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Medaka (*Oryzias latipes*) as a sentinel species for aquatic animals: Medaka cells exhibit a similar genotoxic response as North Atlantic right whale cells*

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Abstract

Hexavalent chromium (Cr(VI)) is emerging as a major concern for aquatic environments, particularly marine environments. Medaka (*Oryzias latipes*) has been used as a model species for human and aquatic health, including the marine environment, though few studies have directly compared toxicological responses in medaka to humans or other aquatic species. We used a medaka fin cell line to compare the genotoxic response of medaka to Cr(VI) to the response observed in North Atlantic right whale cells to see if responses in medaka were similar to those of other aquatic species, particularly aquatic mammals. We used the production of chromosomal aberrations as a measure of genotoxicity. We found that in medaka cells, concentrations of 1, 5 and 10 μ M sodium chromate damaged 17, 32 and 43% of metaphases, respectively and these same concentrations 1, 2.5, 5 and 10 μ M sodium chromate damaged 14, 24 and 49% of metaphases, respectively, in North Atlantic right whale lung cells and 11, 32 and 41% of metaphases, respectively, in North Atlantic right whale testes cells. These data show that genotoxic responses in medaka are comparable to those seen in North Atlantic right whale cells, consistent with the hypothesis that medaka are a useful model for other aquatic species.

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Keywords

Chromate; Chromium; Genotoxicity; Medaka; North Atlantic right whale; Hexavalent chromium

1. Introduction

Genotoxicity is an important endpoint for understanding the potential health threat that a chemical may pose. Genotoxicity is most commonly used as a short-term predictive measure for carcinogenicity as cancers may take decades to emerge. However, genotoxicity can also provide important insight into potential adverse reproductive outcomes. DNA damage, including chromosomal alterations, are known to cause abnormal development, loss of gametes and decreased reproductive success, which can be devastating for individuals, but may also impact populations (Diekmann et al., 2004; Anderson and Wild, 1994). Genotoxicity is not the only mechanism for causing reproductive toxicity, but it can be an important contributing factor.

Cr(VI) is a potent genotoxic agent that is a carcinogen and a reproductive toxicant (United States Department of Health and Human Services, 1993). Exposure to Cr(VI) is an increasing ocean health concern. Cr(VI) enters the marine environment primarily from anthropogenic sources such as fuel combustion (natural gas, coal, and oil), metal industry emissions and industrial wastewaters including releases from electroplating, leather tanning and textiles (US Department of Health, 1993). Cr(VI) is the predominant form of Cr found in marine waters, and can have residence times that last up to 18 years (Pettine and Millero, 1990; US Department of Health, 1993). Total dissolved Cr(VI) levels in marine waters range from 1.2 nM in unpolluted areas, to 365 nM in areas influenced by wastewater effluents (Aboul Dahab, 1989; Kamala-Kannan et al., 2008; Georgescu et al., 1988). Marine air measurements are infrequent but range from 0.0002–6.84 $\mu\text{g}/\text{m}^3$ (Borai et al., 2002; Li et al., 2002). Because oceans are a sink for pollutants, and the Cr(VI) form of Cr predominates in the marine environment, the threat of chromium exposure to marine life is concerning and likely to increase in the future (Pettine and Millero, 1990; US Department of Health, 1993).

Acute toxicity of Cr(VI) for aquatic species has been reported for fish and effects have been studied in a variety of species and environmental conditions. Cr(VI) lethality (96 h LC_{50}) to marine fish ranges from 20.1–91 mg/L (Eisler, 1986). Other studies show that Cr(VI) can also cause sublethal effects in fish, including altered enzyme activity, decreased disease resistance, oxidative stress responses and histological alterations to gill and liver tissue (Thaker et al., 1996; Roberts and Oris, 2004; Prabakaran et al., 2006). Cr(VI) genotoxicity has been measured in fish and includes DNA-protein crosslinks, micronuclei induction, and chromosomal aberrations (Leonard and Lauwerys, 1980; Krishnaja and Rege, 1982; Torres de Lemos et al., 2001; Kuykendall et al., 2006).

The major concern for Cr(VI) exposure, however, is its genotoxic and carcinogenic effects. Cr(VI) is a known human carcinogen (IARC, 1990) and longer lived species such as humans and aquatic mammals may be at risk for carcinogenesis if sufficiently exposed. In addition, rodent studies indicate that Cr(VI) targets the reproductive system in mammals. It accumulates in the testes where it damages sperm among other effects (Witmer et al., 1989,

1991; Chowdhury and Mitra, 1995; Mancuso, 1997; Bataineh et al., 1997; Al-Hamood et al., 1998).

Aquatic animals are known to be suffering from cancer and reproductive issues. St Lawrence beluga whales are known to suffer from cancer likely from environmental exposures (Martineau et al., 2002). The North Atlantic right whale (*Eubalaena glacialis*) used to be abundant in the northwestern Atlantic Ocean. Their population was diminished by hunting to a few hundred individuals in the early 1900s (Kenney et al., 2001; Reeves et al., 2007). International protection banned whale hunting; however, the species has remained at precariously low levels (National Marine Fisheries Service, 2005). The population is too small and dispersed to assess its cancer rate, but one part of the recovery problem for these whales is an underlying reproductive problem, which may be either caused by or exacerbated by exposure to environmental contaminants (Kraus et al. 2001). However, studying these aquatic mammals is complicated by the inability to conduct controlled-toxicology studies on them due to the impracticalities of bringing large endangered marine mammals into the laboratory. It would be helpful to have an aquatic organism to use in the laboratory to model the potential impacts of environmental chemicals on the health of these aquatic species and to have a sentinel species that could be used to predict risk.

Medaka is a small, teleost fish that is a well-established research model organism for carcinogenesis testing (Hawkins et al., 2003). Although medaka is primarily a freshwater fish, they tolerate a wide range of salinity and have been used as a marine model as well (Tachikawa et al., 1991; McElroy et al., 2006). Most research to date has been conducted *in vivo*; few studies have been done on cultured medaka cells, and none have compared genotoxicity with other marine species. Because of their utility as a laboratory model, medaka may be a potential model for understanding the threats of contaminants to aquatic mammals. However, no direct comparisons have been made so far to determine if responses in medaka reflect responses in aquatic mammals. It is not possible to do these comparisons with whole animals, and thus it is necessary to perform comparisons through cell culture models. Here, as a first step towards testing this possible relevance of medaka as a model organism for aquatic mammals, we compared the genotoxicity of Cr(VI) in a medaka fin cell line to two cell lines from the North Atlantic right whale. The data support the hypothesis that medaka may be a relevant model for aquatic mammals.

2. Methods

2.1. Chemicals and reagents

Dulbecco's minimal essential medium and Ham's F-12 (DMEM/F-12) 50:50 mixture was purchased from Mediatech Inc. (Herndon, VA, USA). Penicillin/streptomycin, L-glutamine, Gurr's buffer, trypsin/EDTA and sodium pyruvate, were purchased from Invitrogen Corporation (Grand Island, NY, USA). Crystal violet, sodium chromate, demecolchicine and potassium chloride (KCl) were purchased from Sigma/Aldrich (St. Louis, MO, USA). Giemsa stain was purchased from Biomedical Specialties Inc. (Santa Monica, CA, USA). Acetic acid and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Fetal Bovine Serum (FBS) and Cosmic calf serum (CCS) were purchased from Hyclone, (Logan,

UT, USA). Tissue culture dishes, flasks, and plasticware were purchased from Corning Inc. (Acton, MA, USA).

2.2. Cell culture

A cell line established from adult medaka (*Oryzias latipes*, Adrianichthyidae, Beloniformes) fin (OLHNI2) with a normal chromosome number of 48 was used as a medaka model. The establishment of these cells in culture are described elsewhere (Komura et al., 1988). Medaka cells were maintained as subconfluent monolayers in DMEM/F12 medium plus 20% fetal bovine serum, 100 U/mL penicillin/100 µg/mL streptomycin, 1% L-glutamine, and 0.1% sodium pyruvate. For North Atlantic right whale (*Eubalaena glacialis*) cell models we used primary lung and testes fibroblasts. The isolation of these cells is described elsewhere (Wise et al., 2008). Right whale cells were maintained as adherent subconfluent monolayers in DMEM/F-12 supplemented with 15% CCS, 100 U/mL penicillin/100 µg/mL streptomycin, 2 mM L-glutamine and 0.1 mM sodium pyruvate. All cells were incubated at 33 °C in a 5% CO₂-humidified environment and subcultured using 0.5% trypsin/EDTA. A separate stock of all reagents from the same lot (medium, trypsin, CCS, antibiotics, etc.) was maintained for each cell type to prevent the opportunity for cross-contamination. In addition, only 1 cell-type was handled at a time to further avoid the potential for cross-contamination. Cells were tested routinely for Mycoplasma contamination. All experiments were conducted on logarithmically growing cells. Medaka cells had a doubling time of 24 h and right whale cells had a doubling time of 36 h.

2.3. Chemical preparation

Sodium chromate (CAS #7775-11-3, ACS reagent minimum 98% purity) was administered as a solution in water as previously described (Wise et al., 2002).

2.4. Clastogenicity

Clastogenicity was determined by measuring the production of chromosomal aberrations based on our published methods with minor modifications (Wise et al., 2002). Briefly, logarithmically growing fish or whale cells were seeded in 100 mm dishes; allowed 48 h to plate down and resume normal cycling; and then treated for 24 h with sodium chromate. Shortly before the end of the treatment time, 0.1 g/mL demecolchicine was added to block the cells in metaphase. At the end of treatment, the culture medium and adherent cells were collected. Cells were then resuspended in 0.075 M KCl hypotonic solution for 17–23 min to swell the cells and the nuclei. Next, cells were fixed in 3:1 methanol:acetic acid, the fixative was changed twice. Finally, cells were dropped onto clean wet slides and uniformly stained with 5% Giemsa stain in Gurr's buffer.

Chromosomal aberrations were scored blind using standard criteria by two different scorers (Wise et al., 2002). Because deletions can only be unequivocally distinguished from achromatic lesions if the distal acentric fragment is displaced, these aberrations were pooled. This approach also helps avoid artificial discrepancies between scorers due to different perceptions of the width of an achromatic lesion relative to the width of its chromatid. Furthermore, recent data show a much stronger correlation between DNA strand breaks and chromosomal aberrations if these gaps and breaks are pooled (Paz-y-Miño et al., 2002).

Accordingly, chromatid deletions and achromatic lesions were pooled as chromatid lesions and isochromatid deletions and isochromatid achromatic lesions were pooled as isochromatid lesions. One hundred metaphases per data point were analyzed in each experiment. Each experiment was repeated at least three times.

3. Results

3.1. The frequency of metaphases with Cr(VI)-induced damage in medaka cells reflects the frequency observed in both whale lung and testes cells

The percent of metaphases with damage measures the frequency of metaphases to incur Cr(VI)-induced chromosome damage. It uses the metaphase as a base unit and describes the frequency of damage in a population of cells. Sodium chromate induced a concentration-dependent increase in the frequency of metaphases with chromosome damage in medaka cells (Fig. 1). Specifically, treatments of 1, 5 and 10 μ M damaged 17, 32 and 43% of metaphases, respectively. Remarkably, this level of damage was very similar to the amount of damage induced in whale lung and testes fibroblasts. In whale lung cells, 1, 5 and 10 μ M sodium chromate damaged 14, 24 and 48% of metaphases, respectively (Fig. 1). In whale testes cells, these concentrations damaged 11, 31 and 41% of metaphases, respectively (Fig. 1). The amount of apoptosis was low (<5%) after a 24 h exposure to these concentrations and there was little difference in cytotoxicity among all three cell lines (data not shown).

3.2. The amount of Cr(VI)-induced chromosome damage in medaka cells reflects the amount induced in both whale lung and testes cells

As a second measure of damage, we determined the total aberrations in 100 metaphases. This measure considers the chromosome as a base unit and describes how much damage occurs in an individual cell and thus accounts for cells with multiple aberrations. Sodium chromate also induced a concentration-dependent increase in the total amount of chromosome damage in medaka cells (Fig. 2). Specifically, treatments of 1, 5 and 10 μ M induced 23, 44 and 69 aberrations per 100 metaphases, respectively. This level of damage was very similar to the amount of damage induced in whale lung and testes fibroblasts. In whale lung cells, 1, 5 and 10 μ M sodium chromate induced 15, 27 and 63 aberrations per 100 metaphases, respectively (Fig. 2). In whale testes cells, these concentrations damaged 12, 39 and 58 chromosomes per 100 metaphases, respectively (Fig. 2).

The spectrum of damage was similar for all three cell types and included predominately chromatid and isochromatid lesions (Fig. 3). Chromatid exchanges, double minutes and centromere spreading occurred sporadically in treated cells and did not occur in controls, but did not reach significant levels.

4. Discussion

We found that Cr(VI) was genotoxic to medaka cells in culture and that the levels induced in medaka cells are comparable to the levels induced in whale lung and testes cells by two measures of chromosome damage: percent metaphases with damage and total aberrations per 100 metaphases. It is interesting to note that the amount of damage we observed in medaka cells was similar to the level previously reported for human lung fibroblasts.

Specifically, this study found that a 24 h exposure to 1 μM sodium chromate damaged 17% of metaphases in medaka cells compared to a previous report of 18% damage at this dose and time in human lung fibroblasts (Wise et al., 2002). These data suggest that medaka cells may also predict the genotoxic response of human cells. By contrast, our medaka data differ from previous reports of Cr(VI) genotoxicity in Chinese hamster ovary (CHO) cells as 1 μM sodium chromate for 24 h damaged 8% of metaphases in CHO cells (Grlickova-Duzevik et al., 2006.). This observation is interesting, as CHO cells are currently used as a standard tool by regulatory agencies to predict human response. Our data suggest that medaka cells may be more predictive of human cell and marine mammal cell response than CHO cells.

Cr(VI) tumorigenicity has not been investigated in medaka although cancer has been reported in other species of fish in highly polluted waters (Black 1983; Hawkins et al., 2003; Logan, 2007). Cancer incidence has also not been investigated in the North Atlantic right whale population likely due to the difficulty of detecting cancer in such a small population and the more immediate threat of ship collision and fishing gear entanglement. Other whales such as the St Lawrence beluga whales are known to suffer from cancer likely from environmental exposures, though Cr exposure has not been considered in this species (Martineau et al., 2002). Our data, however, do indicate that Cr(VI) may have the potential to cause cancer in either species if exposure is sufficient enough. There are no data of Cr(VI) levels in wild medaka species, but data in right whales indicate that this species has accumulated skin levels of Cr (mean level of 7.1 $\mu\text{g/g}$) much higher than levels seen in other marine mammals and humans without occupational exposure suggesting that their exposures to Cr may be significant (Wise et al., 2008). More work is needed to assess the potential risk of Cr(VI) induced carcinogenesis in these and other aquatic species.

In this study we focused on a standard 24 h exposure. Previous chromium levels in water have been reported as high as 0.365 μM (Aboul Dahab, 1989; Kamala-Kannan et al., 2008; Georgescu et al., 1988). Our lowest concentration studied here was 1 μM , which was weakly genotoxic and thus we would not expect a genotoxic response to Cr(VI) at 0.365 μM for 24 h. It is tempting then to speculate that fish may not face a risk of adverse effect as they are not in water that reaches a genotoxic effect. However, such a possibility is unlikely as we only considered 24 h exposures and longer exposure will likely cause damage at even lower doses.

Our genotoxicity data are consistent with the proposed mechanism of Cr(VI) toxicity, in which Cr(VI) enters cells via anion transport then is rapidly reduced to Cr(III) via a series of intermediates. Then Cr(III) and/or one or more of the metabolic intermediates induce chromosome abnormalities, DNA adducts and double-strand breaks (Singh et al., 1999; Xie et al., 2004; Wise et al., 2004; Holmes et al., 2005; Xie et al., 2005). The use of cell lines allows for deeper investigation into the cellular mechanism of Cr(VI) toxicity in fish and marine mammals, decreases the need to use living research animals, and adds to a powerful set of medaka-based tools for molecular to whole animal comparison and genotoxicity biomarker development. Our observations suggest that medaka is an excellent model for other aquatic species as well as humans. Further, studies to determine the extent of Cr (VI) exposure and its threat to aquatic animal health are needed along with comparisons of

medaka and whales with other endpoints to see if medaka are a good model in general or are limited to genotoxicity.

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Fig. 1.

The Frequency of Metaphases with Cr(VI)-Induced Damage in Medaka Cells Reflects the Frequency Observed in Both Whale Lung and Testes Cells. This figure shows sodium chromate induced a concentration-dependent increase in the percent of metaphases with damage in medaka (OLNHI-2) cells after a 24 h exposure. The level of damage in medaka cells reflected the amount of damage in North Atlantic right whale lung and testes fibroblasts. Data represent the mean of a minimum of 3 independent experiments $\pm$ standard error of the mean.

Fig. 2.

Cr(VI)-Induced Chromosome Damage Levels in Medaka Cells Reflects the Amount Induced in Both Whale Lung and Testes Cells. This figure shows sodium chromate induced a concentration-dependent increase in the percent of metaphases with damage in medaka (OLNHI-2) cells after a 24 h exposure. The level of damage in medaka cells reflected the amount of damage in North Atlantic right whale lung and testes fibroblasts. Data represent the mean of a minimum of 3 independent experiments $\pm$ standard error of the mean.

Fig. 3.

Representative Cr(VI)-induced chromosome aberrations in North Atlantic right whale and in medaka fin cells. This figure shows representative sodium chromate-induced chromosome aberrations in North Atlantic right whale and in medaka fin cells (1000× magnification). Chromatid lesions represented by dashed arrows and isochromatid lesions represented by solid arrows. (A) chromatid lesion in North Atlantic right whale lung cells; (B) isochromatid and chromatid lesion induced in North Atlantic right whale testes cells. (C) chromatid lesion induced at 10 μ M SC in medaka cells; (D) isochromatid and chromatid lesion induced in medaka cells.