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Effects of corexit oil dispersants and the WAF of dispersed oil on DNA damage and repair in cultured human bronchial airway cells, BEAS-2B

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Abstract

Large quantities of dispersants were used as a method to disperse the roughly 210 million gallons of spilled crude oil that consumed the Gulf of Mexico. Little is known if the oil-dispersant and oil-dispersant mixtures on human airway BEAS-2B epithelial cells. Here we present the cytotoxic and genotoxic *in vitro* effects on the human lung cells BEAS-2B following exposure to and oil-dispersant mixtures on human airway BEAS-2B epithelial cells. Here we present the cytotoxic and genotoxic *in vitro* effects on the human lung cells BEAS-2B following exposure to Corexit dispersants EC9500 and EC9527, Water Accommodated Fraction (WAF) -crude, WAF-9500 + Oil, and WAF-9527 + Oil. Cellular cytotoxicity to WAF-dispersed oil samples was observed at concentrations greater than 1000 ppm with over 70% of observed cellular death. At low concentration exposures (100 and 300 ppm) DNA damage was evidenced by the detection of single strand breaks (SSBs) and double strand breaks (DSBs) as measured by alkaline and neutral comet assay analyses. Immunoblot analyses of the phosphorylated histone H2A.X (γ-H2A.X) and tumor suppressor p53 protein confirmed activation of the DNA damage response due to the exposure-induced DNA breaks. Although, many xenobiotics interfere with DNA repair pathways, *in vitro* evaluation of the nucleotide excision repair (NER) and DSB repair pathways appear to be unaffected by the oil-dispersant mixtures tested. Overall, this study supports that oil-dispersant mixtures induce genotoxic effects in culture.

Keywords

DNA damage; DNA repair; Double strand breaks (DSBs); Lung epithelial cells; Oil-dispersant mixtures

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Declaration of interest

No.

1. Introduction

Over an 87 day period, an estimated 4.9 million barrels (210 million gallons) of Louisiana Sweet Crude Oil spilled into the Gulf of Mexico, making the Gulf Oil Spill the worst oil spill in U.S History to directly impact human and marine life. During the Gulf Oil Spill remediation effort, extensive dispersant use created uncertainty regarding human toxicity to dispersed oil and, more specifically, constituents from this mixture released in aerosolized form. Primary emission of air pollutants following remediative efforts stemmed from evaporative hydrocarbons that produced secondary aerosols (SOA) (Middlebrook et al., 2012). Studies have characterized the SOA plume travelling downwind from the site of the DW (Camilli et al., 2010; Bahreini et al., 2012). SOA consists of intermediate volatiles with a higher presence of ozone and lower presence of polycyclic aromatic hydrocarbons (PAHs) (Middlebrook et al., 2012). Results from chemical SOA plume fingerprinting is similar to the organic hydrocarbons (*i.e* BTEX volatiles, low and mid molecular PAHs) identified in WAF-dispersed oil fingerprinting (Major et al., 2012; Siron et al., 1987, Couillard, 2005). Although the originating formation of SOA and WAF-dispersed oil may differ, understanding the health implication associated with prolonged exposure to this inhalable mixture, containing constituents known to be genotoxic, remains to be seen.

The application of chemical dispersants helps to accelerate dilution and biodegradation of the oil (Swannell and Daniel, 1999), and can reduce the environmental and economic impact of spilled oil. However, use of dispersants can also lead to increased short-term toxicity to aquatic biota and risk of exposure to coastal populations and remediation crews due to higher aqueous concentrations of crude oil bioavailable to the environment (Lin et al., 2009, Anderson et al., 2011, Wang et al., 2012). Dispersants consist of a formulation containing a mixture of substances including, but not limited to, surfactants (anionic/nonionic) and solvents. Surfactants are dual-affinity chemical groups possessing both a hydrophilic head and lipophilic tail that actively function to increase the solubility of oil. By using its hydrophilic head to remain stable in the water while aligning its lipophilic tail with the oil phase, a decrease in oil–water interfacial tension occurs (Lessard and Demarco, 2000). During the Gulf Oil Spill Corexit EC9527 dispersant was applied in smaller quantities through aerial application due to its toxic nature. Corexit EC9500A dispersant was administered through both deep-water injection and aerial application, both at higher quantities (Wise and Wise, 2011). Although the fate of chemicals released during the Gulf Oil Spill has been studied, the lack of information regarding potential genotoxic effects of the WAF of crude oil dispersed by Corexit EC9500A and EC9527 is limited.

Genomic integrity is critical for cellular homeostasis and survival; the fidelity of DNA repair following genomic damage is just as crucial. Exposure to toxicants can cause various types of DNA damage (Helleday et al., 2014). Some of the more common types of exposure-induced damage include the introduction of bulky adducts, intercalating agents, DNA crosslinking and fragmentation: both single strand DNA breaks (SSBs) and double strand breaks (DSBs). When a cell is exposed to toxicants, the resultant DNA damage induces a cell-signaling cascade leading to apoptosis, DNA repair and cell cycle arrest. With highly extensive and non-repairable damage, the apoptotic pathway will be triggered leading to

programmed cell death (Fulda et al., 2010). Specifically, when the type of damage is a double strand break (DSB) ATM activation of H2A.X, initiates phosphor-H2AX co-localization around the damage site for targeted repair (Burma et al., 2001). ATM and γ -H2A.X are capable of phosphorylating the tumor suppressor protein, p53, triggering cell cycle arrest that facilitates either the process of DNA repair or death of the cell (Fragkos et al., 2009). DNA bulky adducts and DNA crosslinks are usually repaired by the nucleotide excision repair (NER) pathway (Noll et al., 2006). Lastly, a cell that experiences either SSBs or DSBs can be repaired by homologous recombination (HR) or the non-homologous end joining DNA repair pathways. NHEJ (Jackson, 2002). Thus, most cells have mechanisms to repair exposure-induced DNA damage. However, many toxicants in addition to causing damage, they have also been shown to inhibit DNA repair increasing the detrimental effect of the exposure (Hengstler et al., 2003). This becomes significant as a compound that alters DNA repair in conjunction with a DNA damaging agent (like those present in oil-mixtures), will likely have a more profound effect on alterations of genetic integrity. In particular, exposure at concentrations that inhibit DNA repair needed to correct the toxicant induced DNA damaged cells may result in the accumulation of genomic mutations. It is the accumulation of these mutations, over time, can lead to deleterious consequences. Thus, evaluating the inhibitory capability of a toxicant is of high interest.

The objective of this study was to provide an initial assessment of the genotoxicity (DNA damage) induced by Corexit dispersants EC9500A and EC9527A and WAF-dispersed oil on human airway BEAS-2B epithelial cells. Because dispersants increase solubility and increase cell penetration ability of different compounds, we hypothesized that the WAF-dispersed oil causes an increase in genotoxic measurable endpoints, when compared to the WAF-crude oil exposure. Three parameters were selected as the measurable endpoints for evaluation of genotoxic effects following WAF-dispersed oil exposure: cell survival, induction of DNA damage, and their effect on DNA repair capability, *i.e.* ability to inhibit known repair pathways. We selected to use the BEAS-2B human bronchial cell line as the lung is a likely target organ of the dispersed oil based on previous human studies showing persistent oxidative stress in the pulmonary system (Rodriguez-Trigo et al., 2010) after participation in crude oil clean-up work. Derived from normal human bronchial epithelium, representing one of the target organs of exposure (Forbes, 2000), and possessing efficient metabolic capability with limited cytochrome p450 activity (Courcot et al., 2012; Davila et al., 1998; Garcia-Canton et al., 2013), this cell line is a highly reliable and considered a suitable model for the study.

2. Materials and methods

2.1. Cell culture

Human bronchial epithelial cells (BEAS-2B cells), purchased from American Type Culture Collection (ATCC, #CRL-9609, Wiltshire, USA), were cultured following standard guidelines. Thawed cells initially grew in a pre-coated flask containing fibronectin (0.01 g/ml), bovine collagen type 1 (0.03 mg/ml), and bovine serum albumin (0.01 mg/ml). Following overnight growth in this pre-coated flask, BEAS-2B cells were sub-cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 10%

fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were cultured at 37 °C in a 100% humidified atmosphere of 5% CO₂ in air.

2.2. Chemicals

Louisiana Sweet Crude Oil was kindly provided by The Architecture, Engineering, Consulting, Operations and Management Company (AECOM, CO). This oil was obtained from the site of the Macondo well during the Gulf Oil Spill disaster. Commercially available Corexit EC9500A and EC9527 dispersants were kindly provided by a contract between Nalco/Exxon Energy Chemicals, L.P. (Sugar Land, TX, USA) and Tulane University (New Orleans, USA). The dispersants are liquid solutions ready for use.

2.3. Water accommodated fraction of crude oil

To prepare WAFs from three different samples, the following procedure was adapted from published protocols (Hemmer, 2010; Major et al., 2012). Aliquots of 10 mL of deionized water were placed into volumetric flasks. The following volume ratios were used: (1) a 1:20 ratio (crude oil: Dulbecco's Modified Eagle Medium/10%FBS/5%PenStrep) for the WAF of crude oil only, (2) a 1:40 ratio (Corexit dispersant: Dulbecco's Modified Eagle Medium/10%FBS/5%PenStrep) for the WAF of Corexit only, and (3) a 2:1:40 ratio (crude oil: Corexit dispersant: Dulbecco's Modified Eagle Medium/10%FBS/5%PenStrep) for WAF-dispersed oil. The water to Corexit ratio is within Nalco manufacturer guidelines for dispersant application. Each sample was stirred, with a magnetic bar, at a rate where it was observable that the mixing volume would not exceed a vortex depth greater than 25% of the sample volume. Samples were stirred for 18 h. Once mixed, the sample was allowed to settle overnight in a separation funnel. Following overnight settling, the WAF layer was separated from the mixture, filtered with a Stericup (Millipore, USA), and stored for treatment. Cells were treated with either the WAF of crude oil or the WAF-dispersed oil mixture for 0 h, 2 h, 8 h, and 24 h at different dilutions (PPM volume to volume). Cells had to be viable (80%) in order to be selected as stock solutions for all biological assays.

2.4. Cell survival

The MTT assay kit (TOX-1) was conducted following manufactures instruction, with modification, (Sigma, St. Louis, MO) to determine percent BEAS-2B cell viability following exposure to WAF-dispersed oil. 1×10^4 cells were seeded in 96-well plates for 24 h to ensure 80% confluence. After treatment with different concentrations of WAF-dispersed oil for 2 h, 8 h and 24 h, 10ul of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide solution (5 mg/ml) was added to each well and incubated for 3 h at 37 °C. *Media* was discarded and 100 µl MTT solubilization solution (Dimethyl Sulfoxide) was added. When the formazan was completely dissolved, absorbance was measured at a wavelength of 584 nm (Fluostar Optima, Cary, NC).

2.5. Detection of DNA breaks by neutral and alkaline comet assays

The comet assay is a sensitive and standard technique used to detect the degree of DNA damage (breaks) in individual eukaryotic cells. BEAS-2B cells were seeded in T-75 flasks at

a density of 1×10^6 for 24 h. Cells were treated with WAF-dispersed oil, WAF-crude oil, and Corexit dispersants EC9500/EC9527 for 2 h and 24 h at 100 ppm and 300 ppm concentrations. After treatment, cells were trypsinized and re-suspended in 1X PBS to a final concentration of 1×10^4 cells/ml. The comet assay was performed through the use of the Comet Assay Reagent Kit for Single Cell Gel Electrophoresis (Trevigen, Gaithersburg, MD) following the manufacturer's instructions. As a positive control, untreated cells were gamma irradiated (5Gy). Cellular DNA, on the alkaline and neutral comet assay slide, was stained with SYBR Green I (Trevigen, Gaithersburg, MD) and analyzed using an epifluorescence microscope at 20 $\times$ magnification (Nikon, Melville, NY). The digital images, acquired with a SensiCam QE digital camera (Cooke Corporation, Romulus, MI), was used to score tail moments in at least 75 random cells using the Comet Assay IV software (Perceptive Instruments, United Kingdom). A tail moment was defined as the %DNA in the comet tail multiplied by the tail length.

2.6. Detection of DNA Damage Response Signals γ -H2A.X and phospho-p53

BEAS-2B-cells were treated with various concentrations (100 ppm or 300 ppm) of WAF-9500 + Oil, WAF-9527 + oil, or WAF-crude at 2 h, 8 h, and 24 h time points. Cells were washed with ice-cold PBS (pH 7.4) and lysed in ice-cold Triton-X/SDS Lysis buffer containing 150 mM NaCl, 50 mM Tris, 10 mM EDTA, 0.5% SDS, 0.5% TritonX-100 (pH = 7.2) for 5 min on ice. Cells were scraped off from the dish and harvested for sonication. The cells were sonicated at 4 °C with the high setting (30s on/30 s off) for 30 min using the Biorupter UCD-200 ultrasonic water bath (Invitrogen, Grand Island, NY). The sample was spun at 13,000 $\times$ rpm for 15 min at 4 °C to remove undissolved debris. Sample supernatants were removed and stored at -80 °C. Protein concentration was determined by the Bradford colorimetric assay (BioRad). Proteins were separated by electrophoresis in pre-cast Bis Tris (Nupage 4–12%, Life Technologies) gels. Electrotransfer was performed using the dry iBlot Transfer system (Invitrogen, Grand Island, NY). After electrotransferring, the membrane was blocked in 5% BSA for 1 h at room temperature, and then washed 3 times at 5 min each with 1X phosphate buffer saline- 0.5% Tween 20 (PBST). For probing, the membrane was incubated with different primary antibodies overnight on a rocker at 4 °C. The following primary antibodies were used: phospho-Histone H2A.X (15 kDa, Ser139, Rabbit mAb, cat #9718), phospho-p53 (53 kDa, Ser15, Mouse mAb, cat #9286) from Cell Signaling (Cell Signaling Technology, Beverly, MA). The loading control was p84 (a 84 kDa nuclear matrix protein, mAb, cat# 5E10, GeneTex, Irvine, CA) and the secondary antibodies were: donkey anti-rabbit IgG HRP (cat# sc-2317) and goat anti-mouse IgG HRP (cat# sc-2031) (Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were detected using the West Pico Chemiluminescent kit (Thermo Scientific, Rockford, IL) and visualized by the ChemiDoc Gel Imaging System (Bio-Rad Laboratories, Hercules, CA) after incubation with the appropriate secondary antibody for 1 h at room temperature. Image Lab software (Bio-Rad Laboratories, Hercules, CA) was used for western blot image acquisition and analysis.

2.7. DNA repair capability: UV and IR sensitivity assays

BEAS-2B cells were seeded at 1×10^5 cells per 60 $\times$ 15mm cell culture dish and allowed to grow for 24 h. The UV sensitivity assay [measure NER repair capability] was adapted from a previously published protocol (Emmert et al., 2000). Cells were washed once with 1X PBS

(pH 7.4), and irradiated in the presence of 1 ml 1X phosphate buffer saline (PBS; pH 7.4) with the dish cover removed. Dishes were exposed to 0, 3, 6, 9 and 12 J/m² UVC (254-nm UVC at an incidence fluence rate of 1.57 J/m²). Similarly, for the IR sensitivity assay [measure DSB (NHEJ/HR) repair capability], cells were exposed at 5 and 7.5Gy of gamma radiation. Exposure time was pre-determined using the irradiator calibration value (0.817Gy/min). Following UV or gamma irradiation, the 1X PBS was removed and replaced with WAF-dispersed oil media at varying concentrations or medium (no treatment control). After 3 days, viable cells per dish were counted with a hemocytometer. Cell survival was calculated as the percent of live cells in the irradiated sample relative to the unirradiated sample.

2.8. Statistical analysis

All results were based on the completion of at least three independent experiments. Data was expressed as mean and error bars represented the standard error (SE). Intergroup differences were tested by analysis of variance (ANOVA) with Tukey test for comparison. The criterion for statistical significance was $p < 0.05$. Linear regression (least squares) was used to derive statistical differences between the slopes of the lines. Error bars represented the SEM ($n > 3$) with a ($p < 0.05$). The software used for all analyses was GraphPad Prism 5 (GraphPad Software, Inc., San Diego, California), and SPSS (IBM, New York, New York).

3. Results

3.1. Cytotoxicity of crude oil and oil-dispersant mixture exposures on the human lung cells, BEAS-2B

Previous data demonstrated that exposure of BEAS-2B cells to low concentrations (100–300 ppm) of Corexit EC9527A and EC9500A dispersants significantly reduced cell viability after 2 h and eliminated almost 100% of the cell culture by 24 h (Shi et al., 2013). To determine the influence of dispersants the WAF-dispersed oil samples, cell viability of BEAS-2B cell following treatment with the WAF-crude, WAF-9500 + oil and WAF-9527 + oil mixed samples was investigated using the MTT assay. BEAS-2B cells were treated at varying concentrations (0–30,000 ppm) with 2 h, 8 h, and 24 h exposure times for all WAF samples (Fig. 1). At 2 h, there was a significant decrease ($p < 0.05$) in cell survival at 10,000 ppm for the WAF-9500 + oil (89.5 ± 3.79) and WAF-9527 + oil (58.1 ± 10.1) when compared to the WAF-crude (89.5 ± 3.79). Significant ($p < 0.05$) results were also observed at a concentration of 30,000 ppm where cell survival for the WAF 9500 + oil (30.9 ± 1.63) and WAF-9527 + oil ($34.1 \pm .818$) decreased in comparison to the WAF-crude (87.3 ± 5.05).

Cell viability loss was significantly higher at the 8 h time period ($p < 0.05$). Specifically at 10,000 ppm, cell survival decreased in the WAF-9500 + oil (38.4 ± 1.34) and WAF-9527 + oil (44.5 ± 8.17) samples when compared to the WAF-crude (98.4 ± 8.96). Significant ($p < 0.05$) reductions in cell viability were also observed at the higher concentration of 30,000 ppm when treated with the WAF-9500 + oil (33.5 ± 1.78) and WAF-9500 + oil (40.2 ± 2.94) when compared to WAF-crude (100 ± 6.93).

Similar to cell viability loss occurring at 8 h, the same pattern in cell death was observed at 24 h ($p < 0.05$). With a treatment concentration of 10,000 ppm cell death decreased in WAF-9500 + oil (39.6 ± 1.51) and WAF 9527 + oil (40.2 ± 4.68) samples when compared to the WAF-crude (93.2 ± 4.75). Again, at 30,000 ppm cell survival was also greatly reduced ($p < 0.05$) for the WAF 9500 + oil (28.6 ± 2.42) and WAF 9527+ oil (29.5 ± 2.44) samples when compared to the WAF-crude (100 ± 4.11). For lower concentrations (<1000 ppm), treatment with WAF-9500 + oil or WAF-9527 + oil showed no significant reduction in cell viability. The threshold for cell death appeared to be significant at concentrations greater than 10,000 ppm. Although the exposure to WAF-dispersed oil is not as cytotoxic as dispersants alone (Shi et al., 2013), these mixtures show higher toxicity than crude oil alone. Overall, none of the WAF crude oil concentrations evaluated reduced cell viability, while the higher concentrations of the WAF –dispersed oil reduced cell viability by ~70% at all exposure time points evaluated.

3.2. Exposure to WAF of oil-dispersant mixtures promote DNA strand breaks

DNA breaks, specifically DSBs, are not only dangerous to a cells genome but can lead to cell death, genomic instability and malignant transformation (Van Gent et al., 2001). Thus, alkaline and neutral comet assays were used to evaluate the induction of SSBs or DSBs, respectively, in BEAS-2B cells that were treated at concentrations (100 and 300 ppm) where WAF exposure resulted in cell viability greater than 85%. As expected, exposure to Corexit dispersants alone promote DNA breaks, as observed for both alkaline and neutral comet assay analyses (Fig. 2) of a 2 h exposure with a concentration of 100 ppm and 300 ppm. Although not as pronounced, WAF-dispersed oil also showed a significant amount of DNA breaks (Fig. 3) which were significantly higher than crude alone. Interestingly, under these conditions, exposure to crude alone was not significantly different than the no treatment control ($p = 0.488$). Although, both concentrations for the WAF - dispersed oil samples show an increase in the tail moment of the alkaline comet assay analyses, only the 100 ppm concentration was significantly different from the (NoTrt) control. Only the 2 h treatment at 100 and 300 ppm of Corexit EC9527 + oil exposure revealed significant increases in DSBs in cells (Fig. 3B).

3.3. DNA damage response signals: detection of phosphorylated histone H2Ax and the tumor suppressor p53

DNA damage triggers a complex signaling pathway that may lead to apoptosis, cell cycle arrest, or DNA repair. Thus, the activation and regulation of DNA signaling pathways in response to this damage was investigated. Phosphorylation of H2A.X (SER139) and p53 (SER15) were probed in BEAS-2B cells exposed to 100 ppm of Corexit dispersants EC9500, EC9527 and dispersed oil samples (WAF-9500 + oil and WAF-9527 + oil) for 2 h and 8 h. Shown in Fig. 4, 2h treatment with Corexit EC9500 and EC9527 resulted in a small increase in (A, B) γ -H2A.X and (C,D) p53 phosphorylation when compared to the (NoTrt) control. γ -H2A.X phosphorylation increased in cells treated with WAF-9527 + oil only, when compared to (NoTrt) control and WAF-crude (Fig. 4 A). Immunoblot quantification confirmed the largest difference to be more than 20% between treated and control cells for both Corexit dispersant and dispersed oil treatment groups. At 8 h γ -H2A.X and p53 phosphorylation remained high in both Corexit EC9500 and EC9527 treated cells. However,

these protein levels slightly decreased in cells treated with dispersed oil, a ~ 6% decrease when compared to 2 h treatment results. p53 phosphorylation generally followed the same pattern at both 2 h and 8 h time points for all treatment groups.

3.4. Assessment of DNA repair in BEAS-2B cells, UV sensitivity and gamma irradiation

Many xenobiotics like heavy metals and cigarette smoke have been identified to not only induce DNA damage but to also adversely alter DNA repair (Hartwig et al., 2002; Laffon et al., 2010; Mehta et al., 2008). As a proxy to measuring NER and HR/NHEJ repair capabilities we used UV and IR sensitivity assays to evaluate the ability of oil-dispersant mixtures to alter DNA repair. BEAS-2B cells were treated with the low concentration of 100 ppm of the WAF-dispersed oil samples (9527 + oil and 9500 + oil), WAF-Crude, and Corexit dispersants EC9500, EC9527 following UV radiation and Gamma radiation (IR). Due to the inherent toxicity of the dispersants, the low concentration of 100 ppm shown to induce breaks was selected. Following treatment, the ability for cells to repair the UV and Gamma induced DNA damage was evaluated as cell survival. Cells were exposed to 0–12 J/m² UVC and 5/7.5Gys IR; after four days of treatment, survival was measured. Not surprisingly, BEAS-2B UVC exposed cells, treated with 100 ppm Corexit dispersants EC9500 and EC9527, exhibited a concentration dependent decrease in cell survival (Fig. 5A). Specifically, the slope of the line for Corexit EC9500 survival loss was significantly different from the (NoTrt) negative control ($p < 0.05$, nonlinear regression). Similar to UVC exposed Corexit dispersant treated BEAS-2B cells, a concentration dependent survival loss was also observed in cells that were gamma irradiated (Fig. 5C). At 7.5Gys, Corexit EC9527 had a 3.7 fold ($p < 0.05$, ANOVA) lower survival rate than the negative control. Slope of the lines for both Corexit EC9500 and EC9527 were significantly different from the negative control ($p < 0.05$, nonlinear regression). A reduction of cell survival between untreated cells and cells treated with WAF-crude oil or WAF-dispersed oil samples (9500 + oil and 9527 + oil) were not observed following UV and gamma irradiation (Fig. 5B Fig. 5D). On the contrary cell survival appears to increase in treated samples. Cells exposed WAF-9500 + oil appear to grow faster than unexposed cells (NoTrt) skewing the results to give the appearance of an increased capability of DNA repair by the NHEJ pathway.

4. Discussion

The goal of this study was to assess the genotoxicity (DNA damage) induced by Corexit dispersants EC9500A and EC9527A and WAF-dispersed oil on human airway BEAS-2B epithelial cells. As previously demonstrated, WAF-dispersed oil is more toxic than WAF-crude oil (Shi et al., 2013), and exposure to Corexit dispersant is toxic (Shi et al., 2013) and causes DNA damage (Fig. 2). Our data indicates that toxicity is manifested after exposure to WAF-dispersed oil (Fig. 1). However, due to the likely dilution of the amount of dispersant in the WAF-dispersed oil mixture only exposure to the higher concentrations of dispersed oil tested resulted in toxicity. Interestingly, no toxicity was observed even at higher concentrations of WAF-crude alone indicating that the addition of dispersants introduce an added negative effect. Similarly, when evaluating DNA breaks, the added adverse effects were also observed in cells treated with low concentrations of WAF-dispersed oil exposed (Fig. 3). Again, the WAF-dispersed oil samples showed higher amounts of DNA damage

when compared to WAF-crude alone. However, there were differences between the dispersants-oil mixtures. DSB formation was highest in cells treated with solutions containing WAF-9527 + oil only (Fig. 3B). The dominance of WAF-9527 + oil in eliciting a higher amount of SSB and DSB damage, when compared to the WAF-crude and WAF-9500 + oil, may be due to chemical compositional differences between the WAFs. This is suggestive that hydrocarbon solubility varies between the dispersants and may directly impact bioavailability of these toxicants into the environment (Su, 2006; Wu et al., 2012). Given that WAF-9527 + oil induced DSB damage exhibited comparable levels to WAF-9527 only treatment, these findings corroborate similar studies confirming differences in toxic response between dispersant only and dispersed oil exposure (Hemmer, 2010; Kim et al., 2012).

DNA damage will cause a cell to trigger a signaling cascade geared towards repairing and protecting the cell from further damage. (Su, 2006). Although small increases in the amount of the phosphorylated H2A.X and p53 proteins were observed, we were able to determine that after 2 h and 8 h, γ -H2A.X levels were the highest in cells treated with the WAF-9527 + oil and Corexit EC9527 samples (Fig. 4). As expected, samples that induced the highest DNA damage in the comet assays also had the highest induction of DNA damage response. These results corroborate that the exposures evaluated introduce damage that elicits a response from the cell at low concentration exposures.

Environmental genotoxicants with the ability to alter a cell's competence to repair genetic damage can have profound effects on human health. In our assays, cell survival was only reduced following treatment with Corexit dispersants. Due to the toxic nature of the dispersants, it is possible that this loss of cell survival is reflective of toxicity and does not reflect an interference with DNA repair capabilities. However, the percent of surviving cells exposed to dispersant is much lower in UV irradiated cells when compared to IR irradiated cells (Fig. 5A and C). Further studies need to be performed to verify if this indeed is indicative of an effect of dispersant exposure on NER repair functions. Our observations indicate that although the WAF-dispersed oil exposures induced DNA damage, they do not appear to alter the cell's DNA repair ability (Fig. 5). On the contrary, the presence of oil in the sample appears to promote cell survival. We speculate this may be an unexpected limitation of the assay due to a "protective" interference of the oil to this artificial assay system in the laboratory setting. However, further studies are needed to further determine if there is an impact of exposure on DNA repair.

Some of the limitations of this study include the fact that it is impossible to determine the exact concentration of dispersants used in response to the *Deepwater Horizon* disaster. However, establishing the potential ramifications that dispersants have on cellular health is a first step to help provide future guidance for decision making to select the best practices and protection procedures during an oil spill. Overall, these results suggest that the use of dispersants could increase the genotoxic effects of exposure as low exposures to WAF-dispersed oil have the ability to induce DNA damage. Because the majority of the cells survive at these low concentrations, it is likely that many of these cells would have damaged DNA that may lead to mutagenic events. Thus, there is the potential that many of the

exposed surviving cells will accumulate mutations and placing them in a position to potentially serve as sources for future disease states such as cancer.

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Abbreviations

DSBs	Double Strand Breaks
FBS	Fetal Bovine Serum
HR	Homologous Recombination
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
PAH	Polycyclic aromatic hydrocarbon
SOA	Secondary Aerosols
SSBs	Singe Strand Breaks
UV	Ultraviolet light
WAF	Water Accommodated Fraction.

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Fig. 1.

Exposures to WAF-dispersed oil show increased toxicity relative to WAF crude. MTT assay was used to determine the percent cell viability of BEAS-2B cells following exposure the WAF of two oil dispersant-oil mixtures (9500 + oil and 9527 + oil) dispersed oil or WAF of crude at 2 h, 8 h, and 24 h time points. Data shown represent a subset of the different concentrations tested at varying time points. Results are reported as percent cell viability (mean $\pm$ SEM). Results are representative of three biological independent experiments, with each concentration conducted at least four times. The asterisk (*) indicates significant

difference from the WAF-crude ($p < 0.005$; one way ANOVA-Tukey test for multiple comparison).

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Fig. 2.

Exposure to corexit dispersants promotes DNA breaks. Alkaline (A, C) and Neutral (B,D) Comet assays show that 2 h exposure of the human lung cell BEAS-2B to 100 and 300 ppm of Corexit dispersants 9500 (purple) and 9527 (blue) promotes DNA breaks. Untreated cells were used as the negative control (NoTrt) and cells treated with 5Gys of ionizing radiation (IR) as positive control. At least three biological independent experiments were completed. Columns represent the mean tail moment and error bars represent the standard error of the mean. Statistical differences are indicated (above each column) as * $p < 0.05$ (one way

ANOVA with Tukey test for comparison) relative to the no treatment (NoTrt) negative control. C and D show representative images of the comet assay results (Magnification: 20×).

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Fig. 3.

Exposure to dispersant-oil mixtures promotes more DNA damage than crude alone. Alkaline comet assays (A and C) analyses show that 2 h exposure to the WAF of 9500 + oil (orange) and 9527 + oil (green) at 100 ppm induces more DNA damage in human BEAS-2B cells than the WAF of crude (brown) alone. Neutral comet assays (B and D) analyses show that only the 2 h exposure of Corexit 9527 + oil mixtures (100 and 300 ppm) significantly induced breaks. Columns represent the mean tail moment and error bars represent the standard error of the mean. Statistical differences are indicated (above each column) as * $p < 0.05$.

0.05 (one way ANOVA with Tukey test for comparison) relative to the no treatment (NoTrt) negative control. C and D show representative images of the comet assay results (Magnification: 20×).

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Fig. 4. Detection of phosphorylated H2Ax and p53. The activation of DNA damage was investigated by immunoblot detection of γ -H2A.X protein (A) and phospho-p53 (B). BEAS-2B cells were treated at varying time points (2 h or 8 h) with either Corexit Dispersants EC9500 and EC9527 or WAFs 9500 + Oil, 9527+ Oil, and Crude, at a concentration of 100 ppm. A representative blot is shown. For quantification, band relative density was normalized to p84 (loading control). Data expressed as the fold change relative to NoTrt (negative control). The mean from three independent experiments is shown plotted on the graphs. Differences in

protein expression compared to NoTrt, for each time point, were analyzed by One Way ANOVA with Tukey test for comparison.

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Fig. 5.

Effects of exposures on UV sensitivity and IR sensitivity. For the sensitivity assays, BEAS-2B cells were exposed to 0, 3, 6, 9 and 12 J/m² UVC concentration or 0, 5 or 7.5 Gy concentration and allowed to recover in the absence (NoTrt, negative control) or presence of 100 ppm of either Corexit dispersants (EC9500 and EC9527), WAF-dispersed oil (9500 + oil and 9527 + oil), or WAF of crude oil only (schematic shown at the top). Cell survival was determined four days after radiation (see materials and methods). Data points are the logarithm of the mean of the percentage of cell survival plotted against the radiation

concentration (UV or IR). Error bars represent the SEM ($n = 3$). Statistical differences in comparisons between each data point, relative to 0 (no radiation) and are indicated (above each data point) as * $p < 0.05$ (two way ANOVA –Tukey test). \$ $p < 0.05$ (indicates statistical differences between the slopes of each line, using regression analysis).