Figure 1. DE indicates diesel exhaust.

Table 4. Analysis of the effects of pyknotic cells on the percentage of DNA in comet tail of rats exposed for 3 months^a.

	SAMPLES WITH PYKNOTIC CELLS				SAMPLES WITHOUT PYKNOTIC CELLS			
	MALE		FEMALE		MALE		FEMALE	
Control	41.4	7.6 ^a	46.8	4.1 ^a	41.4	7.6 ^a	46.8	4.1 ^a
Low	45.5	6.6	41.7	10.3	45.5	6.6	50.8	6.2
Mid	41.7	10.8	27.0	11.5	52.0	4.4	44.8	6.8
High	34.5	8.5	50.1	9.8	34.5	8.5	59.4	4.1

^aData are mean±SEM.

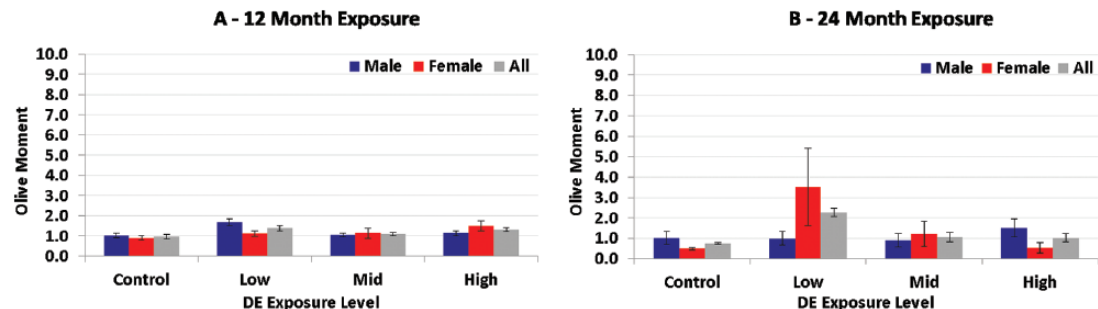


Figure 4. Olive moment data from modified comet assay in rat lung tissue. (A) For 12-month exposure group. (B) For 24-month exposure group. Data shown are the mean olive moment±SEM; $P>.05$, number of animals, data normalization and DE exposure levels are as described in Figure 1. DE indicates diesel exhaust.

subsequent statistical testing of remaining variables, as described below.

DNA damage: 8-OHdG enzyme-linked immunosorbent assay in serum. Comparison of control and treated, as well as between sexes in 1- and 3-month groups, showed no significant differences ($P>.05$, Figure 5) in 8-OHdG. The 3-month group exposure measurements were significantly higher than those for other exposure-duration groups ($P<.001$). Analysis of serum 8-OHdG in serum was significant within the mid-concentration and high-concentration groups (12-month group) and within the high-concentration group in the 24-month group ($P<.05$). Sex difference was observed in the high-concentration group ($P<.05$). There was no concentration-dependent relationship to these

differences, and other interactions were likewise, negative ($P>.05$, Figure 5).

Oxidation/reduction assessment (via TBARS)

Lipid peroxidation assay in the hippocampus. In the 1-month group, sex differences were noted in both the low-dose and high-dose exposures ($P<.05$), for measurements of lipid peroxidation. In the 3-month group, there was a statistically significant difference between control and the low-dose group ($P<.05$). However, no dose-dependent pattern was observed (Figure 6A and B). Analysis of the 12- and 24-month groups was nonsignificant for exposure effects (Figure 6C and D, $P>.05$). Overall TBARS levels in the 24-month group were significantly higher than the other duration groups ($P<.05$), and the overall TBARS level in

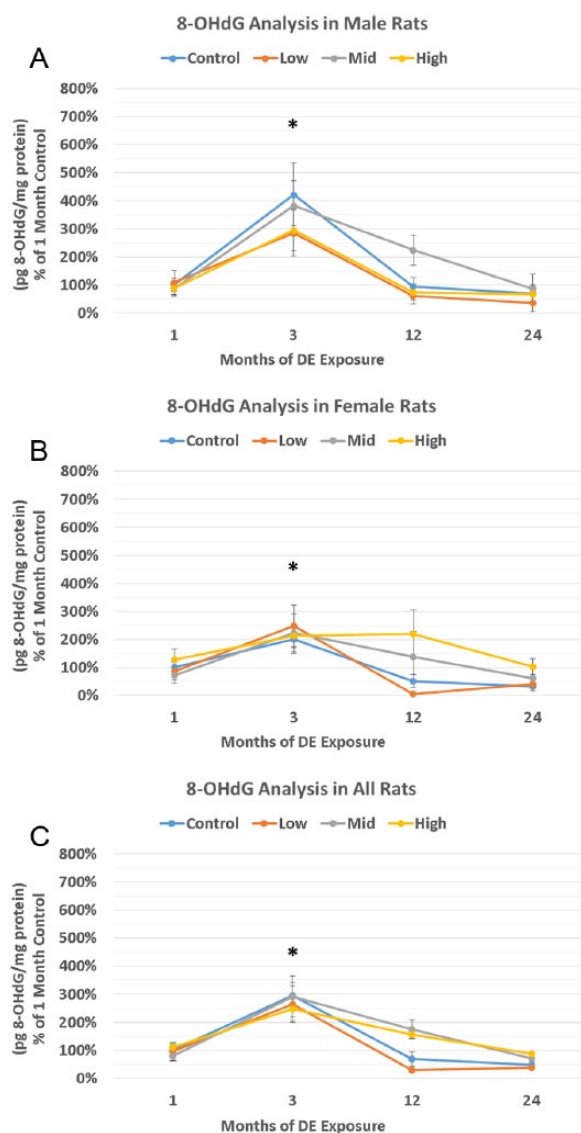


Figure 5. 8-OHdG assay data in rat serum. For (A) male-exposure group, (B) female-exposure group, and (C) all (male and female combined)-exposure group. In the 12-month group, exposure vs control in mid-dose and high-dose groups, $P < .05$, and between sexes at high-dose group, $P < .05$, no dose-dependent pattern was observed. There was a significant increase over time in both male and female samples when comparing 1- versus 3-month groups ($P < .05$). Data shown are the mean 8-OHdG concentration $\pm$ SEM, number of animals, data normalization, and DE exposure levels are as described in Figure 1. * $P < .05$, showing statistical significance. 8-OHdG indicates 8-hydroxy-2'-deoxyguanosine; DE, diesel exhaust.

the 12-month group was also notably higher, in contrast to the 1-month group ($P < .05$). We speculated that this observation was a result of a possible aging effect, as has been observed in other studies³⁸; however, more experiments would be needed to demonstrate this possibility with adequate statistical confidence.

Discussion

Genotoxic components of DE

It is important to reemphasize that the adverse health effects of air pollution are classically attributed to oxidative stress, in

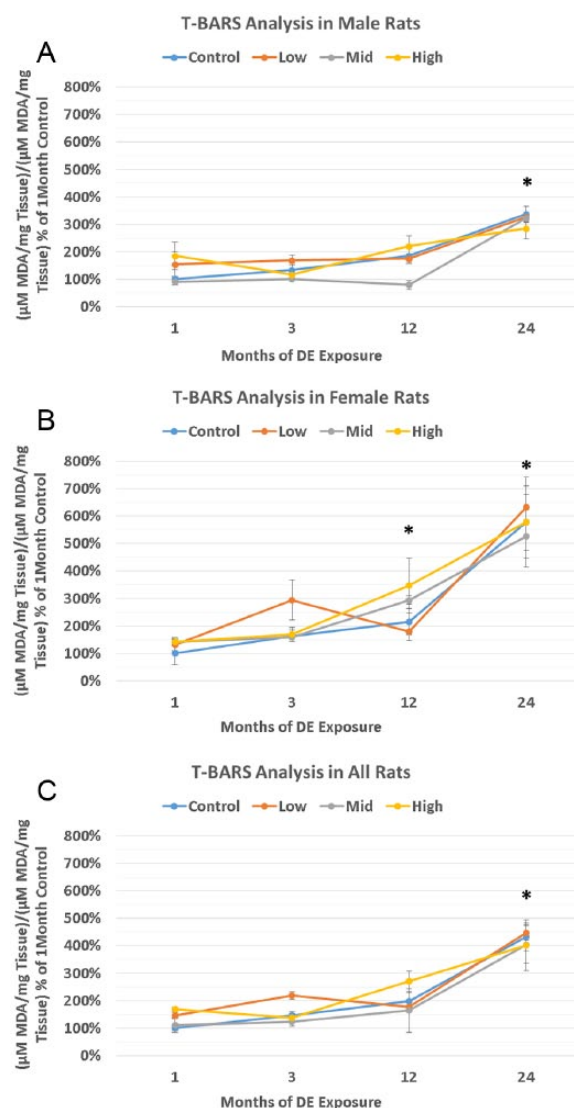


Figure 6. TBARS assay data in rat hippocampal tissue. For (A) male-exposure group, (B) female-exposure group, and (C) all-exposure group. In the 1-month group, a significant difference was noted between sexes in the low-dose and high-dose groups ($P < .05$), and in the 3-month group, there was a significant difference from control in the low-dose group ($P < .05$). No dose-dependent pattern was observed. Analysis of the 12- and 24-month groups was nonsignificant ($P > .05$). There was a significant increase over time in both male and female samples when comparing 1-month versus 12- and 24-month groups ($P < .05$). Data shown are the mean TBARS concentration $\pm$ SEM, number of animals, data normalization, and DE exposure levels are as described in Figure 1. * $P < .05$, showing statistical significance. DE indicates diesel exhaust; TBARS, thiobarbituric acid reactive substances.

large part due to chemical reactions that are secondary to gas-phase to wet-phase chemistry, resultant of effects of particulate exposure.^{39,40} However, DE health effects have also been linked to the induction of genotoxicity associated primarily with effects produced by DE's particulate fraction.^{36,41,42} This is based on the idea that particulates or chemical adherents are able to directly damage tissues, lipids, proteins, or DNA through oxidative stress induction or indirectly through inflammatory responses, particularly the recruitment of inflammatory

cells, which, in turn, produce oxidative mediators. Diesel exhaust particulates, both fine and ultrafine particles (UFPs), are deposited deep in the lungs and, in the case of UFP, capable of entering the blood stream and tissues, themselves. Hundreds of chemicals including poly-aromatic hydrocarbons (PAH), which are a significant contributor to vehicular PM health effects, also are adsorbed onto DEPs.^{40,43} For these reasons, understanding the effects of particulates and their adherents and the study of the effects of potential particulate mitigation by the 2007-diesel engine modifications are of importance and should be considered.

The studies by both Lee et al⁴⁴ and Harri et al⁴⁵ found increases in 8-OHdG in the urine of DE emission inspectors and workers exposed to PAHs. The studies by Rodríguez-Romero, Lee, and Harri all implicate the PAHs in producing genetic damage. It could be argued that by decreasing particulate levels, there would be a concomitant decrease in the manifestation of PAH damage. It is possible that results of the ACES showing the benefits of reduced emission levels could have been due to reductions in PAH, associated with the NTDE mitigation mechanisms.

In the studies outlined above, we investigated NTDE emissions and their effects on DNA damage and oxidation of lipids. Our original hypothesis suggested that oxidative stress was the main mechanism of damage. We postulated that the oxidative mediators promoting stress and subsequent damage originated from DE gases and/or DEP. As the characterization of the NTDE proceeded, it was determined that due to the addition of new after-treatment technology, DEPs were near or similar to ambient levels, potentially reducing the health risk from this component of DE, within the particulate size range that was mitigated. However, there would remain some concern that there were other gaseous components still present, such as NO_x and PAHs, which also might have produced oxidative mediators or inflammatory responses within our study.

Genotoxicity of DE in rats and humans

The comet assay is an appropriate test for DNA damage because it examines the effects on DNA on a broad range of oxidized purines and pyrimidines.⁴⁶ For example, use of the comet assay illustrated that DNA double-strand break levels were greater in a study of DE-exposed bus drivers and garage workers compared with control, in the city of Prague, in the Czech Republic.⁴⁷ Similarly, a Danish study found a correlation between UFP (measured at fixed stations) and increased probability of DNA damage in individuals who bicycled in traffic.⁴² Furthermore, in a study by Muller et al,⁴⁸ after oral ingestion of DEP, elevated levels of strand breaks in the lungs of Big Blue rats were likewise reported, using the comet assay. Thus, we believe that the comet assay was appropriately applied in our search for DNA damage, within our studies.

Interestingly, our investigation did not find significant exposure-related, dose-dependent elevation in DNA damage, in

exposed animals, compared with controls (comet assay, 8-OHdG assay). We noted that control background levels in the 1- and 3-month groups, and in the 12- and 24-month groups, were different, suggesting that the differences between those study groups have some age-related explanation. However, we were unable to locate any study, either in support or refutation, of this suggestion of age-related differences in our rodent lungs.

Environmentally induced lipid peroxidation in brain tissue

Acute, subchronic, and chronic exposures to PM and pollutant gases can cause detrimental effects in people.⁴⁹ Furthermore, respiratory-tract inflammation can produce mediators such as inflammatory cytokines, such as interleukin (IL)-6, tumor necrosis factor α (TNF- α), and C-reactive protein, in addition to ROS.^{50–54} Furthermore, a combination of systemic PM circulation and damaged nasal and olfactory barriers has been reported to have detrimental health effects in the brains of DE-exposed populations.³⁷ Several studies support this possibility, including studies by Calderón-Garcidueñas et al,^{37,52,55–57} which dealt with the effects of Mexico City's air pollution. The investigators observed that in otherwise healthy dogs exposed to Mexico City's air pollution demonstrated increased levels of DNA damage in nasal, respiratory, and olfactory epithelium. Furthermore, they detected chronic brain inflammation and neurodegeneration in these dogs providing evidence that deterioration of the olfactory barrier might play an important role in the observed neuropathology.⁵⁵ In addition, Calderón-Garcidueñas et al⁵⁷ also found that the frontal cortex of the hippocampus in human brain tissue of individuals exposed to the same Mexico City air pollution expressed considerably greater COX-2 levels⁵⁶ implying an association between air pollution and inflammation. Moreover, they also reported endothelial hyperplasia in the olfactory bulb and observed UFPs in olfactory endothelial cytoplasm and basement membranes.⁵⁷ Finally, Gerlofs-Nijland et al⁵⁸ found that in rats exposed to DE, baseline concentrations of pro-inflammatory cytokines, TNF- α , and IL-1 α were brain region dependent and differed in expression, with increases pronounced within the striatum.

Hence, the prior studies mentioned above provide evidence that the brain can be affected by air pollutant exposure and the response may vary by brain region. Observations from our study of the health effects on the rat hippocampus treated with NTDE emissions mainly showed no significant effects. However, in the 1-month group, we did observe that males in the low-concentration and high-concentration categories trended toward an effect of DE exposure, which was not statistically significant ($P = .06$).

Study limitations

Additional control groups. A potential limitation of the ACES was a lack of a pre-2007-compliant diesel engine emission

control within the experimental design. Concurrent testing of pre-2007-compliant diesel engine samples, along with 2007-compliant diesel engine samples, could have added strength to our study. However, this was not included within the experimental design based on the earlier studies of DE exhaust by Mauderly and colleagues,^{30,59,60} in which the use of NO₂ levels was considered a good surrogate of DE exposure, allowing comparisons between the studies, and allowing some inferences to be made from the data collected.

Effects of particulates and NO_x. Although the 2007-compliant diesel engine significantly reduced emissions, including particulates, some small particulate and gaseous (eg, NO_x) agents were present in the exhaust of 2007-compliant diesel engines. It is possible that those remaining particles and gases directly or indirectly induced some oxidative damage and inflammatory responses. The end points of the ACES suggested a minimal amount of oxidative damage, inclusive of remaining particulates and gaseous components, as mentioned above, and provided support for the concept of reduced health risk from the cleaner running 2007-compliant diesel engine.^{26,61,62}

NO₂ levels as an experimental determinant. It could possibly be argued that the ACES did not truly assess the effects of DEP, in that the NO₂ surrogate levels used to monitor exposure atmosphere concentration were without regard to actual particulate concentration. However, particulates continued to be monitored in these studies, as reported by McDonald et al. The daily average Dekati particle mass measured inside the chamber ranged from 0.4 µg/m³ (controls) to 12.3 µg/m³ (high dose) for all collected data over the entire exposure duration, as compared with those of the 1987 study by Mauderly et al, which ranged from 10 µg/m³ (control) to 7080 µg/m³ (high dose)^{13,26,62} (Table 3). Thus, we cannot directly argue that there are no effects from PM from 2007-compliant diesel engines in our study, but we can infer from our findings that the hazard associated with diesel emissions is likely lower than pre-2007-compliant diesel engines. Furthermore, it can also be argued that the effects of NO₂ exposure, in the range of our study (0.1–4.2 ppm), also showed little to no measurable changes in our end points. Thus, although a limited number of observations displayed some randomly distributed significant differences from the standpoint of DE, we believe that it is unlikely that they are of biological importance, in consideration of the overall effects.

Tissue harvest timing. The length of time from end of DE exposure to the harvest of tissue could be a factor, perhaps explaining the lack of significant effects in our findings. An important question was related to the speed and extent of DNA repair, in that there may have been a possibility that DNA repair processes over the time we studied could reduce the measured DNA damage. Table 5 illustrates the harvest duration from end of exposure to harvest completion. This

Table 5. Comparison of harvest duration between males and females in the 1- and 3-month exposure groups from Advanced Collaborative Emissions Study.

	AVERAGE HARVEST DURATION, H	
	MALES	FEMALES
1 month	3.75	2.50
3 months	3.40	4.20 ^a

^aLonger than the averages for the other groups because 1 female was processed earlier in the day than the rest.

ranged from 2.5 (females) to 3.75 hours (males) in the 1-month group and 4.2 hours (females, 1 female harvested earlier than the rest) to 3.4 hours (males) for the 3-month group, which was consistent, and a relatively narrow window, with an average of approximately 3 hours after DE exposure. It is likely that some DNA repair mechanisms were active during that time, resulting in some loss of magnitude of detectable DNA damage-associated changes. However, we point out that (1) as oxidatively damaged DNA was repaired, some of that repair would have released 8-OHdG lesions which should accumulate in serum, but no increase in serum 8-OHdG was observed in our study and (2) DNA damage, repaired in an efficient, error-free fashion in less than 4 hours, would return cells to their original condition and consequently would be biologically immaterial.

Potential for nonoxidative DNA damage. In our study, we concentrated on measuring oxidative DNA damage as DNA strand breaks, 8-OHdG adducts, presence of excision repair products, and lipid peroxidation. The possibility still exists that other types of DNA damage may have occurred that could not be measured by our assays. For example, DNA-DNA or DNA-protein cross-linking by alkylating agents in DE and formation of bulky adducts by PAHs, micronuclei formation, and sister chromatid exchange.⁶² As stated above, if the DNA damage caused by any of these possibilities is correctly repaired, they become biologically immaterial. However, if this DNA damage remains unrepaired, then persistent DNA damage could pose a continuing hazard, allowing for possible DNA damage fixation and production of mutations.^{63–65} Potentially, sufficient and persistent DNA damage may develop into a DNA instability cellular phenotype resulting in continued adverse health consequences.^{6,66–68} Furthermore, the potential for those effects assumes that the complexity of human DNA repair processes are sufficiently modeled by rodents. Although this assumption may be important, it is not different from other studies in which rodents are used as models of investigation of those effects. However, ours were not the only biomarkers measured in the ACES; the agreement of the other various and different biomarkers measured within the main ACES, and those of other auxiliary study investigators, would seem to provide support for our conclusions regarding reduced effects associated with emissions of 2007-compliant diesel engines.^{69–72}

Possible exposure adaptation effects. The ACES modeled the DE exposure in a chronic and continuous manner. It is possible that the animals may have adapted to the DE exposure, mitigating the effects of DE early in the experiment, possibly through upregulation of antioxidant capacity and/or DNA repair. If so, such adaptations may have been sufficiently protective, reducing DNA damage to equivalent levels as that found in control animals. This possibility would be suggestive that (1) the level of DEP exposure produced by 2007-compliant engines, which approaches that of ambient air, may be so low that there may be only a small stimulus for a stress response and subsequent DNA damage and/or repair or (2) the levels of DEP exposure were easily handled by oxidative stress mitigation and DNA repair mechanisms, resulting in no measurable stress and damage responses.

Conclusions

Based on our portion of the ACES, we concluded that emissions from 2007-compliant diesel engines resulted in a lack of measurable DNA damage as measured in lung tissue, serum, and the hippocampus. Other components of the ACES reported a similar lack of measurable effects. For example, Bemis et al⁷⁰ found that exposure to DE in rats for 24 months did not increase the frequency of micronuclei in reticulocytes. Conklin and Kong's investigation likewise indicated that neither there was little change, in more than 20 cardiovascular biomarkers from plasma, nor were there exposure-related changes in cardiac fibrosis and aortic remodeling.⁷² Thus, within the limitations of our measured end points, and those of others within the ACES group, the findings support that measurable ROS-associated tissue derangements and other pathologies typically associated with 2007-compliant diesel engines were mainly absent, as compared with other studies of pre-2007 diesel engines. We have inferred that 2007-compliant diesel engine emission control systems and reformulated fuels can be effective and can potentially reduce the health effect hazards posed by DE exposure. In addition, with the subsequent implementation of the 2010 emission standards for NO_x, one would expect that the NO_x levels should also decrease, further reducing health risk from DE exhaust exposure. With these considerations, it would be reasonable to expect that replacement, or retrofit, of the current aging diesel fleet should improve future air quality, as affected by diesel engine emissions.

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Author Contributions

LMH and JKW conceived and designed the experiments and analyzed the data.

LMH, JKW, and BTA wrote the first draft of the manuscript and jointly developed the structure and arguments for the paper. LMH, JKW, JBW, and BTA contributed to the writing of the manuscript; agree with manuscript results and conclusions; made critical revisions and approved final version; and reviewed and approved the final manuscript.

Disclosures and Ethics

As a requirement of publication, author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality, and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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