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Hexavalent chromium induces oxidative stress and mitochondria-mediated apoptosis in isolated skin fibroblasts of Indo-Pacific humpback dolphin

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ABSTRACT

The increasing gas emissions and industrial wastewater discharge of anthropogenic hexavalent chromium (Cr (VI)) have been growing health concerns to the high trophic level marine mammals. Our previous studies showed that Indo-Pacific humpback dolphin (*Sousa chinensis*), stranded on the Pearl River Estuary region, contained exceedingly high levels of Cr in their skin-tissues. Unfortunately, the molecular toxic mechanisms on this mammal are absent, limiting our understanding of the eco-physiological impacts of Cr(VI) on dolphins. Thus, the cytotoxicity effects of Cr(VI) were analyzed on fibroblasts we isolated from the skin of *S. chinensis* (ScSF). This study showed that Cr(VI) markedly inhibited the viability of ScSF cells via induction of apoptosis accompanied by an increase in the production of reactive oxygen species and the population of G2/M arrest or apoptotic sub-G1 phase cells, up-regulation of *p53*, and activation of caspase-3. Further investigation on intracellular mechanisms indicated that Cr(VI) induced depletion of mitochondrial membrane potential in cells through regulating the expression of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) proteins, resulting in decrease of the ATP level, cytochrome c release from mitochondria into cytosol, and the activation of caspase-9. Furthermore, antioxidants N-acetylcysteine and vitamin C displayed chemoprotective activity against Cr(VI) via suppression of *p53* expression, indicating that the Cr(VI)-induced cell death may be mediated by oxidative stress. Overall, these results provide insights into the potential mechanisms underlying the cytotoxicity of Cr(VI) in Indo-Pacific humpback dolphin skin cells, offer experimental support for the proposed protective role of antioxidants in Cr(VI)-induced toxicity, and suggest that Cr(VI) contamination is one of key health concern issues for the protection of Indo-Pacific humpback dolphin.

1. Introduction

In coastal environments, heavy metal contamination has become a great public concern (Holmes et al., 2008; Martino et al., 2013; Savery et al., 2014; Young et al., 2015). Chromium(Cr) is considered as one of the ubiquitous pollutants in the marine environment, with its major source coming from industrial application and wastewater discharge. Although the two common stable oxidation states of Cr appear as trivalent chromium (Cr(III)) and hexavalent chromium (Cr(VI)) (Bregnbak et al., 2015), Cr(VI) seems to be the predominant state in the oceans (Chen et al., 2012). The toxicity of Cr(VI) as a carcinogen is generally significantly higher than that of Cr(III) (Elshazly et al., 2015; Huang et al., 2014), emphasizing Cr(VI) as the major health concern in the marine environment. In vitro cytological investigations have revealed that Cr(VI) exposure could induce oxidative stress, which might lead to the production and accumulation of reactive oxygen species (ROS) production accumulation via multiple pathways (Banu et al., 2011; Wise et al., 2008; Yao et al., 2008). High levels of ROS production could directly target lipid and DNA, causing lipid peroxidation and DNA damage with other cellular injuries, and finally leads to cell death by both apoptosis and necrosis (Bagchi et al., 2001; Zhitkovich, 2005).

Cetacean as top predators in marine ecosystems of the South China Sea have subjected to high levels of exposure to heavy metals including Cr. Heavy metals with high body burden are increasingly linked to the population decline or slow population recovery of cetaceans (Borrell et al., 2014). Indo-Pacific humpback dolphins (*Sousa chinensis*) is an endangered species in the Chinese coast with its population dramatically declined in recent years for uncertain reasons (Huang et al., 2012). The causes of population loss are considered mostly due to changes of environmental conditions, such as nutritional stress, reduced fecundity,

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and environmental contaminant. Cr(VI) could cause cytotoxic and genotoxic effects on whale and sea lion cells as well (Chen et al., 2009, 2012; Wise et al., 2008; Sr et al., 2009, 2010, 2011). Our previous study on *S. chinensis* from the Pearl River Estuary (PRE) reported a mean skin-tissue level of 3.01 μg g^{-1}, which is 9.7 times higher than the mean skin Cr level (0.31 μg g^{-1}) in humans who have no previous occupational exposure to Cr (Schroeder et al., 1970; Sun et al., 2017a). It suggests that the Indo-Pacific humpback dolphins might be exposed to Cr at potentially cytotoxic levels. However, the toxic mechanism and potential contamination effects of Cr(VI) on the cetaceans are little studied.

In addition to the dietary uptake by oral ingestion of contaminated food, the primary route of Cr exposure for cetaceans in seawater pollution is direct dermal contact with the aqueous contaminant. Among metals commonly coming into contact with the skin, Cr shows the highest sensitization and strongest reactions (Rudolf et al., 2005). Skin is a major target organ for Cr toxicity (Shelnutt et al., 2007). Cr(VI) could cause chrome ulcers, dermatitis and skin cancers (Bregnbak et al., 2015; Teklay, 2016). Human skin cells have been widely used as an in vitro experimental model to study the potential mechanisms underlying Cr(VI), showing that Cr(VI) induces cytotoxicity, clastogenicity, DNA double-strand breaks, and anchorage independence in the skin cells (Ha et al., 2004). However, the toxic mechanisms of marine mammal skins to Cr exposure are not well understood. Less studies have conducted the assays of cellular toxic effects of heavy metals in marine mammals, a rapid and straight forward method to unravel the toxic mechanisms. From our previous studies, Sun et al. (2017a) reported that the levels of Cr in epidermis of Indo-Pacific humpback dolphin from the PRE were exceedingly high. Thereof, the need to acquire the susceptibility responses of dolphins to Cr led us to investigate the cytotoxic potential of Cr(VI) on humpback dolphin cell lines, obtained from skin biopsies collected from free-ranging specimens from our group (Jin et al., 2013). This study is the first to explore the cytotoxic effects of Cr(VI) on humpback dolphin skin cells, a novel cell line from the marine mammal (Jin et al., 2013).

In this study we hypothesize that environmental Cr(VI) exposure to skin-tissues of cetaceans results in Cr accumulation, which could increase skin cells oxidative stress by disrupting antioxidant machinery and inducing apoptosis. The hypothesis was tested by (1) examining the cytotoxic effects of Cr(VI) on Indo-Pacific humpback dolphin skin fibroblasts; (2) assessing a correlation between Cr(VI) burden and apoptosis; (3) analyzing the relationship between Cr(VI) burden and responses of oxidative stress, and (4) uncovering the cytotoxic mechanisms of ROS and mitochondrial apoptosis.

2. Materials and methods

2.1. Materials

Potassium chromate (K_2CrO_4), propidium iodide (PI), N-acetylcysteine (NAC), vitamin C (VC) and bicinchoninic acid (BCA) for the protein determination were purchased from Sigma (St. Louis, MO, USA). The primary antibodies used against caspase 9, *p53*, Bax, Bcl-2, cyt c, and α-Tub were purchased from Beyotime Biotechnology (Shanghai, China). The ultrapure water used in all experiments, was supplied by a Milli-Q water purification system from Millipore (Billerica, MA, USA).

2.2. Cell culture and treatments

The ScSF cell lines, which were previously derived from the skin samples of a healthy Indo-Pacific humpback dolphin, were maintained as previously described (Jin et al., 2013) in Dulbecco's modified eagle's medium supplemented with penicillin-streptomycin (1×), non-essential amino acids (1×), and 15% fetal bovine serum, at 37 °C in a humid atmosphere containing 5% CO_2. The cell lines had been characterized by short tandem repeat profiling, cell morphology and karyotyping assays. The cells were tested routinely for mycoplasma contamination. All experiments were conducted on logarithmically growing cells. For all Cr(VI) exposure experiments, the cells were adjusted to 1×10^6 cells mL^{-1}. Incubations took place at 37 °C in a humidified 5% CO_2 atmosphere. Cells were exposed to Cr(VI) at concentrations of 0, 6.25, 12.5 or 25 μM in complete medium up to 24 h.

2.3. Cell viability assay

Cytotoxicity assays were carried out using the cell counting kit-8 (Beyotime, Shanghai, China). Briefly, ScSF cells were seeded with a density of 1×10^4 cells/well in a 96 well plate. After completion of the treatment period, 10 μL of CCK-8 solution was added to the plate and further incubated at 37 °C for 2 h, and then the absorbance was read at a wavelength of 450 nm using microtiter plate reader.

2.4. Cell cycle distribution analysis

Cell cycle distribution was monitored by flow cytometry. Briefly, cells treated with or without Cr(VI) were harvested by centrifugation and washed with PBS. Cells were stained with PI after fixed with 70% ethanol at −20 °C overnight. Labelled cells were washed with PBS and then analyzed by the flow cytometer. The proportions of cells in G0/G1, S, and G2/M phases were represented in DNA histograms. Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G1 peak. For each experiment, 10,000 events per sample were recorded.

2.5. Determination of mitochondrial membrane potential ($\Delta\Psi m$)

The $\Delta\Psi m$ of ScSF cells was determined using JC-1 assay and analyzed using flow cytometry. Cells in 6-well plates were trypsinized and resuspended in 0.5 mL of PBS (pH 7.4) buffer containing 10 μg mL^{-1} of JC-1. After incubation for 10 min at 37 °C in the dark, cells were immediately centrifuged to remove the supernatant. Cell pellets were suspended in PBS and then analyzed by flow cytometry under the excitation/emission at 488/525 (green) and 590 nm (red). The percentage of the green fluorescence from JC-1 monomers was used to represent the cells that lost $\Delta\Psi m$.

2.6. Caspase activity assay

Harvested cell pellets were suspended in cell lysis buffer and incubated on ice for 1 h. After centrifugation at 11,000 × *g* for 30 min, supernatants were collected and immediately measured for protein concentration and caspase activity. Briefly, cell lysates were placed in 96-well plates and then specific caspase substrates (Ac-DEVD-AMC for caspase-3 and Ac-IETD-AMC for caspase-8) were added. Plates were incubated at 37 °C for 1 h. Caspase activity was determined by fluorescence intensity under the excitation and emission wavelengths set at 380 and 440 nm, respectively.

2.7. Western blot analysis

First RIPA lysis buffer (50 mM TriseHCl, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF, 100 mM leupeptin, and 2 mg/mL aprotinin, pH 8.0) was used to extract total cellular proteins and then the protein extracts were resolved by loading equal amounts of protein, in 10% SDS-PAGE gel, per lane. They were then put onto Immobilon-P PVDF transfer membranes (Millipore, Bedford, MA, USA) by electroblotting. As a final step, they were blocked with 5% non-fat milk in TBST on a shaker at room temperature for 1 h. After that, the membranes were probed by primary antibodies (Cell signaling, Danvers, MA, USA) at a dilution ratio of 1:1000 in 5% nonfat milk at 4 °C overnight, and by secondary antibodies, conjugated with