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Heavy metal toxicity of kidney and bone tissues in South Australian adult bottlenose dolphins (*Tursiops aduncus*)

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ABSTRACT

Metallothioneins (MT) concentration, renal damage, and bone malformations were investigated in 38 adult *Tursiops aduncus* carcasses to determine any associations with cadmium, copper, zinc, mercury, lead and selenium. Significantly higher concentrations of cadmium, copper, and zinc in the liver were observed in dolphins showing evidence of more advanced renal damage. No significant differences in metal or selenium concentrations in the liver were observed between groups differing in level of bone malformations. Some dolphins displayed evidence of toxicity and knowledge of metal toxicity pathways were used to elucidate the cause of these abnormalities. Two dolphins had high metal burdens, high MT concentrations, renal damage, and evidence of bone malformations, indicating possible severe and prolonged metal toxicity. One dolphin showed evidence of renal damage, but the lack of any other symptoms suggests that this was unlikely to be caused by metal toxicity. We recommend examining a range of metal toxicity symptoms simultaneously to aid in distinguishing metal toxicity from unrelated aetiologies.

1. Introduction

Metal concentrations have been recorded in over 60 species of cetaceans (O'Shea, 1999) but few studies have explored the relationship between metals and toxicity. Metal toxicity causes irregular metallothioneins (MT) protein synthesis, renal damage, and disruption of bone structure in humans and wildlife (Beiglbock et al., 2002; Jarup, 2002; Sato and Kondoh, 2002). Studies of metal toxicity in cetaceans, particularly beach-stranded carcasses, are hindered by difficulties arising from examining tissues that have undergone post-mortem autolysis. Since these are usually the only specimens available, it is important that methods of measuring metal toxicity in decomposed carcasses are developed to allow determination of dolphin health, even in degraded specimens.

Metallothioneins (MT) are metal-binding proteins that preferentially bind to Cd (cadmium) and Zn (zinc), reducing the bioavailability of these elements (Sato and Kondoh, 2002; Das et al., 2006) and providing an early indicator of cellular responses to metal toxicity (Petering et al., 1990). Although MT are often correlated with liver and kidney metal burdens, few attempts have been made to correlate them with markers of heavy metal toxicity in cetaceans. This is an absolute prerequisite to support the use of MT overexpression as a bioindicator of metal toxicity.

Metals are toxic to mammalian renal cells (Wang and Pfeiffer, 2001), causing damage that leads to leakage of phosphates, calcium, glycogen and proteins (proteinuria) from the kidney (Loghman-Adham, 1997). Histological indicators of proteinuria have been used as a preliminary measure of renal pathology in *Tursiops aduncus* in South Australia (Long et al., 1997) but may have been impacted by post-mortem autolysis. In contrast, metal-induced swelling of the Bowman's capsule, and protein leakage within an intact capsule, are not affected by post-mortem decomposition (Beiglbock et al., 2002).

While MT and renal structure swelling may determine toxicity in mildly degraded specimens, bone structure is resistant to post-mortem degradation. Loss of bone mineral structure occurs in cases of Cd, Pb (lead), and Hg (mercury) toxicity in humans

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(Escribano et al., 1997; Jarup, 2002; Suzuki et al., 2004) while increased and decreased bone mineral density have been observed in response to Zn and Se (selenium) excess and deficiency, respectively, (Turan et al., 2000; Yun and Zeng-Li, 2002). Loss of bone density is a clinical endpoint marker of chronic metal toxicity and indicates accompanying sub-clinical behavioural and immunological deficits (Staessen et al., 1999) which are difficult to examine directly in free-living cetaceans.

The causes of elevated MT, renal damage, and loss of bone density and complexity are probably interrelated. MT induced by metal toxicants form a large metal-MT complex which leads to damage of renal structures. This results in leakage of calcium, phosphate and proteins from the kidney, hindering bone remodelling and leading to a loss of bone density (bone mineral density) and complexity (bone histomorphometry) (Alfven et al., 2002). Thus, dolphins displaying metal-induced bone malformations should show symptoms of elevated MT synthesis and damage of renal structures, assuming these markers are reflecting metal toxicity and not extraneous influences (e.g., post-mortem cellular degradation, unrelated parasitism, unrelated disease, normal mammalian ageing process). Consistency between MT, renal damage and bone structure should provide a valuable tool for helping to distinguish metal-induced toxicity from other aetiologies.

South Australian adult Indo-Pacific bottlenose dolphins, *T. aduncus,* have been shown previously to have high concentrations of Cd, Hg, and Se in the liver, moderate concentrations of Pb, Cu, and Zn in the liver and moderate concentrations of Cd and Pb in bone compared to dolphins elsewhere (Lavery et al., 2008). This study aims to explore any links between MT, renal damage, bone density and structure, and liver tissue concentrations of Cd, Hg, Pb, Zn, Cu and Se in adult bottlenose dolphins.

2. Materials and methods

2.1. Necropsy procedures and sample selection

Necropsy procedures and determination of Se and metal concentrations by inductively coupled plasma atomic emission spectrometry in 71 *T. aduncus* from South Australia are detailed elsewhere (Lavery et al., 2008). All dolphins were collected by the South Australian Museum (SAMA) and given a museum identification number. Some dolphins had multiple analyses conducted on one tissue, and the mean metal or Se value was used in these cases. Since animal age impacted metal accumulation in these dolphins, only a subset of 38 sexually and/or physically mature adults of tooth category three and above (see Kemper and Gibbs, 2001, for details of dolphin development compared to tooth category) were examined in the present study. This sample of 38 adult dolphins consisted of 17 male and 21 female adult dolphins, 16 of which had kidney samples with intact nuclei and glomeruli available for histopathological examination (Geraci and Lounsbury 1993, decomposition code two and three). Kidney ($N = 15$) and liver ($N = 14$) samples from a subsample of these animals were analysed for MT. Bone density was measured in four vertebrae of 30 animals and the histomorphometry of costal rib tips examined from 15 animals.

2.2. Metallothionein quantification

Soft tissues were collected following procedures that minimise cross contamination (Geraci and Lounsbury, 1993). Subsampling of frozen (−20 °C) tissue entailed collecting approximately 0.5 g of liver and kidney for MT analysis. MT was quantified using the modified cadmium-haemoglobin affinity assay (Eaton and Toal, 1982). Briefly, this methodology requires adding radioactively labelled ^{109}Cd to the tissue supernatant, allowing it to bind to MT present. A 'Cobra Auto-gamma' counter (Packard Company) was used to determine the Cd content of the resultant supernatant, which allows for determination of the equivalent MT concentration, expressed as nmol of Cd bound g^{-1} wet weight.

2.3. Histological examination

Histology was carried out on 16 formalin-fixed kidney tissues: 15 samples were previously frozen (−20 °C) and one fixed without prior freezing (SAMA: M21243). Haematoxylin and eosin (H and E) preparations were carried out on each of the 16 kidney samples using standard histological methodology (Brancroft and Stevens, 1982). An Olympus BH-2 light microscope was used with 20× magnification. Digital microscope images of 60 glomeruli and Bowman's capsules observed were captured, and software (Motic Images Plus, Version 2.0) was used to measure the area of 20 randomly selected glomerulus and Bowman's capsules from each slide. The area of the space between the glomeruli and Bowman's capsule, and the presence of proteins within each Bowman's capsule was recorded.

2.4. Quantification of bone density and structure

Bone strength depends on the amount of bone and the organisational structure of the trabeculae (Kleerekoper et al., 1985), so two measures of bone density were obtained; Dual energy X-ray Absorptiometry (DXA) and histomorphometry. DXA measures the total amount of bone (cortical and trabecular bone), but skeletal metal stores can bias results (Puzas et al., 2002). Histomorphometry directly quantifies the three-dimensional micro-architectural structure and organisation of bone and is not biased by metal burden.

DXA scans were performed on a GE Lunar Prodigy Vision Dual X-ray Absorptiometry, GE Lunar Madison, Wisconsin. Four caudal vertebrae from each of 30 animals were analysed to obtain measurements of bone mineral density, BMD (g cm^{-2}), and bone mineral content, BMC (g). Caudal vertebrae were selected on the basis of being the most posterior four caudal vertebrae with small transverse processes. Small animal software (version 8.10.027) on standard settings was used to examine a maximum area of 25 × 25 cm. Vertebrae were laid longitudinally to best simulate the longitudinal stress placed on the caudal region of the spine by powerful dorsal and ventral muscles associated with swimming. Bones were laid on a 1 cm thick Lucite tissue equivalent block and scanned with parameters set at 76 kilovolts and 150 μA. Pixel size was 1.05 × 0.6 mm. BMD and BMC of the vertebrae and posterior process (but not the transverse processes) were determined.

Histomorphometry was performed using a Skyscan 1072 X-ray Microtomograph on the largest costal rib available from each of 15 animals. A piece 2 cm long was sampled from the largest end of the bone and placed in the X-ray Microtomograph with the cut facing down. Pixel size was 18.95 μm while the X-ray parameters were set at 16× magnification, 100 kilovolts and 98 μA. A ring artefact correction was used and a beam hardening correction of 100% was set. Cone reconstruction software was used to reconstruct the three-dimensional structure of the bone from 1,000 separate two-dimensional cross section images. Bone density parameters from the three-dimensional reconstructions were calculated using CTan software (Skyscan). Density parameters calculated by CTan are shown in Table 1.

2.5. Statistical analyses

Our primary aim was to determine if metal concentrations were associated with markers of toxicity. To examine the influence of metal concentrations on kidney structures a hierarchical cluster analysis (data not shown) was employed which sorted 14 individ-