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Investigation of silver (Ag) deposition in tissues from stranded cetaceans by autometallography (AMG)☆

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ABSTRACT

Silver, such as silver nanoparticles (AgNPs), has been widely used in commercial products and may be released into the environment. The interaction between Ag deposition and biological systems is raising serious concerns because of one health consideration. Cetaceans, as the top predators of the oceans, may be exposed to Ag/Ag compounds and suffer negative health impacts from the deposition of these compounds in their bodies. In the present study, we utilized autometallography (AMG) to localize the Ag in the liver and kidney tissues of cetaceans and developed a model called the cetacean histological Ag assay (CHAA) to estimate the Ag concentrations in the liver and kidney tissues of cetaceans. Our results revealed that Ag was mainly located in hepatocytes, Kupffer cells and the epithelial cells of some proximal renal tubules. The tissue pattern of Ag/Ag compounds deposition in cetaceans was different from those in previous studies conducted on laboratory rats. This difference may suggest that cetaceans have a different metabolic profile of Ag, so a presumptive metabolic pathway of Ag in cetaceans is advanced. Furthermore, our results suggest that the Ag contamination in cetaceans living in the Northwestern Pacific Ocean is more severe than that in cetaceans living in other marine regions of the world. The level of Ag deposition in cetaceans living in the former area may have caused negative impacts on their health condition. Further investigations are warranted to study the systemic Ag distribution, the cause of death/stranding, and the infectious diseases in stranded cetaceans with different Ag concentrations for comprehensively evaluating the negative health effects caused by Ag in cetaceans.

1. Introduction

Commercial products containing silver (Ag) have been used for over 100 years, and awareness of Ag contamination in the environment has markedly increased due to the extensive use of silver nanoparticles (AgNPs) (Nowack et al., 2011). AgNPs have been used in numerous commercial products, such as water filters, textiles, cosmetics, food packaging and medical items, mainly due to their strong antimicrobial properties (Yu et al., 2013). AgNPs also have unique physicochemical properties, such as high electrical and thermal conductivities, so they are increasingly applied in electronic devices and medical imaging (Ajmal et al., 2016; Ge et al., 2014). The production of AgNPs and the number of AgNP-containing products has dramatically increased in the last decade and is expected to increase over time (Hansen et al., 2016; Vance et al., 2015). AgNPs can be released during the production, transport, erosion, washing, and/or disposal of AgNP-containing products, subsequently draining into the aquatic environment and ultimately accumulating in the ocean (Farre et al., 2009; Walters et al., 2014). The fate of AgNPs in the aquatic environment is complicated and variable. Previous studies indicated that AgNPs in the aquatic environment can remain as individual particles in suspension, aggregate, dissolve, react with different species in the environment, or be regenerated from silver ions (Levard et al., 2012; Massarsky et al., 2014). Furthermore, different types of Ag speciation, such as AgCl, Ag_2S, and Ag_0, can be found in marine

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sediments contaminated with AgNPs, where they are ingested by benthic organisms and subsequently enter the food chain in marine environments (Wang et al., 2014). The increasing use and growing production of AgNPs, as potential sources of Ag contamination, raise public concern about the environmental toxicity of Ag.

Ag can be transferred from one trophic level to the next via the food chain and may cause negative effects on the animals at higher trophic levels, such as cetaceans (Buffet et al., 2014; Farre et al., 2009; Wang et al., 2014), and cetaceans have been thought to suffer potentially detrimental impacts from excessive silver exposure (Chen et al., 2017). The Ag concentrations in cetaceans have been investigated by several studies in different countries, and their results have varied among different organs, age classes, animal species, and habitats (Dehn et al., 2006; Reed et al., 2015; Romero et al., 2017; Seixas et al., 2009). The levels of unhealthy and critically dangerous concentrations of Ag in small cetaceans of the North Pacific Ocean have been established, and the suggested threshold concentrations of Ag are 0.43 ± 0.28 and 0.08 ± 0.03 μg/g dry weight for liver and kidney tissues, respectively (Chen et al., 2017). A recent study conducted in Taiwan found an extremely high Ag concentration (726.11 μg/g dry weight) in the liver tissue of a stranded Fraser's dolphin (*Lagenodelphis hosei*), which implies that dolphins in the marine environment of the North-western Pacific Ocean may have severe Ag contamination (Chen et al., 2017). Cetaceans are longevous, and being at the highest trophic levels of marine ecosystem, and they share common food resources with humans. The bioaccumulation effect of anthropogenic contaminants in cetaceans may eventually occur in humans (Bossart, 2011). These reasons further support that cetaceans are ideal sentinel animals for evaluating the health of marine environments and humans. Hence, it is crucial to determine the contamination status (e.g., tissue concentrations and distribution) of Ag in cetaceans.

Generally, the concentrations of trace metals in cetacean tissues are determined by inductively coupled plasma mass spectroscopy (ICP-MS) (Caceres-Saez et al., 2013; Chen et al., 2017; Mendez-Fernandez et al., 2014; Romero et al., 2017). The advantages of using ICP-MS include the provision of quantitative data with favourable detection limits (0.01–0.1 μg/L), simple specimen preparation, and the capability of simultaneous measurement of several elements (Nuttall et al., 1995). However, ICP-MS still has some disadvantages. The capital cost for establishing the ICP-MS and sample storage (including instruments, electricity charges and consumables) are relatively high (Bornhorst et al., 2005; Nuttall et al., 1995). In addition, ICP-MS detects target contaminants only on the organ level, and not the histological location or cell level (Miller et al., 2016). The standard procedure of tissue samples collection from stranded cetacean for ICP-MS analysis usually requires a relatively large sized frozen tissue sample ($6 \times 6 \times 6$ cm, approximately 200 g) due to the possibility of contamination during sample collection in the field environment (Geraci and Lounsbury, 2005), and these frozen samples may not be easy to store in limited refrigeration space. Furthermore, complete sample collection from the stranded cetaceans were seriously limited by several factors including difficulties of logistics and shortage of manpower. Therefore, the samples can be collected are usually the formalin fixed samples. If a relatively rapid, easy to use and inexpensive methodology by using the formalin fixed samples is developed, it will facilitate investigation of the suborgan distribution and concentration of target contaminants in cetaceans.

Formalin-fixed, paraffin-embedded (FFPE) tissues can be a sample resource for molecular analysis (such as polymerase chain reaction) and metal measurements (Bischoff et al., 2008; Bonta et al., 2017; Kokkat et al., 2013; Tran et al., 2014). Bonta et al. (2017) found that the FFPE process caused severe alteration in the suborgan distributions and concentrations of alkali and alkaline earth metals but led to lesser effects on those of transition metals. In addition, previous studies have indicated that heavy metals can be amplified in FFPE tissue sections by autometallography (AMG), which is a histochemical process, and thereby can be visualized under light microscopy (Anderson et al., 2015; Danscher, 1991; Kim et al., 2009; Miller et al., 2016). Although the AMG method may have a relatively low sensitivity (comparing to ICP-MS), difficulty to unveil a homogenously diffused material, underestimation of the content in case of a great concentration of heavy metals in a narrowed surface, it is still a valuable method to study the suborgan distribution of heavy metals. Furthermore, AMG method may amplified a group of trace metals, including gold, silver, mercury, bismuth and zinc, and thus the results of AMG method may be interfered by other trace metals (Stoltenberg and Danscher, 2000). Therefore, the interpretation of AMG positivity signals in the tissue from wild animals (which are not an intentional and well-controlled exposure to a single product) should be incorporated with other specific methods to monitor the actual composition of heavy metals, such as ICP-MS (Stoltenberg and Danscher, 2000). The quantitative analysis of histological tissue sections with histochemical staining has been developed by the use of digital image analysis software, such as imageJ (Deroulers et al., 2013; Jensen, 2013; Parlee et al., 2014; Shu et al., 2016). The present study utilized the histochemical technique (autometallography; AMG) to localize Ag in cetacean tissues, investigated the histopathological lesions possibly caused by the Ag, and developed an assay to estimate the Ag concentration in the liver and kidney tissues of cetaceans by a regression model based on the data from image quantitative analysis and ICP-MS.

2. Materials and methods

2.1. Sample source

The research permit (104-07.1-SB-62) for the cetacean sample collection was provided by Council of Agriculture of Taiwan. From 1999 to 2016, liver and kidney tissues from 110 stranded cetaceans of 7 different species, including 22 *Feresa attenuata* (Fa), 5 *Grampus griseus* (Gg), 38 *Kogia* spp. (Ko), 13 *Lagenodelphis hosei* (Lh), 13 *Stenella attenuata* (Sa), 8 *Steno bredanensis* (Sb), and 11 *Tursiops truncatus* (Tt), were collected. A field number was given to each cetacean for individual identification. The liver and kidney tissues used in the present study were from freshly dead and moderately autolysed stranded cetaceans (Geraci and Lounsbury, 2005). Some liver and kidney tissues were collected from live stranded cetaceans after they died during rescue or rehabilitation efforts. Each individual was classified into 1 of 2 age classes (young or adult) by relative measures of age, such as body length, tooth wear, the presence of hair follicles on rostrum and lingual marginal papillae, skin colour, the status of reproductive organs, and/or fusion of cranial sutures (Hohn, 2009), since age determination by the growth layers of teeth was not done in all individuals. The biological characteristics of each cetacean species are summarized in Table 1.

In total, 220 formalin fixed tissue samples (110 from liver and 110 from kidney) were collected for subsequent histological analysis. Among these 110 stranded cetaceans, only 12 frozen tissue samples (6 from liver and 6 from kidney) were collected, put into zip-lock plastic bags, and stored at −20 °C for determination of Ag concentrations by ICP-MS.

2.2. AMG reactivity of formalin-fixed tissues

The representative formalin-fixed tissues of liver and kidney