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Hexavalent chromium is cytotoxic and genotoxic to American alligator cells

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ABSTRACT

Metals are a common pollutant in the aquatic ecosystem. With global climate change, these levels are anticipated to rise as lower pH levels allow sediment bound metals to be released. The American alligator (*Alligator mississippiensis*) is an apex predator in the aquatic ecosystem and is considered a keystone species; as such it serves as a suitable monitor for localized pollution. One metal of increasing concern is hexavalent chromium (Cr(VI)). It is present in the aquatic environment and is a known human carcinogen and reproductive toxicant. We measured the cytotoxicity and genotoxicity of Cr(VI) in American alligator cells derived from scute tissue. We found that particulate and soluble Cr(VI) are both cytotoxic and genotoxic to alligator cells in a concentration-dependent manner. These data suggest that alligators may be used as a model for assessing the effects of environmental Cr(VI) contamination as well as for other metals of concern.

1. Introduction

The American alligator (*Alligator mississippiensis*) is a long lived, apex predator inhabiting primarily coastal areas of the southeastern US. Once listed as endangered due to overhunting, careful conservation efforts led to delisting in 1987. Localized populations of alligators are exposed to environmental contaminants through a variety of sources. Development in and near their ecosystem can lead to chemical and agricultural runoff. Since the alligator populations have successfully recovered after being listed as endangered in the 1970s, they can be used as a suitable monitor of environmental pollution (Delany et al., 1988).

Chromium (Cr) has recently been shown to be a metal of global concern (Wise et al., 2009). Hexavalent chromium (Cr(VI)) is a known human carcinogen and can damage DNA and impair reproduction and development (Al-Hamood et al., 1998; Bataineh et al., 1997; Chowdhury and Mitram, 1995; Holmes et al., 2008; IARC, 1990; Mancuso, 1997; Wise et al., 2008a,b; Witmer et al., 1989). A few studies have measured Cr in alligators and reveal a concern. A study of alligators in South Carolina found a cluster of alligators had relatively high concentrations of Cr in liver tissue with several animals having levels over 30 μg/g (Campbell et al., 2010). Horai et al. (2014) showed that Cr accumulates in adult alligators based on comparisons between juvenile and adult alligator livers at 3 different sites in Florida. Interestingly of the three sites tested the Cr levels in adult alligators from Merritt Island National Wildlife Refuge (MINWR) were the highest and were 3 times higher than the other two sites suggesting localized pollution (Horai et al., 2014).

However, while these studies show alligators may be exposed to significant levels of Cr, no studies have considered potential adverse effects as a result of Cr exposure in alligators. In fact, consideration of the available literature shows Cr is poorly studied in aquatic reptiles and appears to be limited to two reports. One study, in green sea turtle cells, found Cr(VI) to be one of the most cytotoxic of four

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metals tested (Tan et al., 2010). The other considered hawksbill sea turtle cells and found both particulate and soluble Cr(VI) were cytotoxic and clastogenic (Wise et al., 2014).

The explanation for the lack of data is presumably due to the lack of access to experimental models of aquatic reptiles. Many species are endangered and protected. However, it is possible to gain important species-specific insights into potential toxicological impacts through aquatic reptile cell cultures. Accordingly, to begin developing a better understanding of pollution impacts on alligators and crocodilians in general, we investigated the cytotoxicity and genotoxicity of chromium in fibroblasts developed from American alligator scute tissue. Because the major health concern in the environment is the hexavalent form of chromium, and since particulate Cr(VI) is considered to be a more potent carcinogen than soluble Cr(VI) (IARC, 1990; Holmes et al., 2008; Wise et al., 2008a,b), we focused our study on particulate and soluble Cr(VI) compounds.

2. Materials and methods

2.1. Materials

All plasticware was purchased from BD Falcon. Dulbecco's Phosphate-Buffered Saline (DPBS), RPMI media with Glutagro was purchased from orning. Potassium chloride, demecolcine, lead chromate, and sodium chromate were purchased from Sigma/Aldrich. Crystal violet, methanol and acetic acid were purchased from JT Baker. Microscope slides were purchased from Thermo Scientific. Giemsa stain was manufactured by Rica Chemical Co. Fetal Bovine Serum (FBS), Gurr's buffer, trypsin, penicillin-streptomycin and sodium pyruvate was purchased from GIBCO Invitrogen orporation.

2.2. Cell culture

Alligator fibroblasts were isolated from a scute sample obtained from a free-ranging alligator from the Yawkey Wildlife Preserve in South Carolina. The scute sample was placed in L-15 medium supplemented with 100 g/ml streptomycin, 100 U/ml penicillin, and 10 mg/ml gentamicin and then transported on cold packs to the laboratory. Upon receipt, tissue explants were rinsed several times in PBS with penicillin-streptomycin and gentamicin. Tissue samples were then sliced with a scalpel into small pieces (~1 mm), rinsed repeatedly and placed into T-25 flasks containing RMPI with Glutagro and supplemented with 10% FBS, 100 U/ml penicillin, 100 g/ml streptomycin. Flasks with tissues were placed in a 33 °C (determined to be optimal growth temperature) humidified incubator with 5% CO_2 and fibroblast cells were observed growing out of the explants. Cells were maintained as adherent subconfluent monolayers. They were fed at least twice a week and subcultured at least once a week. Cells were tested routinely for mycoplasma contamination.

2.3. Chemical preparation

Lead chromate (CAS# 7758-97-6, ACS reagent minimum 98% purity), was used as a representative particulate Cr(VI) compound and 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 soluble hexavalent chromium compound and was administered as a solution in water as previously described (Wise et al., 2002). Lead chromate is an insoluble compound and therefore treatment concentrations are expressed as weight per surface area (μg/cm^2). Sodium chromate is fully soluble and therefore treatment concentrations are expressed as μM. Water was used as the vehicle control. Final concentrations ranged from 0 to 5 μg/cm^2 for lead chromate, and 0–5 μM for sodium chromate. Based on comparisons with a potential sea turtle and whale exposures, we believe these concentrations to be environmentally relevant (Wise et al., 2014, 2008a,b).

2.4. Cytotoxicity

Cytotoxicity was determined using a clonogenic assay based on our published methods (Wise et al., 2002). Briefly, log phase cells were seeded into a 6-well culture plate and allowed to resume normal growth for 48 h. Cells were then treated with lead chromate and sodium chromate for 24 h. After treatment, cells were resuspended in fresh medium and reseeded at a colony forming density of 1000 cells per 100 mm dish with four dishes per treatment group. When colony formation was sufficient (approximately 14 days) dishes were fixed and stained with crystal violet. Dishes were counted and averaged together to get a mean value for each dose in each experiment. Treatment dishes were compared to the control. Each experiment was repeated at least three times.

2.5. Clastogenicity

We used a chromosomal aberration assay to determine the clastogenicity of each chemical, based on our published methods (Wise et al., 2002). Briefly, cells were seeded into 100 mm tissue culture dishes for 48 h. Dishes were treated with either lead chromate or sodium chromate for 24 h. One hour prior to the end of the treatment period cells were arrested in metaphase using 0.1 g/ml demecolcine. After the full 24 h treatment period, cells were harvested and resuspended in a potassium chloride hypotonic solution (KCl) for 17 m then fixed with 3:1 methanol:acetic acid. After two additional fixative changes, cells were dropped onto clean, wet microscope slides and stained with 5% Giemsa stain in Gurr's Buffer. Slides were analyzed for chromosome aberrations in 100 metaphases per treatment concentration and reported as both percent of metaphases with damage where the metaphases is the unit of measure and as the total amount of damage seen in 100 metaphases where the chromosome is the unit of measure according to our published methods (Wise et al., 2002).

2.6. Determination of intracellular chromium levels

Intracellular ion levels were measured using the ion uptake assay, as described previously (Holmes et al., 2005), with minor changes. Briefly, logarithmically growing cells were seeded into 100 mm tissue culture dishes, allowed to rest for 48 h and then treated with lead chromate and sodium chromate for 24 h. At the end of the treatment, 3 ml of treated culture media was saved for extracellular chromium analysis; cells were collected and the number and volume of cells were determined. Cells were washed twice with PBS, resuspended in 1 ml hypotonic solution followed by 1 ml 2% SDS. Finally, the solution was sheered through and 18 g needle and filtered to remove cellular debris. Samples were stored at −20 °C until analysis.

Intracellular Cr ion levels were determined using an Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES), equipped with a gem cone low flow nebulizer, according to previously published methods (Holmes et al., 2005). Solutions were introduced to the nebulizer using a peristaltic pump operating at 2 ml/min. Samples of intracellular fluids were diluted 5× in 0.16 M aqueous HNO_3 prior to analysis. Chromium was determined using emission wavelength at 267.716 with a minimum detection limit of 2 ppb. Yttrium(Y) was used as an internal standard for chromium determinations. The intracellular concentrations were converted from μg/l to μM by dividing by the volume of the sample, the