

Supplementary Issue: Occupational Health and Industrial Hygiene

Evaluation of Pulmonary and Systemic Toxicity of Oil Dispersant (COREXIT EC9500A®) Following Acute Repeated Inhalation Exposure

Jenny R. Roberts, Stacey E. Anderson, Hong Kan, Kristine Krajnak, Janet A. Thompson, Allison Kenyon, William T. Goldsmith, Walter McKinney, David G. Frazer, Mark Jackson and Jeffrey S. Fedan

Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV, USA.

ABSTRACT

INTRODUCTION: Oil spill cleanup workers come into contact with numerous potentially hazardous chemicals derived from the oil spills, as well as chemicals applied for mitigation of the spill, including oil dispersants. In response to the Deepwater Horizon Macondo well oil spill in the Gulf of Mexico in 2010, a record volume of the oil dispersant, COREXIT EC9500A, was delivered via aerial applications, raising concern regarding potential health effects that may result from pulmonary exposure to the dispersant.

METHODS: The current study examined the effects on pulmonary functions, cardiovascular functions, and systemic immune responses in rats to acute repeated inhalation exposure of COREXIT EC9500A at 25 mg/m³, five hours per day, over nine work days, or filtered air (control). At one and seven days following the last exposure, a battery of parameters was measured to evaluate lung function, injury, and inflammation; cardiovascular function; peripheral vascular responses; and systemic immune responses.

RESULTS: No significant alterations in airway reactivity were observed at one or seven days after exposure either in baseline values or following methacholine (MCh) inhalation challenge. Although there was a trend for an increase in lung neutrophils and phagocyte oxidant production at one-day post exposure, there were no significant differences in parameters of lung inflammation. In addition, increased blood monocytes and neutrophils, and decreased lymphocyte numbers at one-day post exposure also did not differ significantly from air controls, and no alterations in splenocyte populations, or serum or spleen immunoglobulin M (IgM) to antigen were observed. There were no significant differences in peripheral vascular responsiveness to vasoconstrictor and vasodilator agonists or in blood pressure (BP) responses to these agents; however, the baseline heart rate (HR) and HR responses to isoproterenol (ISO) were significantly elevated at one-day post exposure, with resolution by day 7.

CONCLUSIONS: In summary, acute repeated exposure to COREXIT EC9500A did not alter pulmonary function, lung injury/inflammation, systemic immune responses, or vascular tone, but did cause transient chronotropic effects on cardiac function.

KEYWORDS: inhalation, oil dispersant, pulmonary toxicity, immunotoxicity, cardiovascular effects

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CORRESPONDENCE: jur6@cdc.gov

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Introduction

Workers involved in cleanup responses to oil spills risk exposure to numerous hazardous chemicals from the oil and from agents utilized to mitigate the spills, such as dispersants.^{1,2} Cleanup worker health effects, both physical and mental,

have been documented in response to only a handful of the major oils spills from supertankers that have occurred over the past several decades. As reviewed by Aguilera et al.³ and Goldstein et al.⁴, these include Exxon Valdez (1989; 37,000 tonnes), MV Braer (1993; 85,000 tonnes), Sea Empress



(1996; 72,000 tonnes), Nakhodka (1997; <6,000 tonnes), Erika (1999; 20,000 tonnes), Prestige (2002; 63,000 tonnes), Tasman Spirit (2003; 37,000 tonnes), and Hebei Spirit (2007; 10,900 tonnes).^{5–7} Despite the small number of epidemiological studies that have been performed, some of the acute health effects observed in cleanup workers are similar across incidents and include headache, dizziness, fatigue, nausea, diarrhea, skin and eye irritation, and respiratory symptoms (sore throat, cough/wheeze, breathing difficulty), as well as anxiety and depression.

On April 20, 2010, an explosion on an oil rig, Deepwater Horizon, in the Gulf of Mexico released approximately 210,000,000 gallons of crude oil, or approximately 500,000–660,000 tonnes, over the course of approximately three to five months. Capping of the well occurred on July 15, 2010, and the well was permanently sealed on September 19, 2010.^{8,9} The ongoing National Institute of Environmental Health Sciences (NIEHS) GuLF Study (Gulf Long-term Follow-up Study) estimates that greater than 150,000 people were involved in cleanup efforts to varying degrees.¹⁰ In response to one of the largest spills and cleanup efforts in history, a near record of 1.8 million gallons of oil dispersant were deployed by aerial (~1 million gallons) and by subsea applications.⁹ Two forms of COREXIT were employed in the response, 9527 and EC9500A, the latter being the primary dispersant utilized after supplies of 9527 were exhausted early in the operation.⁴ Both dispersants are currently approved for use in oil spill cleanup operations by the Environmental Protection Agency (EPA) under the National Contingency Plan Product Schedule.¹¹ Although data related to the toxicity of dispersants alone in oil mixtures on aquatic/marine plant and animal life existed prior to the Deepwater Horizon spill as reviewed elsewhere,^{12,13} few mammalian toxicity studies related to dispersant mixture were available at the time of the spill, with only one *in vivo* study of an oral exposure in rats that demonstrated adverse gastrointestinal effects from COREXIT.¹⁴ In addition to the environmental and ecological interests related to the dispersant application, the sheer volume of dispersant applied over the course of the spill now raised concerns about the potential worker health effects that might be triggered by respiratory exposure to aerosols of dispersants.

Oil dispersants are complex mixtures that include surfactants and solvents, and are commonly employed in different operations during oil spill cleanup responses.¹² Although many dispersant formulations contain proprietary information, the components of COREXIT 9527 and EC9500A have been disclosed by Nalco to the U.S. EPA.¹⁵ The list of chemicals includes propylene glycol, dioctyl sodium sulfosuccinate (DSS) as the primary organic sulfonic acid salt, hydrotreated light petroleum distillates, and ethoxylated alcohols (2-butoxyethanol is present in the 9527 formulation only). Although the effects of pulmonary exposure to oil dispersants have not been well characterized, the individual components of COREXIT are known to cause pulmonary toxicity to varying degrees,

including altered lung function, lung irritation, and pulmonary edema and injury, and may also cause cardiovascular, hematological, and immunological toxicities depending on the route of exposure. It is important to note that doses employed in the studies of these materials may be at levels that may exceed that of their presence in COREXIT. Toxicological effects specific to each agent have been reviewed elsewhere.^{16–20}

Soon after the Deepwater Horizon oil spill, a series of studies were conducted at the National Institute for Occupational Safety and Health (NIOSH) that examined the potential toxic effects of a single acute dermal or respiratory exposure to COREXIT EC9500A.^{21–25} The goal of the present study was to characterize pulmonary and systemic responses *in vivo* following an acute repeated inhalation exposure to COREXIT EC9500A. The model utilized an inhalation exposure system designed to deliver an aerosol of COREXIT²¹ to rats at a concentration of 25 mg/m³ for five hours per day over the course of nine work days to evaluate lung function, injury, and inflammation. It is also well understood that pulmonary exposures to aerosols and particulates can result in adverse systemic effects on cardiovascular function^{26–28} and the immune system^{29–31}; therefore, in addition to pulmonary toxicity, hemodynamic measures, peripheral vascular responses, hematologic parameters, splenocyte phenotypes, and responsiveness to T-cell antigen were also measured.

Methods

Experimental design. Male Sprague-Dawley rats (~300 g) were exposed by whole-body inhalation to an aerosol of the oil dispersant, COREXIT EC9500A, using an exposure system²¹ designed for delivery of the particulate portion of the compound at a target dose of 15 mg/m³ (27 mg/m³ of particle and vapor) for five hours per day for nine work days. Control rats were exposed to filtered air. No direct measurements of COREXIT in the air exist from the worksites.² Therefore, the dose was based on the portion of the material that was measurable in real time in the inhalation system, the particulate portion of the aerosol. The exposure was designed to achieve a particulate portion of aerosol at a concentration of 15 mg/m³, the occupational exposure limit for a nuisance dust or a material considered to be relatively nontoxic. We had previously examined a single five-hour exposure. For the purposes of this study, a longer acute repeated exposure design was selected to determine if a subacute exposure to COREXIT would lead to adverse effects. The average daily work shift at the cleanup sites was 12 hours. However, heat was a factor during the operation and the average worker spent 20 minutes in active work with 40 minutes rest for every hour to avoid heat stress.² Therefore, a five-hour per day exposure was selected for the animal study.

Six separate exposures were conducted to assess effect of COREXIT on parameters related to pulmonary, cardiovascular, and immune responses. All the parameters were evaluated at one and seven days following the last day of inhalation exposure. Pulmonary function was measured as



lung resistance (R_L) and dynamic compliance (C_{dyn}); baseline values were measured, and responses to inhaled methacholine (MCh) aerosol were obtained to assess airway reactivity. Bronchoalveolar lavage (BAL) was performed on the lungs to collect cells and fluids to assess inflammation and injury to the lung. Cardiovascular toxicity was evaluated as alterations to hemodynamic parameters (HR, heart rate; LVSP, left ventricular systolic pressure; and MAP, mean arterial pressure) and changes in vascular tone and responsiveness to vasoactive agents *ex vivo*. To assess systemic immunotoxicity, blood cell differential cell counts were obtained, splenocytes were isolated and phenotyped, and the immunoglobulin M (IgM) response to T-cell-dependent antigen was measured.

Materials. COREXIT EC9500A was provided by Nalco. In this report, the use of the abbreviation, COREXIT, refers to the specific formulation of COREXIT EC9500A. Materials used for analysis of lactate dehydrogenase (LDH) and albumin were purchased from Roche Diagnostic Systems. Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Life Technologies. MCh, isoproterenol (ISO), norepinephrine (NE), acetylcholine (ACh), and phenylephrine (PHEN) were purchased from Sigma-Aldrich, and Dulbecco's modified Eagle's medium (DMEM) with glucose was purchased from Invitrogen. All sheep red blood cells (SRBC) were drawn from a single donor animal (Lampire Biological Laboratories). IgM was measured using a commercially available kit (Life Diagnostics), Zap-oglobin for red blood cell lysis was purchased from Beckman Coulter Inc., and antibodies and reagents to identify splenocyte phenotypes were purchased from BD Pharmingen.

Animals. Male Sprague-Dawley (Hla: SD CVF) rats were obtained from Hilltop Lab and were used for *in vivo* studies in accordance with an animal protocol approved by the National Institute for Occupational Safety and Health (NIOSH) Morgantown Institutional Animal Care and Use Committee (IACUC). Animals were given a Teklad 2918 diet and tap water *ad libitum*, and were housed in a clean air and viral- and antigen-free room with restricted access in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International-approved animal facility under a 12-hour:12-hour light:dark cycle. All rats arrived at 52–59 days of age, weighing approximately 300 g. Rats were allowed to acclimate for one week before use. All rats were exposed at the same age and approximately the same weight.

COREXIT exposure. An automated whole-body inhalation exposure system was used to expose 24 individually housed rats to COREXIT ($n = 12$) or air (control; $n = 12$). Goldsmith et al.²¹ provide a detailed description of the exposure system design. Briefly, the oil dispersant aerosol was generated with a collision-type atomizer (TSI 3076; TSI Inc.) and regulated with software feedback to maintain a constant concentration during the exposure period. Temperature and relative humidity were continually recorded during the exposure period with a Vaisala HMP60 humidity and

temperature probe. Real-time measurement of COREXIT aerosol concentration in the chamber was measured with a calibrated light scattering instrument, and gravimetric filters were used to measure the COREXIT aerosol concentration in the breathing zone of animals during exposure. A modified acidified methylene blue spectrophotometric assay for anionic surfactants²¹ was implemented to determine the total aerosol concentration (aerosol + vapor). Particle size distribution of the aerosol was determined using an aerodynamic particle sizer (APS; TSI Inc.) and a scanning mobility particle sizer (SMPS; TSI Inc.). Rats were placed in the inhalation chamber and exposed to filtered air or COREXIT for five hours per day for nine work days (Monday through Friday of one week followed by Monday through Thursday of the next week). A target particulate dose of 15 mg/m³ (not including vapor) was chosen based on exposure limits for nuisance dusts, substances considered to be relatively inert. Desiccation studies performed previously indicated that the filters retained 56% weight of the initial COREXIT liquid over the exposure period per day, indicating that a portion of the material vaporized.²¹ Therefore, a 15-mg/m³ particulate exposure is equivalent to a ~25-mg/m³ total COREXIT exposure (particulate and vapor). Rats were returned to the colony room immediately following each exposure. Six separate sets of exposures were conducted to obtain a value of $n = 6$ –8 for all toxicological endpoints.

Lung function/airway reactivity. At one and seven days following the last exposure to COREXIT or air, rats were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). After exposing the trachea through a midline incision in the neck, a cannula was inserted into the trachea. The animals were given supplemental ketamine (40 mg/kg suffused onto the muscles of the neck), placed into plethysmographs on top of a warming pad, and ventilated (90 breaths/minute, 40 cm H₂O mouth pressure, 5 mL tidal volume). Values were logged at five-second intervals and averaged (Buxco Research Systems). After recording basal values of lung resistance (R_L) and dynamic compliance (C_{dyn}) in air, the animals were challenged with saline aerosol (20 μ L), the vehicle control for MCh challenge ($n = 5$ –6/group/time point). Following baseline measurements, rats were administered aerosols of MCh (20 μ L) in stepwise-increasing concentrations (0.3–10 mg/mL). The peak values for MCh-induced changes in R_L and C_{dyn} responses were quantified.

Lung injury and inflammation. In a separate group of rats, on days 1 and 7, following the last exposure to COREXIT or air, BAL was performed in order to obtain pulmonary cells and fluids for analysis of indicators of lung injury, inflammation, and cellular activity ($n = 6$ /group/time point). Rats were euthanized with an overdose of sodium pentobarbital (>100 mg/kg; Sleepaway; Fort Dodge Animal Health), the trachea was cannulated, the chest cavity was opened, the left lung was clamped off, and BAL was performed on the right lungs. The acellular fraction of the first lavage was obtained by



filling the right lung with 1 mL/100 g of phosphate-buffered saline (PBS), massaging for 30 seconds, withdrawing, and repeating the process one more time. This concentrated aliquot was withdrawn, retained, kept separately, and was designated as the first fraction of BAL fluid (BALF). The following aliquots were 6 mL in volume, instilled once with light massaging, withdrawn, and combined until a 30-mL volume was obtained. For each animal, both lavage fractions were centrifuged (10 minutes, 1,600 rpm), the cell pellets were combined and resuspended in 1 mL of PBS, and the acellular fluid from the first fraction was retained for further analysis.

BALF analysis: LDH activity, albumin, and cytokines. The presence of albumin and LDH activity in the BALF of rats exposed to air or COREXIT was measured to evaluate the loss of integrity of the alveolar–capillary barrier and general cytotoxicity, respectively. Measurements of both albumin and LDH activity in the acellular fluid were obtained using the Cobas Mira analyzer (Roche Diagnostic Systems). LDH activity was quantified by detection of the oxidation of lactate coupled to the reduction of NAD⁺ at 340 nm. Albumin was determined by spectrophotometric measurement of the reaction product of albumin with bromocresol green according to a method by Sigma Diagnostics. Inflammatory cytokines, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-2, interleukin (IL)-6, and IL-10 were measured in BALF using commercially available ELISA kits.

Cell differentials: alveolar macrophages (AM), polymorphonuclear (PMN) cells, lymphocytes, and eosinophils.

The total numbers of BAL cells collected from rats exposed to air or COREXIT were counted using Coulter Multisizer II (Coulter Electronics). Cell differentials were performed to determine the total number of AM, PMN, lymphocytes, and eosinophils. Briefly, 10⁵ cells from each rat were spun down onto slides with Cytospin 3 centrifuge (Shandon Life Sciences International) and labeled with Leukostat stain (Fisher Scientific) to differentiate cell types. In all, 200 cells per slide were counted, and the percentages of AM, PMN, lymphocytes, and eosinophils were multiplied by the total number of cells to calculate the total number of each cell type.

Chemiluminescence (CL). To estimate lung phagocyte oxidant production, luminol-dependent CL was performed on BAL cells as a measure of the light generated by the production of reactive oxygen species (ROS) by AM and PMN using Berthold LB 953 luminometer (Wallace Inc.) as described previously.³² Baseline oxidant production by the cells was measured in the absence of a stimulant. Phorbol 12-myristate 13-acetate (PMA) (10 M), a soluble stimulant of total BAL phagocytes (AM and PMN), or nonopsonized, insoluble zymosan (2 mg/mL), a stimulant of AM only,³³ was added to the assay immediately prior to CL measurement to determine the contribution of both AM and PMN to the overall production of ROS in the lungs of the rats. Measurement of CL was recorded for 15 minutes at 37 °C, and the integral of counts per minute (cpm) per 10⁶ cells versus time was calculated. CL was calculated as the cpm of the stimulated cells minus

the cpm of the corresponding resting cells, and the value was normalized to total number of BAL cells for PMA-stimulated CL and total number of AM in the BAL for zymosan-stimulated CL.

Cardiovascular responses. In all, one and seven days following COREXIT exposure, a set of rats was evaluated for changes in HR and blood pressure (BP) measured as MAP and LVSP. In a separate set of rats, ventral tail arteries were dissected, and vascular tone and responsiveness to vasoactive agents were evaluated *ex vivo*.

In vivo hemodynamic measurements. Rats ($n = 5\text{--}8/\text{group}/\text{time point}$) were anesthetized with inhaled 2% isoflurane mixed with oxygen at a flow rate of 2 L/minute. Using aseptic technique, a custom catheter made according to the method described by Wang et al.³⁴ was inserted into the left ventricle through the carotid artery. The correct position of the catheter tip in the left ventricle was confirmed by the waveform of left ventricular pressure visualized on a computer monitor. Systemic arterial BP was determined using a fluid-filled arterial catheter that was placed in the femoral artery and connected to a pressure transducer coupled to a computerized cardiovascular continuous monitoring system (PowerLab/4SP analog-to-digital converter; ADInstruments). Another catheter (polyurethane, 2 French size) was inserted into the jugular vein for the administration of ISO or NE. All three catheters were exteriorized through subcutaneous tunneling and were sutured on the back. Left ventricular function and BP were measured in unrestrained, conscious rats while ambulating within a small cage. The arterial catheters were connected to a fluid-filled pressure transducer for at least 20 minutes prior to measurements being made (or until rats displayed stable HR and MAP). HR, MAP, and LVSP were recorded and analyzed using cardiovascular continuous monitoring software (PowerLab/4SP; ADInstruments).

Microvascular measurements. Tails were dissected from rats ($n = 8\text{ rats/group/time point}$) after euthanasia and placed in cold DMEM with glucose. Ventral tail arteries from the C14–C15 region of the tail were dissected, mounted on glass pipettes in a microvessel chamber (Living Systems), and perfused with biocarbonated-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer warmed to 37 °C. Arteries were pressurized to 60 mmHg and allowed to equilibrate for approximately one hour. After one hour, the chamber buffer was changed and the pressure was lowered to 10 mmHg. Pressure was gradually increased (5 mmHg every 10 seconds until reaching a final pressure of 110 mmHg), and changes in the internal diameter of the artery were recorded. Arteries were repressurized to 60 mmHg and allowed to stabilize, and then responsiveness to PHEN, an α -1 adrenoreceptor agonist that mediates vasoconstriction, and ACh, which mediates redilation, was assessed. PHEN was applied to the chamber in half-log increments (-8.5 to -6 M), and the internal diameter was recorded after vessels stabilized (approximately five minutes between doses). Because ventral tail arteries usually



display little basal tone, endothelial-mediated redilation was assessed after PHEN-induced constriction by adding ACh in half-log increments (-8 to -5 M).

Systemic immune responses. Two sets of studies were conducted to assess immunotoxicity at one and seven days following the last exposure. Blood cell differentials and splenocyte phenotyping were performed using the set of rats that were employed to study lung injury and inflammation in order to characterize systemic inflammatory and immune responses. In a separate set of rats, IgM response to a T-cell-dependent antigen, SRBC, was evaluated as an index of humoral immune status.

Blood cell differentials. Blood samples were taken from anesthetized rats prior to BAL. Hematology was performed using a Hemavet 950 veterinary multi-species hematology system (Drew Scientific Group). White blood cells were counted and differentiated (PMN, lymphocytes, monocytes, and eosinophils). Additional analysis included erythrocyte counts, platelet counts, hematocrit content, hemoglobin levels, mean corpuscular hemoglobin and hemoglobin concentration, mean corpuscular volume, mean platelet volume, and platelet distribution width.

Splenocyte immune phenotyping. Single-cell suspensions of rat spleens, removed from the same set of rats that were used for blood cell differentials, were prepared by disruption of tissue between the frosted ends of microscopic slides, and spleens were prepared by processing tissue through $70\text{ }\mu\text{M}$ nylon mesh screens in PBS. Cells were counted on Coulter Counter Z1 following lysis of red blood cells with Zap-oglobin. Cells (10^6) were resuspended in PBS containing 1% bovine serum albumin and 0.1% sodium azide (FACS buffer). To prevent non-specific binding to Fc receptors, the cells were incubated with anti-rat CD32 (clone D34-485) in FACS buffer for 10 minutes on ice. Cells were then centrifuged and resuspended in FACS buffer containing fluorescently conjugated monoclonal antibodies, including anti-rat CD45RA-PE, clone OX-33; CD3-FITC, clone G4.18; CD4-APC, clone OX-35; CD8-PerCP, clone OX-8; and CD11b-V450, clone WT.5. Samples were also stained with biotin-labeled anti-CD161a, clone 10/78. The cells were protected from light and incubated on ice for 20 minutes. Samples stained with biotin-labeled anti-CD161a (1:100 dilutions in flow staining buffer) were resuspended in flow cytometer staining buffer containing streptavidin APC/Cy-7, incubated for an additional 30 minutes on ice in the dark, and washed. The cells were then washed and resuspended in BD Cytofix buffer and incubated for 20 minutes on ice. The cells were also then washed and resuspended in 0.3 mL of FACS buffer and analyzed on LSR II flow cytometer (BD Biosciences). Cells were discriminated by forward and side light scatter, and doublets were excluded by side scatter area versus side scatter height. Lymphocyte populations were identified by their phenotype as follows: B-cells (CD45A+ CD3-negative), CD4 T-cells (CD3+CD4+), CD8 T-cells (CD3+CD8+), macrophages (CD11b+), and natural killer cells (CD161a+).

IgM response to T-cell-dependent antigen. To assess alterations in humoral immunity, a separate study was conducted in

which rats were immunized with SRBC (2×10^8 SRBC/rat) in a 1 mL volume by intravenous injection five days prior to the one- or seven-day post COREXIT exposure. The primary IgM response to SRBC was enumerated using a modified hemolytic plaque assay of Jerne and Nordin³⁵ after intravenous challenge with SRBC. The spleen plaque forming cell (PFC) assay was performed as previously described.³⁶ Briefly, splenocytes were isolated and counted on a Coulter counter as described above. Spleen cells were then diluted and added to a warm agar/dextran mixture, followed by further dilution into lyophilized guinea-pig complement (Cedarlane Labs). The mixture was then poured into a petri dish, coverslipped, and incubated for three hours at 37°C . The plaques, representing antibody-forming B-cells, were counted. The results were expressed as specific activity (IgM PFC per 10^6 spleen cells) and total activity (IgM PFC per spleen). Sera collected from the same animals and evaluated for the PFC response were used to determine the serum titers of the primary IgM response to SRBC. Serum samples were analyzed for anti-SRBC IgM using ELISA kits according to manufacturer recommendations as previously described.³²

Statistical analysis. Results are expressed as means $\pm$ standard error of measurements (SEMs). Statistical analyses for systemic immune responses and lung inflammation and injury toxicity data were carried out using SigmaStat 11.0 statistical program. The significance of the interaction among different treatment groups for the different parameters at each time point was assessed using analysis of variance (ANOVA). The significance of difference between individual groups was analyzed using the Student-Newman-Keuls post-hoc test with the significance set at $P < 0.05$. The analysis of COREXIT effects on basal R_L and C_{Dyn} values was compared using Student's paired and nonpaired t -tests, as appropriate. The analysis of COREXIT effects of MCh on R_L and C_{Dyn} , ISO and NE on hemodynamic parameters, and PHEN and ACh on vascular responsiveness was generated using SAS/STAT software, Version 9.1, of the SAS system for Windows (SAS Institute). PROC MIXED was utilized to run a two-way factorial ANOVA with concentration of MCh treated as a repeated measure to account for multiple measures in individual animals. Treatment comparisons were then calculated at each level of MCh utilizing the slice option. All differences were considered significant at $P < 0.05$. n is the number of separate experiments.

Results

Inhalation exposures. Six sets of exposures were conducted at five hours per day for nine work days. Chamber conditions during all exposures averaged temperatures of $71\text{--}72^\circ\text{F}$ and relative humidity of 68–71%. The average chamber concentration of COREXIT particulate remained consistent across sets of exposures and ranged from 14.92 to 15.07 mg/m^3 . The average total concentration (particulate + vapor) for each exposure was 25.21 mg/m^3 (range: 23.29–28.4 mg/m^3).

Particle size analysis from the SMPS and APS indicated that the aerosol had a mass median aerodynamic diameter of 655 nm with a geometric standard deviation of 1.7.

Lung function, inflammation, and injury. Repeated exposure to COREXIT did not result in alterations to baseline values of R_L or C_{dyn} (Fig. 1A and B), nor were any significant changes observed in responses to inhaled MCh at any time point (Fig. 1C and D). LDH activity and albumin levels (Fig. 2) were reduced when compared to air-treated rats one day following the last COREXIT exposure; however, there were no significant differences by seven days following exposure. Increases in the inflammatory cytokines, MCP-1, MIP-2, and IL-6 were observed one day following the inhalation period, however, the increases were not significant and there were no differences in cytokine levels between treated and air control animals by day 7 (Table 1). Cells recovered from the lungs of rats exposed to COREXIT did not differ significantly from those of air controls (Fig. 3). AM accounted for the majority of cells infiltrating the lungs following exposure to COREXIT or air (Fig. 3A). Approximately twice as many PMN were present in the lungs of rats exposed to COREXIT one day following the last exposure (Fig. 3B); however, similar to the cytokine pattern, the difference was not significant when compared to controls. There were also no significant

differences in the number of lymphocytes in the lungs of COREXIT-exposed rats when compared to those of air-treated controls (Fig. 3C). There was a nonsignificant increase (twofold) in ex vivo oxidant production by total phagocytes (AM and PMN) from COREXIT-exposed rats as indicated by PMA-stimulated CL (Fig. 4A); however, there were no significant differences in either PMA- or zymosan-stimulated oxidant production (Fig. 4) by phagocytes recovered from rats treated with COREXIT when compared to controls.

Cardiovascular and peripheral vascular functions. BP, MAP, and LVSP, both basal and in response to stimulation by NE and ISO, respectively, were not significantly altered following repeated exposure to COREXIT when compared to air controls (Fig. 5B and C). However, basal HR and HR responses to ISO were significantly increased one day following the last COREXIT exposure (Fig. 5A). By seven days post exposure, HR had returned to control levels and there were no significant differences in chronotropic responses upon stimulation with ISO when compared to air-exposed rats. There were no significant differences in peripheral vascular responsiveness in rats following repeated exposure to COREXIT when compared to air controls. The baseline internal diameter of the ventral tail artery (Fig. 6A) and the dose-dependent

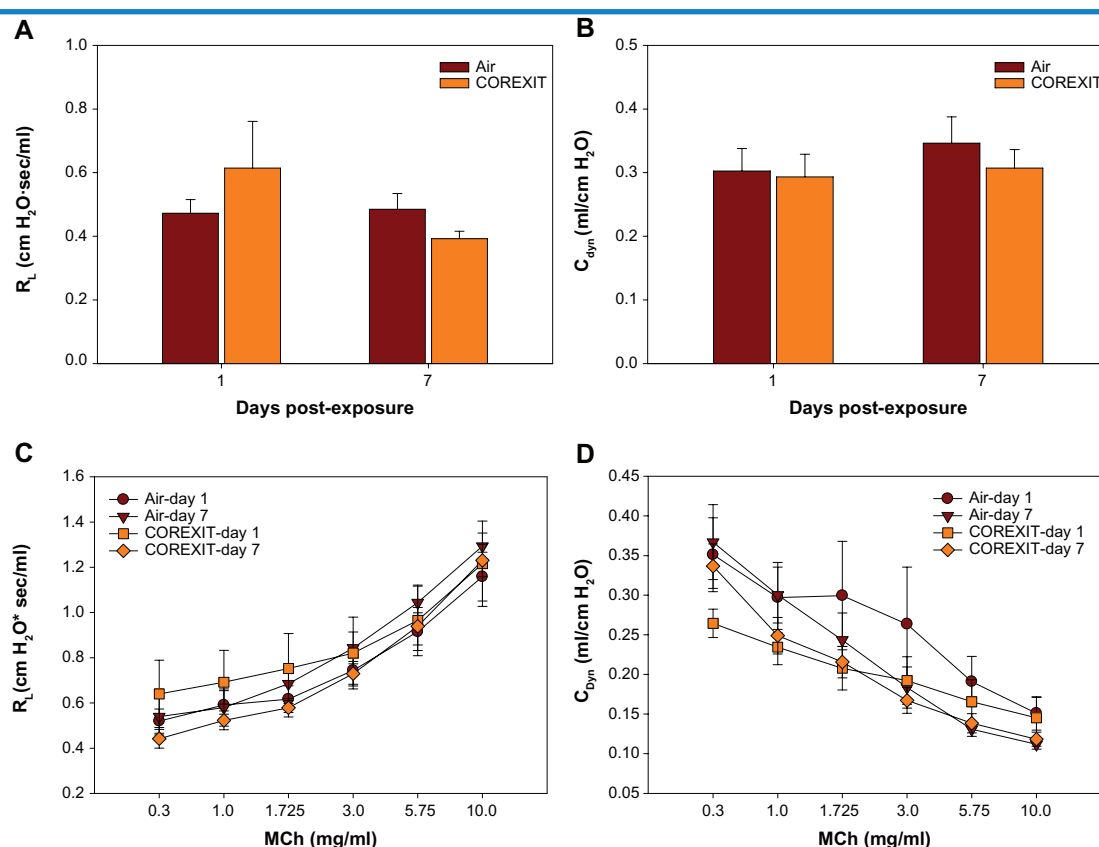


Figure 1. Airway resistance (R_L) and dynamic compliance (C_{dyn}) as measures of lung function and airway reactivity. Basal values of R_L (A) and C_{dyn} (B), and MCh challenge-induced alterations to R_L (C) and C_{dyn} (D) one and seven days post exposure to air or COREXIT (27 mg/m³ for five hours per day for nine work days).

Note: Values are means $\pm$ SEMs; $n = 7-8$ /group/time point.

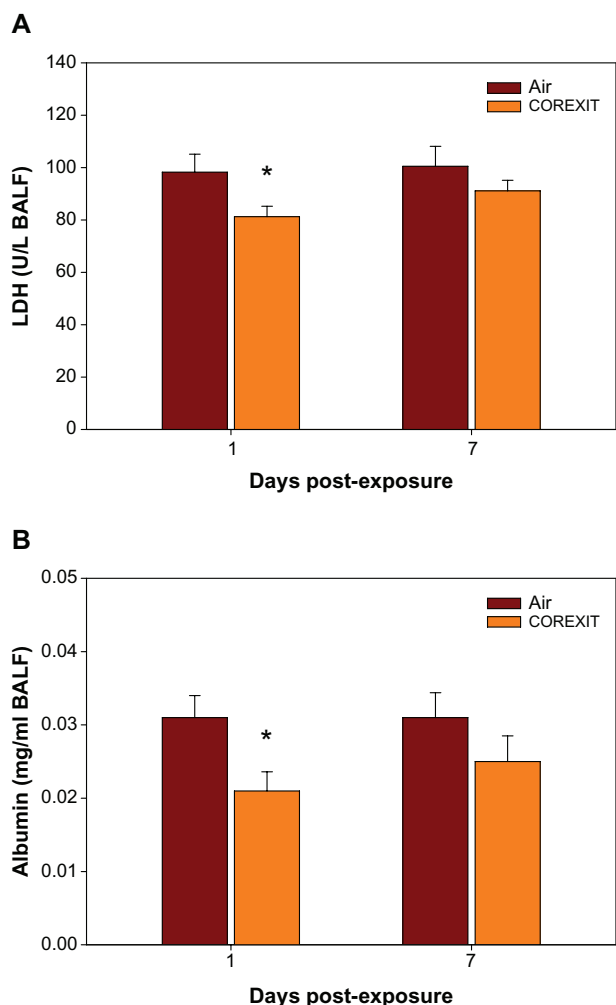


Figure 2. Lung injury. LDH activity (A) and albumin (B), indicators of cytotoxicity and damage to the alveolar air–capillary barrier, respectively, in BALF recovered from rats one and seven days post exposure to air or COREXIT (27 mg/m³ for five hours per day for nine work days).

Notes: Values are means $\pm$ SEMs. *indicates significantly different from air control ($P < 0.05$); $n = 6$ /group/time point.

redilation of the artery to ACh after PHEN-induced vasoconstriction were similar in all groups (Fig. 6B).

Systemic immune responses. Although there was a nonsignificant increase in the neutrophil and monocyte populations (6% and 1.7%, respectively, when compared to control) and a decrease in the lymphocyte population (7.5% compared to control) in the COREXIT-exposed rats on day 7, the differences in the hematology parameters on both day 1 and day 7 were not significant (data not shown). In addition, there were no significant differences in splenocyte populations of lymphocytes, macrophages, and natural killer cells (Table 2). To assess whether exposure to COREXIT was immunosuppressive, the serum and spleen IgM responses following immunization to the T-cell-dependent antigen, SRBC, and spleen cellularity were analyzed. There were no significant differences in the total or specific number of antibody-producing cells in the spleen or serum following

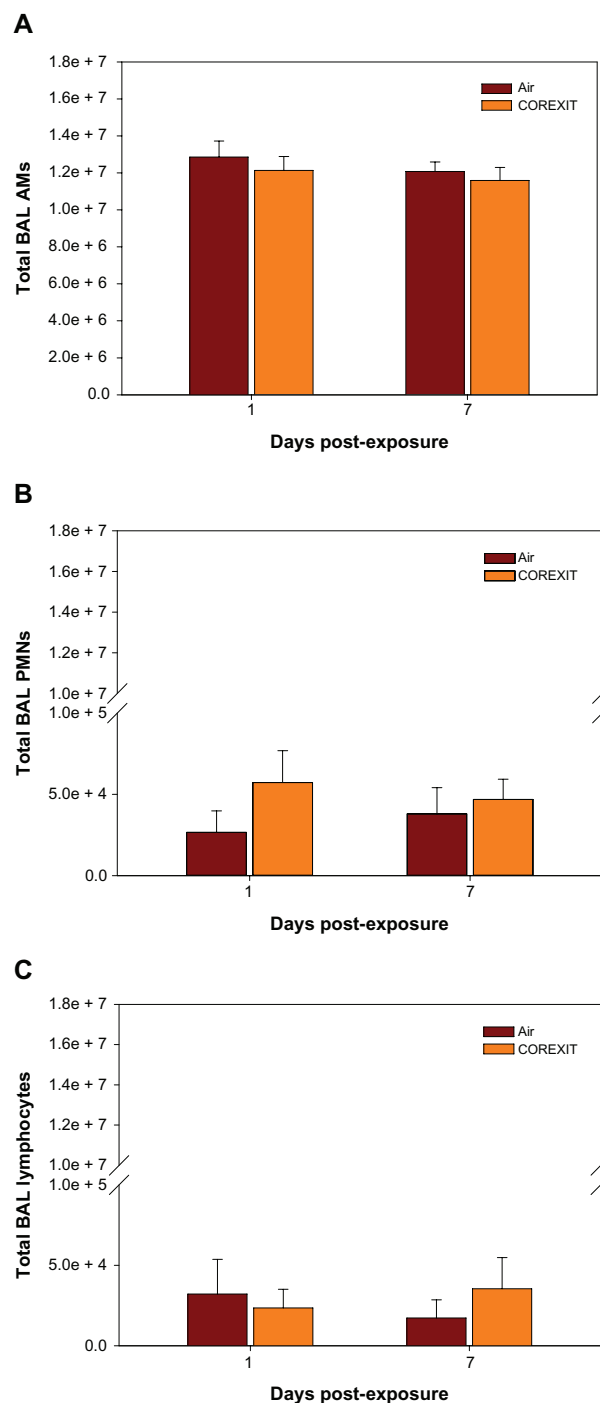


Figure 3. Cellular influx into the lung as an index of lung inflammation. Total AM (A), PMN cells (B), and lymphocytes (C) recovered by BAL from rats one and seven days post exposure to air or COREXIT (27 mg/m³ for five hours per day for nine work days).

Note: Values are means $\pm$ SEMs; $n = 6$ /group/time point.

SRBC immunization (Table 3), indicating that the COREXIT exposure was not immunosuppressive.

Discussion

To date, there has been very little information available related to worker health effects following the Deepwater Horizon oil

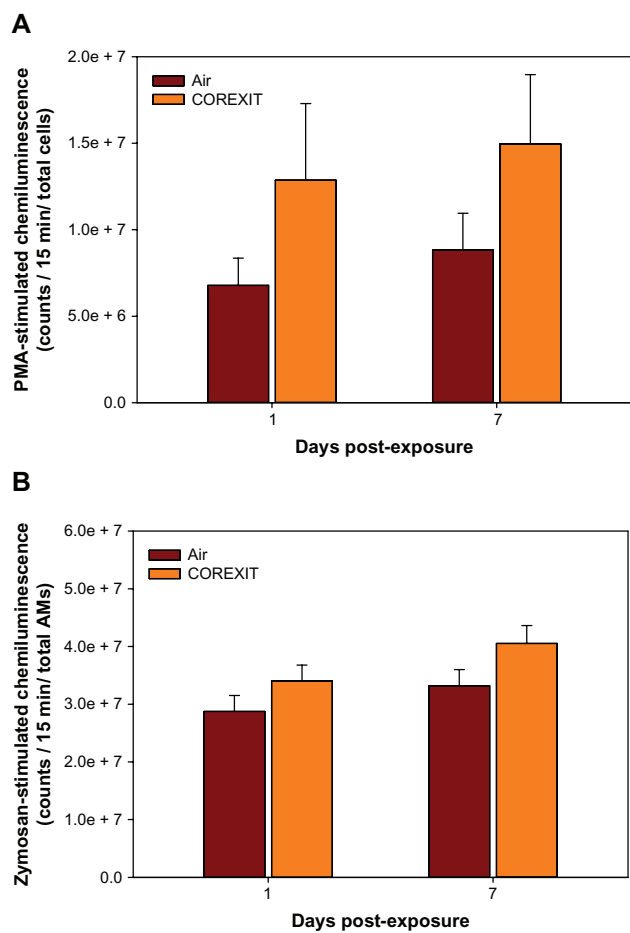


Figure 4. Oxidant production by lung phagocytes ex vivo. PMA-stimulated CL by total phagocytes (AM + PMN) (A) and zymosan-stimulated CL by total AM (B) recovered by BAL from rats one and seven days post exposure to air or COREXIT (27 mg/m³ for five hours per day for nine work days).

Note: Values are means $\pm$ SEMs; $n = 6$ /group/time point.

spill, and there is no information specifically related to the consequences of dispersant exposure. There are currently three studies describing the initiatives undertaken to evaluate response workers' health. Approximately 33,000 workers joined the GuLF Study.¹⁰ There are several reports available on the website about GuLF Study relating primarily to worker demographics, work history and past occupational exposures, health and lifestyle, and the sites at which the workers worked in the Gulf of Mexico. Health exams are currently underway. More recently, a study by D'Andrea and Reddy³⁷ examined hematological and hepatic alterations, as well as somatic symptoms, in exposed (cleanup workers active on the coast of Louisiana) and nonexposed (people residing at least 100 miles from the coast) groups. The investigators found that in cleanup workers, platelet counts were decreased, hemoglobin and hematocrit values were elevated, serum creatinine levels were elevated, and blood urea nitrogen levels were decreased. Hepatic toxicity markers in blood (alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase) were also elevated. In addition, somatic symptoms, includ-

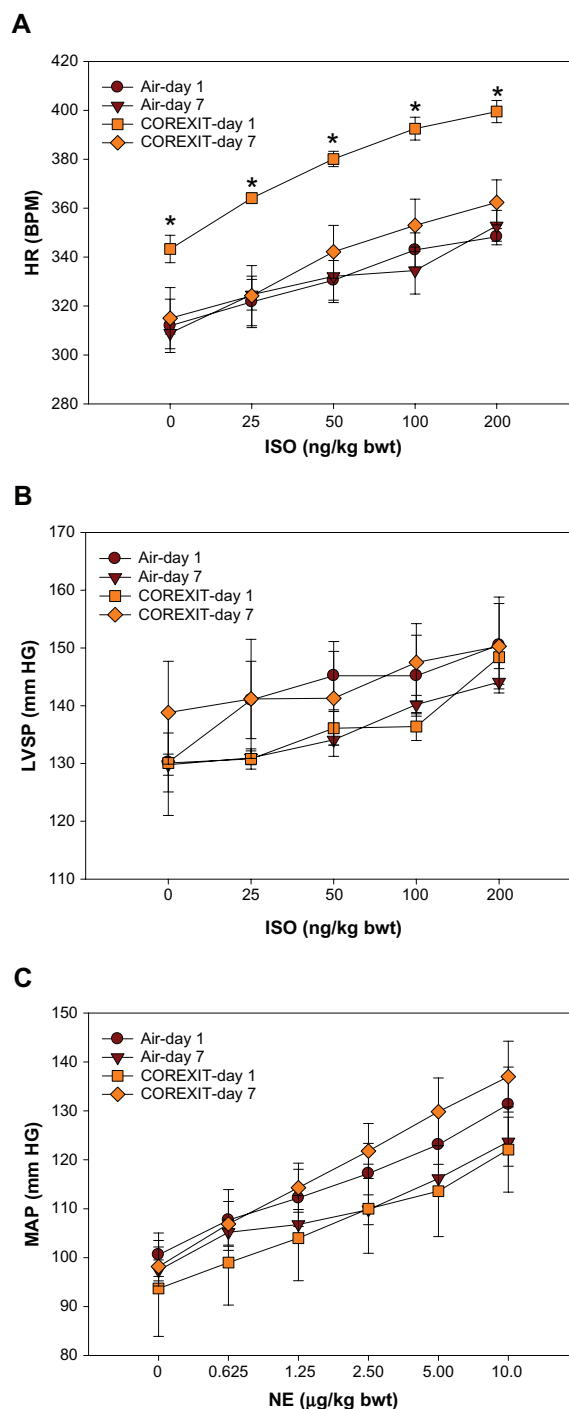


Figure 5. HR, MAP, and LVSP as hemodynamic measures. Alterations in HR (A) and LVSP (B) in response to increasing doses of ISO, and changes in MAP in response to increasing doses of NE in rats one and seven days post exposure to air or COREXIT (27 mg/m³ for five hours per day for nine work days).

Notes: Values are means $\pm$ SEMs. *indicates significantly different from air control ($P < 0.05$); $n = 5$ –8/group/time point.

ing headaches, skin irritation, respiratory and gastrointestinal complaints, vision impairment, pain, and fatigue, were documented. The study also accounted for differences in age and sex of exposed workers; however, correlations to specific chemical exposures could not be ascertained.

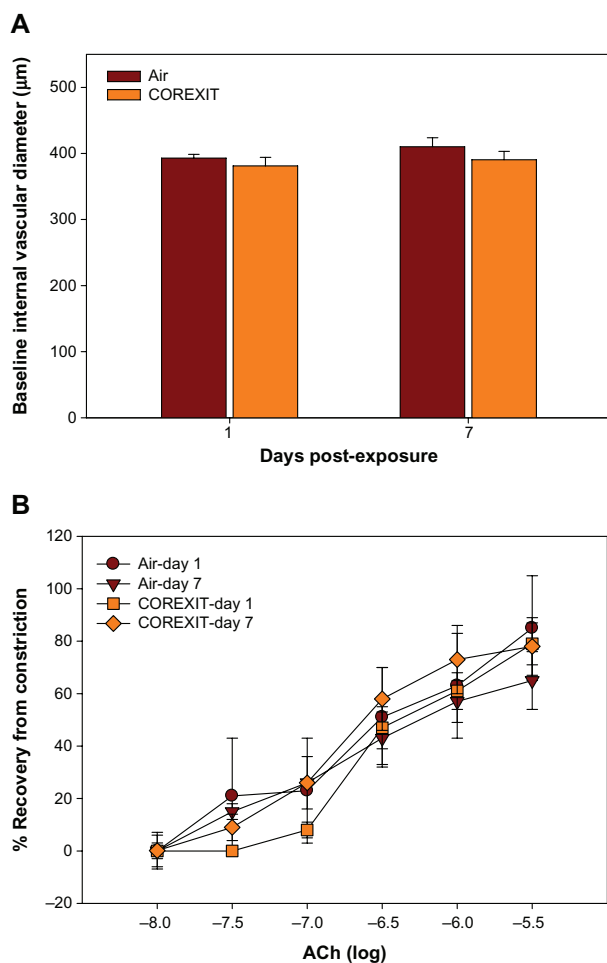


Figure 6. Vascular responsiveness. Baseline internal vascular diameter of the ventral tail artery (**A**) and ACh-induced recovery (vasodilation) following PHEN-induced vasoconstriction (**B**) in ventral tail arteries dissected from rats one and seven days post exposure to air or COREXIT (27 mg/m³ for five hours per day for nine work days).

Note: Values are means $\pm$ SEMs; $n = 8$ /group/time point.

During the spill, two health hazard evaluation (HHE) investigations were performed by NIOSH.² The goal of these investigations was to assess whether acute health effects occurred in workers during response operations. The first was initiated in response to the hospitalization of seven fishermen in the Vessels of Opportunity (VoO) program following booming and skimming operations. The investigation was expanded to include all offshore operations, including application of disper-

sant. The second investigation focused on onshore operations. Investigations included interviews, health symptom surveys, and industrial hygiene assessments of specific work activities (oil dispersant release, in situ oil burning, oil booming/skimming/vacuuming, activities at the oil source, wildlife cleanup, beach cleanup, and decontamination/waste management). Two vessels were monitored during oil dispersant release in an area where additional aerial release was also occurring. Levels of volatile organic compounds, propylene glycol, benzene soluble fraction, diesel exhaust, mercury, carbon monoxide, hydrogen sulfide, and total particulate matter in the personal breathing zone were either not detected or were below occupational exposure limits. Health symptom surveys reported a range of respiratory, musculoskeletal, gastrointestinal, and psychological symptoms. Infirmiry logs at Deepwater Horizon Venice, Louisiana, Branch Infirmiry indicated worker reports of ear, nose, throat, and respiratory complaints; heat-related disorders; and nonspecific symptoms including eye and respiratory irritation, headache, cramps, and fatigue. The seven fishermen hospitalized on the same day reported headache, upper respiratory congestion and irritation, and nausea; however, these men were not working in areas where dispersant was deployed and symptoms could not be related back to a specific chemical. Additional hospitalizations were related to varying degrees of heat-related illnesses and possible chemical exposures; however, data reported at the time of hospitalization did not allow for direct correlations of symptoms to specific oil or chemical exposures.

From the current data, health effects that may have been specific to exposures to oil alone, COREXIT and oil mixtures, or COREXIT alone cannot be discerned. In response to the need to address potential COREXIT-related toxicity in exposure workers, several mammalian toxicity studies have been conducted, including *in vitro* studies on human cell cultures.^{38–42} Additionally, a battery of *in vivo* studies in rodents^{22–25} were conducted at NIOSH. In the *in vitro* study that compared effects of three different formulations of COREXIT (9580A, 9500A, and 9527) on bronchial-epithelial cells (BEAS-2B cells),⁴² COREXIT 9527 was found to be the most cytotoxic of the three and 9580A was the least cytotoxic. Both 9527 and 9500A caused increased ROS production, and only 9527 induced markers suggestive of apoptosis. Judson et al.³⁸ and Bandele et al.³⁹ examined the

Table 1. Inflammatory cytokines in BALF following exposure to COREXIT EC9500A.

TREATMENT GROUP	MCP-1	MIP-2	IL-6	IL-10
Air-day 1	40.4 $\pm$ 9.81	676 $\pm$ 60.8	156 $\pm$ 60.2	15.3 $\pm$ 6.34
COREXIT-day 1	62.0 $\pm$ 12.6	739 $\pm$ 46.0	248 $\pm$ 26.1	15.1 $\pm$ 7.68
Air-day 7	38.7 $\pm$ 6.48	639 $\pm$ 44.4	243 $\pm$ 40.3	48.2 $\pm$ 26.4
COREXIT-day 7	31.0 $\pm$ 3.11	623 $\pm$ 42.1	244 $\pm$ 71.2	15.1 $\pm$ 6.36

Note: Values represent means $\pm$ SEMs.

Abbreviations: MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; IL, interleukin.

**Table 2.** Splenocyte immune phenotyping in rats after inhalation exposure to COREXIT EC9500A.

CELL PHENOTYPE	AIR-DAY 1	COREXIT-DAY 1	AIR-DAY 7	COREXIT -DAY 7
B-cells Total (cells x 10 ⁷) % splenocytes	19.2 ± 0.74 43 ± 2	22.1 ± 0.17 46 ± 3	20.2 ± 0.74 54 ± 1	20.9 ± 0.74 58 ± 2
T-cells Total (cells x 10 ⁷) % splenocytes	15.7 ± 0.19 40 ± 3	19.5 ± 0.92 37 ± 3	17.9 ± 0.13 40 ± 1	13.9 ± 0.17 36 ± 3
CD4 T-cells Total (cells x 10 ⁷) % splenocytes	9.5 ± 0.1 24 ± 0.9	10.2 ± 0.43 23 ± 0.6	11.4 ± 0.74 20 ± 0.8	9.3 ± 0.92 22 ± 1
CD8 T-cells Total (cells x 10 ⁷) % splenocytes	4.4 ± 0.81 10 ± 0.8	5.2 ± 0.50 8 ± 0.6	3.6 ± 0.64 11 ± 1	5.2 ± 0.79 9 ± 1
Macrophages Total (cells x 10 ⁷) % splenocytes	0.88 ± 0.78 2.3 ± 0.27	0.58 ± 0.95 1.8 ± 0.17	1.09 ± 0.55 2.6 ± 0.2	1.05 ± 0.67 2.4 ± 0.2
Natural Killer cells Total (cells x 10 ⁷) % splenocytes	0.58 ± 0.08 1.5 ± 0.04	0.69 ± 0.09 1.1 ± 0.08	0.48 ± 0.06 1.4 ± 0.08	0.61 ± 0.07 1.3 ± 0.01

Note: Values represent means ± SEMs derived from the total number of cells counted in each group and are expressed as the percentage of total spleen cells and the absolute cell number per spleen.

effects of various dispersants in human liver cell lines. Among the eight dispersants examined by Judson et al.³⁸, COREXIT 9500A, along with five other dispersants, had an LC50 of 100 ppm, and did not produce significant effects on androgen or estrogen receptor activity. Bandele et al.³⁹ showed that COREXIT 9500A and 9527 did not differ in their cytotoxicity effects, and they induced oxidant production by hepatocytes, in part because of the DSS component of the mixtures. In a study by Zheng et al.⁴¹, the effects of COREXIT 9500A were examined on skin, neural, glial, and kidney cells. Skin was the most sensitive to cytotoxic effects, followed by glial, neuronal, and then kidney cells. The mechanisms of toxicity were also correlated to oxidative stress responses in this study.

As mentioned above, a battery of *in vivo* studies was performed to assess potential adverse effects associated with dermal and inhalation exposures to COREXIT. Anderson et al.²² evaluated the immunotoxicological responses following dermal exposure to COREXIT EC9500A and its active ingredient, DSS, in mice. Mice were exposed on the surface of the ear to COREXIT EC9500A or DSS at concentrations of 12–100% in propylene glycol (vehicle) or vehicle alone. A series of tests

evaluating hypersensitivity (local lymph node assay, mouse ear swelling test, serum immunoglobulin E (IgE) response, and lymphocyte proliferation and phenotype differentiation) and immune suppression (IgM response to T-cell antigen) were conducted. COREXIT and DSS induced dermal irritation and lymphocyte proliferation, and ear swelling, with COREXIT being classified as a potent sensitizer and DSS as a moderate sensitizer, possibly because of T-cell-mediated mechanisms. COREXIT did not cause immune suppression following dermal exposure.

To date, the only *in vivo* inhalation studies examining potential pulmonary and extrapulmonary responses following inhalation exposure to COREXIT EC9500A were those conducted on rats at NIOSH.^{23–25} These inhalation studies have shown that an acute pulmonary exposure to COREXIT EC9500A, 27 mg/m³ for five hours, resulted in a transient decrease in lung function one day following exposure, but did not produce significant lung inflammation.²³ Basal values of HR, BP, and vascular tone were not altered by the exposure; however, transient increases in agonist-induced HR and BP, as well as reduced vascular responsiveness to stimulation with an agonist were observed at one

Table 3. Effect of COREXIT EC9500A on IgM response to SRBC in rats.

	AIR-DAY 1	COREXIT-DAY 1	AIR-DAY 7	COREXIT -DAY 7
Serum IgM (ng/ml)	8169 ± 1626	7602 ± 2299	10050 ± 2288	8598 ± 2586
PFC/spleen	347 ± 55	375 ± 53	360 ± 85	427 ± 91
PFC/10 ⁶ splenocytes	1421 ± 170	1670 ± 258	1647 ± 380	1802 ± 416
Spleen cellularity (× 10 ⁶)	5.76 ± 0.30	5.65 ± 0.13	5.34 ± 0.14	6.11 ± 0.17

Note: Values represent means ± SEMs.

Abbreviation: PFCs, Plaque forming cells.



day post exposure.²⁴ There were no residual pulmonary or cardiovascular alterations by one week following exposure. Neurotoxicological findings included alterations in proteins related to olfactory signal transduction, axonal function, and synaptic vesicle fusion.²⁵

In the present study, the potential effects of an acute repeated exposure to COREXIT alone were evaluated, and were designed as a follow-up to the acute single exposure model, utilizing the same inhalation exposure system²¹ and concentration of COREXIT EC9500A®, ~25 mg/m³ for five hours per day for nine work days. These studies showed that a short-term repeated exposure to COREXIT did not significantly alter lung function, indicated by no observed alterations in baseline R_L and C_{dyn} , and no differences in reactivity following inhaled MCh. Although there was a trend for an increase in PMN and inflammatory cytokines in the lung at one day following the last exposure, and a trend for an increase in oxidant production by total phagocytes recovered from the lung at one and seven days post exposure, COREXIT did not elicit significant lung inflammation. The significant decrease in baseline lung injury markers in BALF on day 1 following exposure was a trend that was also present in the acute COREXIT exposure study.²³ The reasons for this finding are unclear. It is possible that the volume of COREXIT inhaled contributed to a fraction of the volume of fluid recovered by BAL on day before being cleared to a greater degree at the later time point, thereby decreasing baseline LDH and albumin levels in the fraction of BAL recovered. By day 7, the response had returned to baseline. Despite the lack of a significant inflammatory response in the lung, there is still a possibility that changes in the lung at the molecular level may occur following COREXIT exposure, which may lead to responses at later time points following exposure; however, this was not investigated in the current study. Overall, there was no evidence that the acute repeated exposure to COREXIT caused significant lung toxicity.

In the earlier COREXIT skin exposure study discussed above,²² COREXIT was identified as a dermal irritant and sensitizer in the murine local lymph node assay, possibly through a T-cell-mediated mechanism. However, following the acute repeated respiratory exposure in the present study, there were no significant increases in splenic lymphocyte populations or in circulating white blood cell counts, indicating that COREXIT had little effect on the immune system following the inhalation route of exposure. Consistent with the dermal exposure study, inhalation of COREXIT also did not alter IgM response to T-cell antigen (SRBC), suggesting that COREXIT exposure does not cause immune suppression. These studies taken together, along with those of Zheng et al.⁴¹ *in vitro*, suggest that skin may be more sensitive to COREXIT than other organ systems.

An important finding observed in this study was the increase in baseline HR one day following the repeated exposure and the HR increase in response to stimulation with

an agonist. In the previous study,²³ an increase in HR was also observed, but only at the highest concentration of agonist one day after the acute exposure (five hours). These findings suggest a dose–response effect of COREXIT on HR. Conversely, the acute five-hour exposure to COREXIT caused an increase in BP response to NE at one day post exposure. Concomitantly, a decreased vascular responsiveness to the highest dose of ACh occurred, whereas there was no effect of COREXIT on BP or vascular responsiveness in the current study. The mechanisms involved in these differing effects were not investigated. It is possible that the acute effects that were observed in relation to BP and vascular tone were responses to the initial insult of an inhalant, and were not observed after repeated exposure because of physiological feedback mechanisms in the periphery or in the brain regulating BP and tone. In addition, clearance of the material from the lung has begun during the nine-day exposure period, and once this process and the signal cascade related to it have been activated, responses to the initial deposition of a foreign material into the lung may have attenuated. In both the single acute and acute repeated studies, however, the effects of COREXIT resolved by one-week post exposure, indicating the effects of COREXIT on cardiovascular function were transient.

In conclusion, acute repeated inhalation exposure in rats to 25 mg/m³ of COREXIT EC9500A® for five hours per day for nine work days did not significantly alter pulmonary function and inflammation, immune function, or vascular tone, but did cause transient effects on cardiac function. In addition, components of COREXIT may cause adverse neurological effects following respiratory exposures.²⁵ Questions and concerns still remain regarding the possible effects of longer exposures to dispersant, and there is controversy as to the toxicity of exposures to combinations of oil and dispersant. Further studies are needed to better address the health effects related to these concerns.

Author Contributions

Conceived and designed the experiments: JRR, SEA, HK, KK, JAT, AK, WTG, WM, DGF, MJ, JSF. Analyzed the data: JRR, SEA, HK, KK, JAT, AK, WTG, WM, DGF, MJ, JSF. Wrote the first draft of the manuscript: JRR. Contributed to the writing of the manuscript: JRR, SEA, HK, KK, JSF. Agreed with manuscript results and conclusions: JRR, SEA, HK, KK, JAT, AK, WTG, WM, DGF, MJ, JSF. Jointly developed the structure and arguments for the paper: JRR, SEA, HK, KK, JSF. Made critical revisions and approved the final version: JRR, SEA, HK, KK, JAT, AK, WTG, WM, DGF, MJ, JSF. All authors reviewed and approved the final manuscript.

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