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The Cytotoxicity and Genotoxicity of Particulate and Soluble Hexavalent Chromium in Leatherback Sea Turtle Lung Cells

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Abstract

Hexavalent chromium [Cr(VI)] is a marine pollution of concern as recent studies show it has a global distribution, with some regions showing high Cr concentrations in marine animal tissue, and it is extensively used. Leatherback sea turtles (*Dermochelys coriacea*) are an endangered marine species that may experience prolonged exposures to environmental contaminants including Cr(VI). Human activities have led to global Cr(VI) contamination of the marine environment. While Cr(VI) has been identified as a known human carcinogen, the health effects in marine species are poorly understood. In this study, we assessed the cytotoxic and genotoxic effects of particulate and soluble Cr(VI) in leatherback sea turtle lung cells. Both particulate and soluble Cr(VI) induced a concentration-dependent increase in cytotoxicity. Next, using a chromosome aberration assay, we assessed the genotoxic effects of Cr(VI) in leatherback sea turtles. Particulate

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Contributors

All authors contributed to the preparation of this manuscript. Mark Martin Bras, Mike Barandiaran, Erick Bermúdez, and Lirio Márquez-D'Acunti were integral in the collection and initial processing of sea turtle samples used for this study. Rachel Speer, Catherine Wise, and Jamie Young all contributed to the data collection and analysis in this study. AbouEl-Makarim Aboueissa provided statistical analysis. All authors have approved this statements and the final article.

Conflict of Interests

The authors claim no conflict of interests.

and soluble Cr(VI) induced a concentration-dependent increase in clastogenicity in leatherback sea turtle lung cells. These data indicate that Cr(VI) may be a health concern for leatherback sea turtles and other long-lived marine species. Additionally, these data provide foundational support to use leatherback sea turtles as a valuable model species for monitoring the health effects of Cr(VI) in the environment and possibly as an indicator species to assess environmental human exposures and effects.

Keywords

Hexavalent chromium; cytotoxicity; genotoxicity; leatherback sea turtle; marine pollution; chromate

1. Introduction

The leatherback sea turtle species (*Dermochelys coriacea*) is a long-lived marine reptile that spends the vast majority of its life in the ocean with only females coming on shore for short instances to lay their eggs. Leatherbacks are large turtles growing to over six feet long and weighing up to 1400 pounds (Eckert et al., 2012). They have a leathery shell, while all other sea turtles have hard, bony-plated shells. They have uniquely adapted (among turtles) a thick insulating layer of fat and the ability to eliminate waste gases through their skin, which allows them to dive underwater to great depths for long periods of time (Bickler and Buck, 2007; Dodge et al., 2014). However despite their charisma and immense size, the leatherback sea turtle is considered critically endangered and at risk of extinction (Spotila et al., 2000; Tapilatu et al., 2013).

It is important to study the potential effects of environmental contaminants on leatherback sea turtles in order to understand the potential impact on the health of their population. Leatherback sea turtles are an endangered species that face many pressures due to human activity including plastic in the oceans, fishing entanglement, habitat degradation, and human released pollution and contaminants (Innis et al., 2010; James et al., 2005; Kaplan, 2005; Lewison et al., 2004; Perrault et al., 2011). Leatherbacks are found throughout all of the world's oceans and commonly travel long distances during their lives. The extended amount of time leatherbacks spend in the ocean subjects them to exposure to any marine pollutants and contaminants that may be present (Godley et al., 1999; Guirlet et al., 2008; Guirlet et al., 2010; Perrault et al., 2013; Perrault 2014; Stewart et al., 2011; Storelli and Marcotrigiano, 2003). These pollutants have the potential to lead to detrimental health effects including reproductive issues (Guirlet et al., 2010; Perrault et al 2011; Stewart et al., 2011). Furthermore, leatherbacks may bioaccumulate environmental contaminants exacerbating health issues.

The health status of the oceans has recently been changing at a faster rate due to climate change leading to concerns, such as ocean acidification. This process may lead to the release of hazardous compounds, such as hexavalent chromium [Cr(VI)], that were previously deposited in ocean sediments (Ellis, 2002; Wang et al., 2015; Zeng et al., 2015). Hazardous compounds released from the sediments may suspend in the water column where leatherback sea turtles, a pelagic species, spend a majority of their time (Dodge et al., 2014).

In addition to being released from ocean sediments, Cr(VI) is also released into the environment through the burning of fossil fuels and other industrial processes. Ultimately Cr(VI) released in this manner can travel through air currents around the world with the potential to settle in the oceans. Cr(VI) is a known human lung carcinogen inducing lung tumors characterized by genomic instability (Holmes et al., 2008; Urbano et al., 2008; Wise et al., 2012). However, to date, there are no studies on the effect of Cr(VI) in leatherback sea turtles. Since leatherback sea turtles may experience prolonged exposure to Cr(VI) in the marine environment through the air, water, and food sources it is important to understand the health implications from this potential exposure.

It is increasingly clear that pollution derived from anthropogenic activities has reached even the remotest ocean regions. We recently described metal pollution around the world using sperm whales (*Physeter macrocephalus*) as an indicator species (Wise et al., 2009a). One marine pollutant with particularly high levels was Cr. Data from humans and laboratory animals show Cr(VI) can damage DNA and induce reproductive and developmental toxicity (Al-Hamood et al., 1998; Bataineh et al., 1997; Chowdhury and Mitra, 1995; Mancuso, 1997; Witmer et al., 1989; Witmer et al., 1991). If such outcomes occur it could lead to disease and decreased reproductive success, which could seriously impair a critically endangered species like the leatherback.

Several studies have investigated metal levels in leatherback sea turtles around the world and found their tissues may accumulate metals such as mercury, cadmium, lead, and arsenic (Guirlet et al 2008; Guirlet et al., 2010; Kunito et al., 2008; McKenzie et al., 1999; Perrault et al., 2013; Perrault, 2014; Poppi et al., 2012; Stewart et al., 2011; Storelli et al., 1998). To our knowledge only one study has investigated Cr levels in leatherback sea turtles, but did not measure Cr levels in lung tissue (Poppi et al., 2012). Poppi et al. did find that Cr was present in the kidney, muscle, skin, and, at the highest concentration, in the liver. However, one study showed that in tissues of adult and young loggerhead sea turtles (*Caretta caretta*) Cr accumulated in the highest concentrations in the lung (Storelli et al., 1998). Another study found that Cr levels in the yolk of eggs from green sea turtles (*Chelonia mydas*) were considered above normal compared to levels observed in mammals and birds, however the effects of these levels remain unknown (Lam et al., 2006).

Metal exposure in sea turtle model systems has been considered in two studies: one on green turtles and one on hawksbill. The green sea turtle studies correlated carapace metal levels with adverse health markers and found that cadmium and Cr were the most cytotoxic of four metals tested for cytotoxicity in cell lines (Tan et al., 2010; Wang et al 2013). We found Cr(VI) was both cytotoxic and genotoxic to another marine sea turtle species, the hawksbill sea turtle (*Eretmochelys imbricate*) (Wise et al., 2014; Young et al., 2015). We found no studies of metal toxicity in leatherbacks. Therefore, in this study we investigated the cytotoxic and genotoxic effects of Cr(VI) in leatherback sea turtle lung cells. We focused our study on particulate and soluble Cr(VI) compounds as the marine environment favors the hexavalent form of Cr (Geisler and Schmidt, 1991; Pettine and Millero, 1990) and because in humans the particulate Cr(VI) forms are more potent than soluble ones (Holmes et al., 2008; IARC, 1990; Wise et al., 2002; Wise et al., 2008).

2. Materials and Methods

2.1 Chemicals and reagents

DMEM/F12 (1X), phosphate-buffered solution (PBS) 1X without calcium or magnesium, Corning glutagro supplement (200 mM), tissue culture dishes, flasks and plasticware were purchased from Corning (Corning, NY). Sodium pyruvate (100 mM) was purchased from Lonza (Allendale, NJ). Gurr's buffer and 0.5% trypsin-EDTA (10X) were purchased from Life Technologies Corp (Carlsbad, CA). Crystal violet and acetic acid were purchased from J.T. Baker (Phillipsburg, NJ). Lead chromate (CAS #7758-97-6), sodium chromate (CAS#7775-11-3), and demecolcine were purchased from Sigma-Aldrich (St. Louis, MO). Giesma stain was purchased from Biomedical Specialties Inc. (Santa Monica, CA). Seradigm premium grade fetal bovine serum (FBS), Sodium dodecyl sulfate (SDS), and methanol were purchased from VWR International (Radnor, PA). Potassium chloride (KCl) was purchased from Alfa Aesar (Tewksbury, MA). Attachment factor was purchased from ThermoFisher Scientific (Waltham, MA). Trace-element grade nitric acid was purchased from Fischer Scientific (Hampton, NH).

2.2 Cell line development and cell culture

A leatherback sea turtle primary lung fibroblast cell line was established from explant lung tissue derived from a leatherback sea turtle embryo and were named PGDC9-1LU cells. Primary PGDC9-1LU cells (Figure 1) were maintained as sub-confluent monolayers in DMEM/F12 media supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutagro, and 0.1% sodium pyruvate. PGDC9-1LU cells were incubated in 5% CO₂ at 26°C and media was replaced with fresh, warm media every two to three days. Cells were subcultured every four to seven days using 0.1% trypsin-EDTA. The cell line was evaluated for numerical and structural chromosome normality through sub sequential passaging of the cells. No aneuploidy or cellular phenotypic changes were observed in untreated cells.

2.3 Chromium preparation

Lead chromate (CAS# 7758-97-6, ACS reagent minimum 98% purity) was used as a representative particulate Cr(VI) compound and was administered as a suspension in water as previously described (Wise et al., 2002). Sodium chromate (CAS #7775-11-3, ACS reagent minimum 98% purity) was used as a representative soluble Cr(VI) compound as previously described (Wise et al., 2002). Lead chromate is insoluble in water and was therefore administered as weight per surface area (ug/cm²). Sodium chromate is 100% soluble in water and was administered in micro molar (uM) concentrations. Final chromate concentrations in cell culture ranged from 0–10 ug/cm² for particulate Cr treatments and from 0–10 uM for soluble Cr treatments, which are equal to 0.067–7.191 ppm and 0.026–0.520 ppm, respectively. We believe these concentrations are within environmentally relevant ranges to which humans, sea turtles, and other wildlife species may be exposed.

2.4 Intracellular chromium ion measurements

Intracellular Cr ion levels were determined based on published methods adapted for atomic absorption spectrometry (AAS) (Holmes et al., 2005). Briefly, cells were seeded at a density

of 700,000 cells in a 100-mm tissue-culture dish and allowed to grow for 48 h. Then the cells were treated with increasing concentrations of sodium chromate for 24 h or lead chromate for 0 h and 24 h exposure periods. At the end of the treatment time cells were collected using 0.1% trypsin-EDTA, washed two times with PBS, and placed in a hypotonic solution of 0.075 M KCl followed by 2% SDS to lyse the cells. The cell solution was sheered through a 20 gauge needle seven times and filtered through a 0.2 μ m filter. The samples were then diluted in 2% trace element grade nitric acid and Cr ion concentrations were measured by AAS using a PinAAcle 900Z Atomic Absorption Spectrometer. Each experiment was repeated at least three times, and each sample was analyzed by AAS in triplicate.

To account for the possibility that undissolved Cr particles may pass through the 0.2 μ m filter 0 h treatments were performed for the particulate Cr experiments. The Cr ion concentrations from the 0 h treatment were subtracted from the 24 h treatment Cr ion concentrations. For all samples, the corrected intracellular Cr ion concentrations were converted from μ g/L to μ M by dividing by the volume of the sample, the atomic weight of Cr, the number of cells in the sample, and the average cell volume determined by a Beckman Coulter Multisizer 3.

2.5 Cytotoxicity assay

Cytotoxicity was determined using a clonogenic assay modified from methods previously described (Wise et al., 2002). Briefly, cells were seeded at a density of 1500 cells/cm² in a tissue-culture dish, and allowed to grow for 48 h. The cells were then treated for 24 h with various concentrations of sodium chromate or lead chromate. Cells were removed from the dish using 0.1% trypsin and reseeded at colony forming density (1000 cells per dish) in 100-mm tissue-culture dishes pre-coated with attachment factor. When colonies had formed after approximately two weeks they were fixed with 100% methanol, stained with crystal violet, and the colonies were counted. There were four dishes per treatment group, and each experiment was repeated at least three times. The results are expressed as relative survival derived from the number of colonies within a treatment group divided by the number of colonies in the negative control.

2.6 Clastogenicity assay

Clastogenicity was measured using a chromosome aberration assay modified from methods previously described (Wise et al., 2002). Cells were seeded at a density of 700,000 cells in 100-mm tissue-culture dishes, and allowed to grow for 48 h. The cultures were then treated with various concentrations of sodium chromate or lead chromate for a 24 h exposure period. Five hours before the end of the treatment time, 0.1 μ g/ml demecolcine was added to arrest the cells in metaphase and the cultures were harvested. One hundred metaphases per treatment concentration were analyzed in each experiment and each experiment was repeated at least three times. Metaphases were analyzed for chromatid lesions, isochromatid lesions, chromatid exchanges, dicentrics, double minutes, acentric fragments, fragmented chromosomes, and aneuploidy.

2.7 Statistics

Student's t-tests were conducted to determine statistical significance between data points. Statistical significance was determined to be a p value less than 0.05. Regression analysis techniques were used to fit regression lines for the observed data. The fitted regression lines were used to determine the LC₅₀ and TC₂₀ values. The analyses were conducted in R 3.4.3 software.

3. Results

3.1 Intracellular Chromium Ion Concentrations are Similar Following Particulate or Soluble Hexavalent Chromium Exposure

Particulate and soluble Cr(VI) dissolve in cell culture media to different extents and, thus, cannot be accurately compared based on administered dose. To enable such comparisons, we measured the intracellular levels of Cr following exposure to either particulate or soluble chromate in leatherback sea turtle lung cells to investigate the possibility of differing intracellular accumulation. Intracellular Cr ion concentrations increased in a concentration-dependent manner after particulate or soluble chromate exposure in leatherback sea turtle lung cells (Figure 2A and 2B). For example, 24 h exposure to 0, 0.1, 0.5, 1, 5, and 10 $\mu\text{g}/\text{cm}^2$ particulate chromate resulted in intracellular Cr ion concentrations of 0, 1, 210, 399, 763, and 1076 μM , respectively. Similarly, after 24 h exposure to 0, 0.5, 1, 2.5, 5, and 10 μM soluble chromate intracellular Cr ion concentrations were 12, 75, 142, 471, 868, and 1229 μM , respectively.

3.2 Particulate and Soluble Hexavalent Chromium are Cytotoxic to Leatherback Sea Turtle Lung Cells

We measured cytotoxicity using a clonogenic survival assay to determine the ability of cells to proliferate after exposure to chemicals. Exposure to particulate or soluble chromate induced a concentration-dependent decrease in relative survival in leatherback sea turtle lung cells after 24 h exposure (Figure 3A and 3B). Particulate chromate exposures of 0.1, 0.5, 1, 5, and 10 $\mu\text{g}/\text{cm}^2$ reduced relative survival to 87.5, 66.2, 51.2, 18, and 6.3%, respectively. Soluble chromate exposures of 0.5, 1, 2.5, 5, and 10 μM reduced relative survival to 86.6, 62, 40.3, 17.4, and 4.4%, respectively. LC₅₀ values were calculated based on administered concentration for lead chromate and sodium chromate to be 3.35 $\mu\text{g}/\text{cm}^2$ and 3.37 μM , respectively (Figure 3C and 3D).

In order to compare particulate and soluble chromate exposures more directly we evaluated the cytotoxicity of particulate and soluble chromate using the intracellular Cr ion concentrations determined using the AAS. Based on these intracellular Cr ion concentrations particulate and soluble chromate induce similar levels of cytotoxicity (Figure 4). For example, the highest concentration tested for both particulate and soluble chromate resulted in similar levels of cytotoxicity. For particulate chromate, an intracellular Cr ion concentration of 1076 μM reduced relative survival to 6.3% while an intracellular Cr ion concentration of 1080 μM following soluble chromate reduced relative survival to 4.4%. Additionally, regression analysis shows intracellular chromium ion concentrations were a

significant predictor of relative survival after lead chromate (r^2 value = 90.77%) and sodium chromate (r^2 value = 96.42%) (Figure 4B).

3.3 Particulate and Soluble Hexavalent Chromium are Genotoxic to Leatherback Sea Turtle Lung Cells

We measured the genotoxicity of particulate and soluble chromate in leatherback sea turtle lung cells using a chromosome aberration assay. Both particulate and soluble chromate induced a concentration-dependent increase in genotoxicity in leatherback sea turtle lung cells (Figure 5A and 5B). Treatment with 0, 0.1, 0.5, 1, and 5 $\mu\text{g}/\text{cm}^2$ particulate chromate for 24 h resulted in 10.3, 8, 16.7, 23.7 and 31.3% of metaphases with damage and 14.3, 9.7, 21, 28, and 43.7 total damage in 100 metaphases. Similarly, 24 h exposure to 0, 0.5, 1, 2.5, and 5 μM soluble chromate resulted in 9, 15, 23.3, 31, and 35.3% of metaphases with damage and 9.3, 17.3, 29.7, 40.7, and 50.7 total damage in 100 metaphases. Treatment with the highest concentration for particulate and soluble chromate (10 $\mu\text{g}/\text{cm}^2$ and 10 μM , respectively) resulted in no metaphases in the genotoxicity assays indicating cell cycle arrest or failure to enter mitosis.

Additionally, we calculated TC_{20} values which represent the concentration at which 20% of the metaphases analyzed were damaged or total damage was over 20 aberrations per 100 metaphases. The TC_{20} values based on administered concentration for lead chromate were 0.76 $\mu\text{g}/\text{cm}^2$ for total damage in 100 metaphases and 1.82 $\mu\text{g}/\text{cm}^2$ for the percent of metaphases with damage (Figure 5C). The TC_{20} values based on administered concentration for sodium chromate were 0.58 μM for total damage in 100 metaphases and 1.25 μM for the percent of metaphases with damage (Figure 5D).

The total amount of damage in 100 metaphases was increased compared to the percent of metaphases with damage following both particulate and soluble chromate exposure. This reflects that some cells contained more than one event of damage (e.g. two chromatid breaks). A majority of the damage observed in the leatherback sea turtle lung cells following both particulate and soluble chromate exposure were chromatid gaps or chromatid breaks (Table I and II). Other more complex types of damage, such as centromere spreading or dicentric chromosomes, were either not observed or at a very low frequency in our analysis.

Similarly to the cytotoxicity assay we more directly compared the genotoxicity of particulate and soluble Cr(VI) using the intracellular Cr ion concentrations from the metal ion uptake assays. Particulate chromate was slightly less genotoxic compared to soluble chromate at similar intracellular Cr ion concentrations (Figure 6A and 6B). For example, an intracellular Cr ion concentration of 399 μM following 24 h exposure to particulate chromate resulted in 21 total damage in 100 metaphases while an intracellular Cr ion concentration of 471 μM following soluble chromate exposure resulted in an average of 40.7 total aberrations in 100 metaphases. Regression analysis of these data shows intracellular chromium ion concentrations were a significant predictor of total damage in 100 metaphases after lead chromate (r^2 value = 98.43%) and sodium chromate (r^2 value = 87.68%) (Figure 6C). Similarly, intracellular chromium ion concentrations were calculated to be a significant predictor of the percent of metaphases with damage after lead chromate (97.41%) and sodium chromate (82.83%) (Figure 6D).

4. Discussion

Cr(VI) is an established human carcinogen, reproductive toxicant, and genotoxicant in humans and laboratory animals that primarily targets the respiratory and reproductive systems (Holmes et al., 2008; Mancuso et al., 1997; Wise et al., 2011; Witmer et al., 1989; Witmer et al., 1991). Recently it has also been shown to be a concern for marine species including marine mammals and aquatic reptiles (Li Chen et al., 2009, Li Chen et al., 2012; Wise et al., 2009b; Wise et al., 2014; Wise et al., 2016; Young et al., 2015). We chose to evaluate Cr(VI) in leatherbacks to better understand how Cr(VI) leads to lung carcinogenesis as a part of a larger effort under a concept known as the One Environmental Health Approach. This approach considers toxicological endpoints across several species to better understand the mechanisms and threats of environmental contaminants. Specifically in this study, we found Cr(VI) damaged chromosomes and induced cell death in leatherback sea turtle lung cells. Our data are the first to consider the toxic impacts of a metal pollutant in leatherbacks, and also raises concern about the impact of Cr on leatherback sea turtle reproduction and development. If genotoxicity were to occur during reproduction or development, it could cause offspring loss or impact hatchling development (El-Makawy et al., 2006; Keshava and Ong, 1999; Nayak et al., 1989).

Our data are consistent with the only other two studies that considered metal cytotoxicity in sea turtles (Tan et al., 2010, Wise et al., 2014). One study only considered cytotoxicity and found Cr(VI) was the most cytotoxic of the four metals (cadmium, zinc, copper and Cr(VI)) considered in fibroblasts from a green sea turtle suffering from severe fibropapilloma. The other considered particulate and soluble Cr(VI) in immortalized hawksbill cells (Wise et al., 2014). Our cytotoxicity and chromosome damage outcomes were consistent in both effects and potency with our previous hawksbill study (Wise et al., 2014). By contrast, while our outcomes are consistent with the green turtle study, the potency of Cr(VI) differed.

More specifically, Cr(VI) was more cytotoxic in the leatherback cells in our study. This potency difference could be due to a species effect with green sea turtles more resistant to Cr(VI) than leatherback sea turtles, or it may reflect a difference in cell type, as our study considered lung cells, which may be more sensitive to Cr(VI) than the cells in their study. Alternatively, it may reflect a methodology difference as the green sea turtle study measured cytotoxicity using the MTT and Coomassie blue assays, which are less sensitive than the colony-forming assay used in our present study. Of course, it may reflect the health of the animals from which the cells were isolated. Our cell line was isolated from a leatherback sea turtle embryo with a stable, normal chromosome count, while the green turtle cell lines had abnormal chromosome counts and were isolated from a turtle with severe fibropapilloma. It is possible the aneuploidy or the disease made the cells more resistant to Cr(VI). Our results are consistent with the Cr(VI) cytotoxicity outcomes and potency for other vertebrate cell lines (Li Chen et al., 2009; DeFlora et al., 1990; Wise et al., 2009a; Wise et al., 2009b; Wise et al., 2012) and, thus more likely reflect Cr(VI) cytotoxicity in healthy sea turtles.

We did use lead chromate as a representative particulate Cr(VI) compound because it is commonly used in the literature, and it has widespread use in industry and by the general public. Its use does raise a question about the possible role of lead in the toxic outcomes. We

previously established that lead does not contribute to lead chromate cytotoxicity and genotoxicity because the released lead ions are poorly absorbed by cells (Wise et al., 2004; Wise et al., 2005). Consistent with this conclusion, in these leatherback cells, we found very low intracellular lead levels after lead chromate exposure (i.e. levels were equal to or lower than untreated controls and below 1 uM), and, consequently, we do not believe lead ions were a meaningful contributor to the toxic outcome we observed (data not shown). Lead levels have been found to be low or nondetectable in sea turtle tissues, which may mean lead is not a particular health concern for sea turtles (Garcia-Fernandez et al., 2009; Gardner et al., 2006; Paez-Osuna et al., 2010; Storelli et al., 2005).

The concentrations of chromate compounds used in this study were based on standards in the toxicological field and those previously used in studies of this kind. This was the first study to test the toxicity of chromate compounds in leatherback sea turtle cells in cell culture. Therefore, the concentration range chosen was based off of preliminary testing to determine how leatherback sea turtle cells would tolerate chromate exposure. These concentrations range from what would be considered mildly toxic to having moderate toxicity. Additionally, these concentrations have been previously studied in cells from other wildlife species including hawksbill sea turtles and various whale species as well as in human cells.

Specifically, in our study, we exposed cells to 27–273 ug of total Cr from lead chromate and 0.52 to 10 ug of total Cr from sodium chromate for a 24 h period similarly to our previous study in hawksbill skin cells (Wise et al., 2014). In order to gain a better understanding of the toxicological relevance of these concentrations we related our tested Cr concentrations to possible levels of Cr leatherback sea turtles may experience through food sources in the marine environment. Leatherback sea turtles almost exclusively feed on gelatinous zooplankton such as jellyfish and Ctenophores, and to a lesser extent Urochordata (Davenport and Balazs, 1991; Doyle et al., 2007). Leatherbacks potentially ingest around 200 kg of prey per day or about 20–40% of their total body mass (Jones et al., 2012; Lutcavage and Lutz, 1986; Wallace et al., 2006). Four different studies measured Cr levels in various species of which may be prey for leatherback sea turtles. The range of Cr levels in leatherback prey species when considering these four studies was from 0.030 to 1.923 ug Cr/g wet weight (Duyak et al., 2013; Liu et al., 2012; Muñoz-Vera et al., 2015; Templeman and Kinsford, 2010). Multiplying this amount by the estimated amount leatherbacks eat per day equals between 6,000–384,200 ug total Cr per day (for example 0.030 ug total Cr/g wet weight jellyfish tissue $\times$ 1000 g/kg $\times$ 200 kg jellyfish tissue/day = 6000 ug total Cr/day). This indicates that the amount of Cr used in our study was potentially at least 21 times lower than the lowest level of Cr ingested in one day and up to 1,400 times lower than the highest level of Cr that may be ingested in one day.

While there are many other factors that contribute to the level of Cr(VI) that affects leatherback sea turtle cells, this estimation shows that we are using exposures that are not excessively high. Additionally, review of the prey species for leatherbacks shows that the levels of Cr that leatherbacks are exposed to through food sources varies greatly, and this reflects uncertainty about their exposure in the environment.

While we show Cr(VI) is cytotoxic and genotoxic to leatherback cells, the exposure risk to the animals is uncertain as Cr(VI) exposure in this species is poorly understood. Few studies have investigated levels of metals in leatherbacks, but they show metals do have the potential to accumulate in their tissues and are detectable in blood (Godley et al., 1999; Guirlet et al., 2008; Harris et al., 2011; Kunito et al., 2008; Storelli and Marcotrigiano, 2003). However, of these, only one study investigated Cr and found 0.95 ug/g wet weight in liver, 0.16 ug/g wet weight in kidney, 0.13 ug/g wet weight in muscle, and 0.18 ug/g wet weight in skin (Poppie et al., 2012). However, this study did not consider lung tissue, a primary target of Cr exposure, and a target of concern specifically for humans (Urbano et al., 2008). Interestingly, a hawksbill study showed that the highest levels of Cr were in the lungs compared to liver, kidney, and muscle (Storelli et al., 1998).

Leatherback sea turtles are air breathing animals who hold their breath for long periods of time during deep dives to forage (Dodge et al., 2014). An interesting study of marine mammals suggest marine animals may be exposed to higher levels of air pollutants because these pollutants have a tendency to concentrate at the water-air interface where they breathe (Rawson et al., 1995). The pressure and extended period of time a breath of air is in the lungs of leatherbacks increases the amount of time the lung tissue may be exposed to airborne pollutants like Cr. Considering this behavioral information and the data presented here that show Cr(VI) is cytotoxic and genotoxic to leatherback lung cells, further investigation into the effects and exposure of Cr(VI) in leatherbacks is essential.

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Highlights

- Particulate and soluble Cr(VI) are cytotoxic to leatherback sea turtle cells.
- Particulate and soluble Cr(VI) are genotoxic to leatherback sea turtle cells.
- Cr(VI) may be a health concern for leatherback sea turtles and other long-lived marine species

Figure 1. Image of PGDC9-1LU Cells

This figure shows a representative image of the leatherback sea turtle lung cells used in this study.

Figure 2. Intracellular Chromium Ion Concentrations Increase with Increasing Particulate and Soluble Chromate Treatment

This figure shows that with increasing particulate and soluble chromate treatments, intracellular Cr ion concentrations increase in a concentration-dependent manner. Data represent an average of at least three independent experiments $\pm$ standard error of the mean.

*Statistically significant compared to control ($p < 0.05$). (A) Lead chromate. (B) Sodium chromate.

Figure 3. Particulate and Soluble Chromate are Cytotoxic to Leatherback Sea Turtle Lung Cells
 This figure shows a 24h exposure to particulate or soluble chromate reduced relative survival in a concentration-dependent manner. Data represent an average of at least three independent experiments $\pm$ standard error of the mean. *Statistically significant compared to control ($p < 0.05$). (A) Lead chromate. (B) Sodium chromate. (C) Regression analysis was used to determine r^2 and LC_{50} values for lead chromate ($p = 0.018$). (D) Regression analysis was used to determine r^2 and LC_{50} values for sodium chromate ($p = 0.016$).

Figure 4. Particulate and Soluble Chromate Induce Similar Levels of Cytotoxicity in Leatherback Sea Turtle Lung Cells

This figure shows when comparing intracellular concentrations of Cr ions, particulate and soluble chromate induce similar levels of cytotoxicity. Data represent an average of at least three independent experiments $\pm$ standard error of the mean. (A). Scatter plot showing particulate and soluble chromate induce similar levels of cytotoxicity at similar intracellular Cr ion concentrations. (B) Regression analysis determined r^2 values for lead chromate and sodium chromate to be significant predictors of relative survival, $p = 0.001$ and $p = 0.003$, respectively.

Figure 5. Particulate and Soluble Chromate are Genotoxic to Leatherback Sea Turtle Lung Cells

This figure shows after a 24 h exposure particulate and soluble chromate induces a concentration-dependent increase in chromosome damage. No metaphases were observed at the highest concentration tested for particulate chromate (10 ug/cm²) or soluble chromate (10 uM). Data represent an average of three independent experiments $\pm$ standard error of the mean. *Statistically significant compared to control ($p < 0.05$). (A) Lead chromate. (B) Sodium chromate. (C). Regression analysis was used to determine r^2 and TC_{20} values for the percent of metaphases with damage ($p = 0.052$) and total damage in 100 metaphases ($p =$

0.021) for lead chromate (**D**) Regression analysis was used to determine r^2 and TC_{20} values for the percent of metaphases with damage ($p = 0.031$) and total damage in 100 metaphases ($p = 0.017$) for sodium chromate were also calculated based on regression analysis.

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Figure 6. Particulate and Soluble Chromium Induce Similar Levels of Genotoxicity

This figure shows that at similar levels of intracellular Cr ion concentrations particulate and soluble chromate induce a similar frequency of cells with damage and similar levels of total chromosome damage. Data represent an average of at least three experiments $\pm$ standard error of the mean. (A) Percent of metaphases with damage. (B) Total aberrations in 100 metaphases. (C) Regression analysis determined r^2 values for lead chromate and sodium chromate to be significant predictors of the percent of metaphases with damage, $p = 0.002$ and $p = 0.032$, respectively (D). Regression analysis determined r^2 values for lead chromate

and sodium chromate to be significant predictors of total damage in 100 metaphases, $p = 0.001$ and $p = 0.019$, respectively.

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Table I

Lead Chromate Concentration (ug/cm ²)	Chromatid Break	Chromatid Gap	Isochromatid Break	Isochromatid Gap	Dicentric	Double Minutes	Acentric Fragment	Centromere Spreading	Total Damage
0	8 ± 4.2	5.3 ± 0.9	1 ± 0.6	0	0	0	0	0	10.3 ± 1.3
0.1	4.3 ± 0.7	4.7 ± 2.0	0	0.3 ± 0.3	0	0	0	0	8 ± 2.5
0.5	8 ± 1.5	11.3 ± 0.3	1.3 ± 0.3	0.3 ± 0.3	0	0	0	0	16.7 ± 0.3
1	13.3 ± 3.5	14 ± 1.2	0.7 ± 0.7	0	0	0	0	0	23.7 ± 2.2
5	21.7 ± 2.7	21 ± 4.7	0.3 ± 0.3	0.7 ± 0.3	0	0	0	0	31.3 ± 5

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Table II

Spectrum of chromosome aberrations induced by sodium chromate in leatherback sea turtle lung cells^a

Sodium Chromate Concentration (uM)	Chromatid Break	Chromatid Gap	Isochromatid Break	Isochromatid Gap	Dicentric	Double Minutes	Acentric Fragment	Centromere Spreading	Total Damage
0	5.3 ± 0.7	3.7 ± 0.7	0.3 ± 0.3	0	0	0	0	0	9 ± 0.6
0.5	6.0 ± 1.5	9.7 ± 1.8	0.3 ± 0.3	0.7 ± 0.3	0	0	0.3 ± 0.3	0.3 ± 0.3	15 ± 2.5
1	12.3 ± 0.3	14.7 ± 2.6	0.7 ± 0.3	0.7 ± 0.3	0.3 ± 0.3	0.7 ± 0.3	0.3 ± 0.3	0	23.3 ± 2.2
2.5	21.7 ± 2	17.7 ± 2.8	0.3 ± 0.3	0.7 ± 0.7	0	0	0.3 ± 0.3	0	31 ± 1
5	26.3 ± 5.3	22.7 ± 3	0.7 ± 0.7	1 ± 0.6	0	0	0	0	35.3 ± 1.2

^aData represent an average of at least three independent experiments ± standard error of the mean.