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Determination of the immunotoxic potential of heavy metals on the functional activity of bottlenose dolphin leukocytes *in vitro*

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Abstract

Heavy metals may affect the immune system of cetaceans. But no information exists on their effects on the bottlenose dolphin (*Tursiops truncatus*) immune system, although this species is a coastal top predator which can bioaccumulate high concentrations of them. This work studies the effects of Hg (1, 5 and 10 mg/L), Al (2,5, 25 and 50 mg/L), Cd (1, 10, 20 and 40 mg/L), Pb (1, 10, 20 and 50 mg/L) and Cr (1 and 10 mg/L), on the function of phagocytes and lymphocytes isolated from the peripheral blood of bottlenose dolphins under *in vitro* conditions. Cell viability, apoptosis, lymphocyte proliferation and phagocytosis were evaluated. Viability and lymphoproliferation were measured with Alamar Blue assay, and apoptosis and phagocytosis were evaluated with flow cytometry. Apoptosis was detected as mechanism of cell death after cadmium and mercury exposure. A significant reduction in the lymphoproliferative response was registered by exposure to 1 mg/L of mercury, 10 mg/L of cadmium and 50 mg/L of lead. Decreased phagocytosis was also observed at 5 mg/L of mercury, 50 mg/L of aluminium and 10 mg/L of cadmium. Chromium did not present any effects on any immune assay at the concentrations tested. The concentrations of heavy metals that were found to affect the functional activity of bottlenose dolphin leukocytes are within the environmental ranges reported in the tissues of bottlenose dolphins. These results support the hypothesis that exposure to these contaminants, particularly mercury and cadmium could lead to a reduction in host resistance to disease in these animals.

Keywords: Immunotoxicity; Bottlenose dolphin; Heavy metals; Viability; Apoptosis; Lymphoproliferation; Phagocytosis

1. Introduction

The bottlenose dolphin (*Tursiops truncatus*) is a coastal predator at the top of the marine food chain, so it constitutes an excellent indicator of the health of the marine ecosystem (Wells et al., 2004). This dolphin species is considered a fish and squid eater (Culik, 2004). It tends to bioaccumulate high levels of persistent contaminants present in the marine environment, such as heavy metals (Law et al., 1991). Various studies have documented high levels of these chemicals in bottlenose dolphins in different parts of the world (Cardellicchio et al., 2000, 2002; Frodello et al., 2000; Parsons and Chan, 2001; Roditi-Elasar et al., 2003; Carballo et al., 2004). The available literature mainly reports concentrations in liver and kidney.

Heavy metals are known to produce toxic effects on animals. The most sensitive target tissue affected by some toxins (or toxic components) is the immune system (Black et al., 1992; Raszyk et al., 1997). Extensive experimental investigations have shown that different heavy metals, such as mercury, cadmium, lead,

Abbreviations: PI, propidium iodide; ppm, parts per million; SI, stimulation index.

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chromium or aluminium can cause immunomodulation in laboratory animals and also in humans (Koller, 1979; Descotes, 1988; Bernier et al., 1995; Lawrence and McCabe, 2002; Shrivastava et al., 2002; Synzynys et al., 2004). But to assess a possible immunosuppression induced by environmental xenobiotics in cetaceans, information on the functioning of the immune system of these species is needed. So, in the last few years, important efforts have been made to adapt methods for *in vitro* evaluation of the immune function in cetaceans (Romano et al., 1992; De Guise et al., 1995, 1996a, 1997; Noda et al., 2003; Beineke et al., 2004) and models using *in vitro* exposure of immune cells of marine mammals have been developed. These immunotoxicological assays have shown that different contaminants may cause immunosuppression in different marine mammals using parameters such as mitogen-induced proliferation, phagocytosis and natural killer cytotoxicity (De Guise et al., 1996b; Pillet et al., 2000; Nakata et al., 2002; Lalancette et al., 2003; Levin et al., 2004; Mori et al., 2006).

Most of the studies in cetacean refer to the immunotoxicological potential of organochlorine compounds, but the effect of heavy metals on the immune function of cetaceans was only investigated in the beluga whale (*Delphinapterus leucas*) (De Guise et al., 1996b). But no studies have investigated the effects of heavy metals on the immune system of bottlenose dolphins.

The aim of this study was to determine the action of five heavy metals (Al, Cd, Cr, Hg and Pb), on the function of phagocytes and lymphocytes isolated from the peripheral blood of bottlenose dolphins under *in vitro* conditions. These five heavy metals were chosen due to their previous detection in bottlenose dolphin tissues in many geographical areas, mainly in places of industrialized world (Das et al., 2003). Also their potential immunological impact in terrestrial and marine mammals has been considered for Hg, Cd and Pb, but still very little information is available about the immuno-modulating effect of Cr and Al. The immunotoxicity in this study was assessed using different parameters (cell viability, apoptosis, lymphocyte proliferation and phagocytosis).

2. Materials and methods

2.1. Toxics tested

Five heavy metals were selected for the study: mercury ($HgCl_2$, purity 99.9%), aluminium ($AlCl_3$, purity 99.9%), cadmium ($CdCl_2$, purity 99.9%), lead ($PbCl_2$, purity 99.9%) and chromium ($CrCl_2$, purity 95%). All were purchased from Sigma–Aldrich Chemical Co. Concentrated stock solutions (10 g/L) were prepared by dilution in distilled water. All the toxics were then diluted in cell culture medium (RPMI-1640) with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL sptreptomycin, 2 mM L-glutamine, 10 mM HEPES and 1% non-essential aminoacids (Sigma). The final incubation concentrations of metals were as follows: mercury, 1, 5 and 10 mg/L (ppm); aluminium, 2.5, 25 and 50 mg/L; cadmium, 1, 10, 20 and 40 mg/L; lead, 1, 10, 20 and 50 mg/L; and chromium, 1 and 10 mg/L.

2.2. Animals

Blood samples were collected from nine captive bottlenose dolphins from four aquariums in Spain (Zoo Aquarium Madrid, Zoo Barcelona, L'Oceanográfic Valencia and Marineland). All individuals were healthy animals of both sexes and without haematological signs of disease. Blood samples were drawn from the ventral tail fluke into heparinized tubes and were kept at 4° C until the time of analysis, within 24 h after collection. Each assay was performed three to five times, depending upon blood availability.

2.3. Isolation of cells

2.3.1. Peripheral blood mononuclear cells (PBMCs)

For assessment of lymphocyte viability, apoptosis and proliferation, the PBMCs were isolated following protocols designed by De Swart et al. (1993). Briefly, blood was diluted 1:1 with RPMI-1640 medium. The diluted blood was then carefully layered over a commercially available density gradient (Histopaque 1077, Sigma) and centrifuged at $400 \times g$ at room temperature for 30 min. The PBMC layer was carefully collected, and the recovered cells were washed twice in PBS at $200 \times g$ for 7 min at 4 °C. The cells were then resuspended in cell culture medium described previously, and their viability was determined by the trypan blue exclusion test. Cell viability prior to assays was >95%.

2.3.2. Peripheral blood leukocytes

For evaluation of phagocytosis, leukocytes were isolated from peripheral blood adding a solution of NH_4Cl for lysis of erythrocytes. The cells were then washed three times in PBS and suspended in supplemented RPMI. Viability of the cells was