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REVIEW

A Greater Capacity of Metallothionein Synthesis: Would it be Sufficient as Explanation for the Absence of Pathological Effects in Marine Mammals with Extremely High Cd Concentrations? A Review

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Abstract:

Several studies have shown that marine mammals, especially the oceanic ones, present extremely high Cd concentrations. As the phenomenon of Cd transfer from cephalopods to cetaceans seems to be natural, the most plausible reason for the perpetuation of these predator species is related to adaptations to this high exposure to Cd during the evolution process. The most widely accepted explanation is that these mammals present a greater capacity of metallothionein (MT) synthesis. However, renal physiology studies have shown that the Cd-MT complex is 100-fold more toxic than cadmium chloride. In this context, attributing the resistance of these animals to cadmium to the abovementioned higher capacity of MT production is obviously insufficient and the present study discuss in detail how unsatisfactory this explanation is.

Keywords: cadmium; marine predators; detoxification; adaptations; toxicity

1. Introduction

Cadmium (Cd) is widely distributed, presenting average concentrations varying from 0.1 to 0.2 μg.g^{-1}. These low concentrations turn cadmium into the 67th element on the earth crust in terms of abundance [1]. However, extremely high Cd levels can be found in cephalopods and their predators, such as squid-eating (teutophagous) marine mammals [2, 3, 4, 5].

Naturally, high cadmium concentrations are found in squids and octopuses [4, 6, 7]. Such mollusks accumulate elevated levels of heavy metals, especially Cd, in the digestive gland. Cadmium accumulation by squids occurs regardless of their exposure to anthropogenic contamination [4, 7] and these cephalopods end up transferring this metal to cetaceans, as they constitute an important source of food for these mammals [2]. As the exposure of cephalopod predators to Cd has occurred during the evolution process, adaptations are expected. Considering that there is growing evidence that Cd levels in air, water and soils have been multiplied in many parts of the world as a result of anthropogenic activities [1], understanding how some mammal species are able to deal with a high exposure to this toxic element becomes a subject of increasing importance.

2. Cadmium Toxicity

The interest in understanding the behavior of cadmium in the environment comes from the fact that it is a toxic element. Cadmium has affinity for hydroxyl, carboxyl, phosphatidyl, cysteinyl and histidyl side chain groups of proteins, purines and porphyrins, and may interfere in different

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metabolic processes including oxidative phosphorylation pathways [8]. Many of the toxic effects of cadmium are due to the fact that this element interacts or competes with essential metals. In animal studies, for example, it has been observed that high levels of this element in the diet depress the absorption and alter the tissue distribution of copper [9, 10]. Furthermore, a decrease in weight gain induced by excess cadmium in the diet of mice and rats is largely overcome by zinc and copper supplementation [11]. Cadmium appears to displace zinc in many vital enzymatic reactions, causing interference or even disruption of the enzyme activity [12]. References to the interactions between cadmium and manganese [13, 14], as well as between cadmium and selenium [15] can also be found in the literature. As for cadmium interference in iron metabolism, the development of anemia due to the administration of the former metal in some studies involving birds and mammals has also been demonstrated [9, 11], but the mechanisms for this effect have not been fully elucidated. High cadmium intake interferes with iron absorption, possibly due to competition for binding sites in proteins in the intestinal mucosa. To further explain anemia as one of the effects caused by cadmium, it would be important to consider other relations between the two metals concerning their toxicokinetics. It has been shown that iron-deficient animals may have a higher cadmium uptake [16], and these findings have been confirmed in humans [17, 18]. Women with low body iron stores, as reflected by low serum ferritin levels, had on average a gastrointestinal absorption rate of cadmium (about 10%) two times higher than women from a control group [19]. Iron deficiency causes inhibition of ferritin synthesis and it constitutes stimulus to the synthesis of the receptor protein that binds to the iron-transporting protein (transferrin) [20]. It is then plausible to assume that cadmium binds to the transferrin receptor protein, as this would explain the higher cadmium absorption rate in iron deficient individuals.

A number of toxic effects from exposure to cadmium were observed through laboratory experiments. However, in the most extreme example of the influence of this element on human health, Fanconi's syndrome was the observed effect [21].

3. Cadmium Toxicokinetics and Itai-itai Disease

The most dramatic example of human exposure to Cd involved an endemic disease observed in residents of the Jinzu River Basin, in the Municipality of Toyama, Japan. The infirmity has received the name of Itai-itai disease because it is the way Japanese people vocalize pain. Therefore, for didactic purposes, it can be mentioned that if the disease had occurred in an English-speaking region, it would have been called ouch-ouch disease. The Itai-itai disease represented the most severe stage of chronic intoxication by cadmium from environmental exposure [22]. Although it has been impossible to pinpoint the number of casualties from Itai-itai disease over the years, it is estimated that approximately one hundred deaths have occurred as a result of this disease by the end of 1965 [23].

Although the incidence of Itai-itai disease has been high since 1936 [24], the connection between this condition and the consumption of cadmium contaminated rice was only made in 1968, when the Ministry of Health of Japan attributed Itai-Itai Disease to cadmium pollution originating from zinc mines and refineries located at the head of the Jinzu River. It was concluded that this water system was polluted by cadmium, with its Cd-rich waters being used in the cultivation of rice, the main food in the region. This scenario generated a high cadmium intake by that population, which has led to the development of the Fanconi's syndrome, generating osteomalacia in a number of inhabitants [22].

In the mentioned syndrome, there is a generalized dysfunction of the proximal tubule, without any primary glomerular involvement. When the numerous functions of this segment of the renal tubule are taken into account, it is possible to have a more approximate notion of the damages caused by cadmium intoxication. The proximal tubule is responsible for the reabsorption of almost all the filtered burden of glucose, amino acids and bicarbonate, as well as of most of the filtered burden of sodium, water, chloride and phosphate. Although many grams of protein are filtered daily, under physiological conditions urine is virtually depleted of these molecules due to the highly efficient apical

endocytic apparatus presented by the proximal tubule [25, 26, 27]. Thus, in Fanconi's syndrome the clinical symptoms observed are all related to impairments of the abovementioned functions, *i.e.*, variable degrees of losses of PO_4^{3-}, HCO_3^-, amino acids and glucose by the proximal tubule. The clinical presentation in children includes rickets and poor growth, as well as, in adults, it includes osteomalacia and osteoporosis. In addition, polyuria, renal salt loss, hypokalemia, acidosis, hypercalciuria and low molecular weight proteinuria may also be part of the clinical spectrum. All the mentioned symptoms can be explained by an insufficiency in the absorption of solutes by the proximal tubule. This reabsorption function is, for all the aforementioned solutes, directly or indirectly coupled to the action of Na^+-K^+-ATPase located in the basolateral membrane of the proximal tubule cell [25, 26, 27]. The osteomalacia observed in Itai-itai disease would have occurred as a consequence of Cd-induced damage to the renal cortex, which probably hampered the occurrence of the second hydroxylation of vitamin D in the affected cells [28].

It is known that normal calcium absorption in the intestines and normal bone mineralization are dependent on 1,25-dihydroxycholecalciferol. Once synthesized on the skin by UV rays from 7-dehydrocholesterol, cholecalciferol (vitamin D3) is converted to 25-hydroxy-cholecalciferol in the liver, and then to 1,25-dihydroxy-cholecalciferol in the mitochondria of renal proximal tubule cells. The latter molecule is the biologically active chemical species of the vitamin D cascade. Cadmium accumulates in proximal tubule cells, depressing cellular functions and this may result in reduced conversion of 25-hydroxy-cholecalciferol to 1,25-dihydroxy-cholecalciferol. This is likely to lead to reduced calcium absorption and, consequently, to a progressive demineralization of the bone matrix, due to the hyperparathyroidism generated [29]. Understanding why kidney is considered the target organ requires comprehension of cadmium toxicokinetics in mammals. Once in plasma after gastrointestinal absorption, cadmium is largely bound to albumin and the Cd-albumin complex is absorbed by hepatocytes. In the liver, cadmium can bind to glutathione - a tripeptide consisting of the amino acids glycine, cysteine and gamma-glutamic acid - and be excreted through bile. More importantly, cadmium can bind to metallothionein and be stocked. Part of the cadmium-metallothionein complex (Cd-MT) leaks into plasma and is then rapidly removed therefrom by glomerular filtration, followed by reabsorption by proximal tubule cells. Much of this cadmium once dissociated from the Cd-MT complex by lysosomal digestion is complexed to metallothionein pre-synthesized by proximal tubule cells, but part of it will cause injury, especially when a critical Cd concentration is reached in the kidney [30].

The Itai-itai disease has aroused the world public interest for controlling and better understanding how and to what extent human activities cause redistribution and, consequently, the punctual concentration of cadmium, which occurs naturally in the earth's crust.

Regarding the distribution of cadmium in the tissues of the mammal species submitted to the highest environmental exposure to this metal, the investigations pointed to a general toxicokinetic model for all mammals. All the studies consulted contemplating Cd determination in at least two organs, liver and kidney, demonstrated the latter organ to be the main Cd accumulation site in cetaceans. These studies have dealt with twenty different cetacean species [5, 31, 32, 33, 34, 35, 36, 37] and their findings lead to the idea that the biokinetics model for cadmium adopted for humans and laboratory mammals is also applicable to cetaceans.

4. Cephalopods as Vectors of Cadmium Transfer to Cetaceans

Since food consumption is the main route for environmental exposure to cadmium, the first question would be about which contaminated food items cetaceans are ingesting for generating renal Cd concentrations as high as 960.0 $\mu g.g^{-1}$ (wet weight) [3]. The even higher Cd concentrations (up to 2,562.0 $\mu g.g^{-1}$, w.w.) found in cephalopods seem to constitute an obvious answer [4].

As cephalopods are mollusks, they are phylogenetically-related and have physiological features in common with other members of this taxonomic class. Nevertheless, they present

marked peculiarities related to their way of life. Squids are highly active when compared to the other mollusks. They are voracious predators that not only compete, but also exert predation on teleost fish, which requires extreme mobility and vigor (38). The occupation of such ecological niche by mollusks was only possible due to a series of morphophysiological adaptations in comparison to the "standard mollusk", some of them are related to the digestive system. Adding to the abovementioned aspects the fact that cephalopods are fast-growing animals, it can be concluded that only an intense food consumption, a high digestive efficiency and a low feed conversion rate would allow these mollusks to fulfill the demands of this markedly active way of life. In this context, it is important to mention that the cephalopod species *Eledone cirrhosa*, *Octopus cyanea* and *Illex illecebrosus* present food assimilation efficiencies above 90%. These values are at the top of those indicated for carnivores (85 to 92%). Still regarding food consumption, it is worth mentioning that post-embryonic and juvenile squids may ingest from 50 to 100% of their own body weight per day [38].

The high feed intake and digestive efficiency observed in cephalopods may contribute to the intense bioaccumulation of cadmium verified in these mollusks. Regarding the absorption sites in the digestive tract of cephalopods, different organs are involved including the digestive gland, the appendages of the digestive duct, the cecum and the intestine. However, the main absorption organ is the digestive gland, with the exception of the Loliginidae Family, a taxonomic group in which no food absorption seems to occur in this gland [39]. In the cephalopod species for which the digestive gland is the main food absorption site, the cells of this gland perform intracellular digestion (particle capture by phagocytosis) and this seems to be an important aspect in which concerns Cd absorption by these mollusks.

Using the radionuclide ^{109}Cd, Bustamante et al. [40] observed that *Sepia officinalis* presents an absorption efficiency of 53%, more than ten times higher therefore than the same parameter in mammals [29]. Capturing large molecules in the digestion process would explain the fact that Cd absorption by cephalopods is ten times more efficient than that observed in vertebrates. Digestion in mammals is performed through the secretion of digestive enzymes into the lumen of the gastrointestinal tract. The enzymes are able to break down the molecules, which are then absorbed by intestinal cells. For absorption of essential metals such as iron, for example, mammals need special proteins that act as receptors. Cephalopods, using intracellular digestion and phagocytosis, assimilate all the elements included in the particles. Bustamante et al. [40] observed no excretion of cadmium in *S. officinalis*, concluding for a biological half-life tending to infinity in adult individuals of the species. The occurrence of a cadmium biological half-life of the same magnitude as the lifetime of *S. officinalis*, which in the Mediterranean Sea corresponds to one or two years [41], suggests that cephalopods have followed an evolution path that has favored the storage of this toxic element rather than its elimination [40]. Such high retention capacity was shown to be almost exclusively related to the digestive gland, since this organ retained 97% of the total cadmium body burden [40]. Therefore, the assimilated cadmium, contained in the digestive gland, can be considered as truly stocked.

5. Metalloproteins

It has been speculated that this high capacity of Cd accumulation is related to a high number of protein molecules present in the digestive gland of cephalopods that are capable of binding metals [42], called metalloproteins. Cephalopods present several metalloproteins, including metallothioneins (MTs), a group of proteins that have been extensively studied in different animal groups [28, 30, 43, 44, 45]. The term metallothionein encompasses a family of non-enzymatic low molecular weight proteins (6-7 kDa in mammals), which have an unusual amino acid composition, since they contain practically no aromatic amino acids and exhibit cysteine residues totaling approximately one-third of their amino acids [30, 45]. Metallothioneins were found in eukaryotic microorganisms [46], throughout the animal kingdom and in higher plants [43]. The amino acid sequences of metallothioneins from many mammals reveal that they all contain approximately 61 amino acids of markedly similar composition. In addition, they all contain twenty cysteine residues that remain

invariant throughout the amino acid sequence. It is also known that all cysteines participate in the coordination of seven moles of divalent metals per mole of metallothionein [30]. The omnipresence and great conservation of metallothionein in many living beings leads to the idea that this protein has great importance for life, and its structural conservation has been dictated by its functional requirement [30].

However, in addition to metallothionein, other polypeptides capable of binding to metals have already been observed in cephalopods. In the digestive gland of the squid *Ommastrephes bartramii*, Castillo & Maita [47] found evidence of four of such proteins, which presented either a molecular weight of around 16kDa or very small quantities (or even non-detectable) cysteine residues. These features demonstrated that the metalloproteins found by Castillo & Maita [47] were not MTs. Tanaka et al. [48] and Castillo et al. [49] showed that most of cytoplasmic cadmium in the digestive gland of the cephalopods *Todarodes pacificus* and *Onychoteuthis borealojaponica* was bound to proteins that presented molecular weights higher than 70kDa. Additionally, Finger & Smith [50] reported occurrence of Cd-binding proteins presenting more than 70kDa in the digestive gland of the squid *Nototodarus gouldi*.

These subcellular investigations showed therefore that most of Cd in the digestive gland of cephalopods is associated to soluble cytoplasmic compounds, which constituted unexpected results for cells presenting such high cadmium levels. The observation of metal-rich granules (MRGs) would be the expected results, since trace-element precipitation into such concretions constitute an efficient metal detoxification mechanism reported for a number of invertebrate species [51]. These MRGs play a role in the storage and excretion of essential and non-essential metals and the production of these concretions is observed in all main phyla [51]. Ultrastructural investigations however have not found these insoluble granules in different cephalopod species [38].

These features altogether, *i.e.*, high degree of cadmium retention and the association of this metal to cytoplasmic proteins rather than to concretions, demonstrate how efficiently cephalopods can play the role of vectors of cadmium transfer to their predators. This conclusion is based on the fact that cadmium is present in squids and octopuses not only in high quantities, but also in a bioavailable form. This is corroborated by the extremely high Cd concentrations commonly found in squid-eating odontocetes [3].

6. High Cadmium in Cetaceans Due to Natural Phenomena

An investigation from our research team that included the determination of Cd concentrations in the digestive gland of squids from the Brazilian coast [4] has verified that Cd levels were 20 times higher in *Illex argentinus* (Family Ommastrephidae) than in *Loligo plei* (Family Loliginidae). That finding corroborated data from the literature, since Cd concentrations had been always higher in ommastrephid than in loliginid squids. Bustamante et al. [2] determined Cd concentrations in tissues of ommastrephid and loliginid squids. The authors have found higher mean Cd levels in the former than in the latter cephalopod family in three different regions, *i.e.*, in Faroe Island waters, in the western continental shelf of Ireland, as well as in the Bay of Biscay. Those data were also in agreement with the Cd levels observed by [52] in the digestive gland of cephalopods from North Pacific. The latter authors have found lower mean Cd concentrations in *Loligo opalescens* than in *Ommastrephes bartrami*. It seems that species from the Ommastrephidae Family have a greater Cd accumulation capacity than those from the Loliginidae Family, which may be related to the different digestive physiologies of the two groups. As previously mentioned, the digestive gland of Loliginidae and, consequently, of the Myopsine Suborder, does not present the absorption function [39]. This turns loliginid squids into an exception, since for the other twenty-nine families of the Teuthida Order, including the Ommastrephidae Family, the digestive gland is the main absorption site.

In addition to the mentioned differences in digestion physiology between the two cephalopod families, the two taxonomical groups do not share the same habitats. In general, ommastrephids inhabit the oceanic province while loliginids live on coastal waters. Information

from the literature concerning cadmium concentrations in tissues of odontocete cetaceans also indicates the occurrence of higher concentrations in oceanic species / populations, which probably feed on ommastrephids, when compared to coastal odontocetes, which are well-known to exert predation on loliginids [4, 53, 54, 55]. As it can be extracted by the word “probably” used in the last sentence, the diet of oceanic dolphins is not completely known. However, studies have found significant correlations between cadmium concentrations and carbon stable isotope data, showing that oceanic dolphin populations present higher Cd levels than coastal ones [56]. In other words, the more distant from urban and industrial areas is the region were dolphins live, the higher will be the Cd concentrations in their tissues. This reinforces the idea that the high Cd levels found in marine mammals result from natural phenomena. Several studies involving metal determination in tissues of Arctic marine mammals [57, 58, 59, 60] have led to the same conclusion. Cadmium levels have apparently always been high in the Arctic, as indicated by the absence of temporal changes in dated sediment cores or in historical samples (since the fifteenth-century) of both seal and Inuit hair [60, 61, 62]. In addition, Lane et al. [63] have found out that a marine diatom can synthesize a carbonic anhydrase that is a cadmium enzyme in addition to synthesizing the commonly found zinc enzyme carbonic anhydrase. The discovery of a biological function for cadmium in marine organisms [63] ended up producing an explanation to the nutrient-like behavior of cadmium in oceanic waters [64]. Cadmium is considered a nutrient-like element since it is incorporated into and released from biogenic detritus in direct proportion to the regeneration of phosphate and nitrate [64]. It is plausible to assume this utilization of cadmium is more important in environments that have been poor in nutrients during the evolution process, such as the oceanic province. This use of Cd enables organisms from oligotrophic environments, for which the supply of Zn is low, to use Cd as well. This utilization of cadmium provides an important entrance for this metal into the oceanic food web and helps explaining the wide differences found in Cd concentrations between oceanic and coastal nektonic organisms (specifically cephalopods and dolphins), which can reach a 20-fold difference [4, 54, 55].

7. What are the Adaptations to a High Exposure to Cd Presented by Cetaceans?

The evidences exposed above suggest that cephalopod predators have been subjected to high doses of cadmium on a geological scale. Thus, it has been commonly accepted that squid-eating cetaceans have probably evolved to withstand the toxicity of this metal using efficient detoxification processes [60]. In mammal kidney, cadmium is mainly bound to metallothionein [29], a protein that could allow the storage of this metal in the intracellular environment as a non-toxic product [30]. The presence of metallothioneins (MTs) was detected in liver and kidney of eleven different marine mammal species, including odontocetes and pinnipeds [65, 66, 67, 68, 69]. Das et al. [65] emphasized: (A) that the percentage of cytosolic cadmium bound to MTs can reach almost 100%; (B) that intimate and dynamic interactions occur between cadmium and these proteins; as well as (C) that these animals could use those proteins to mitigate the toxic effects of cadmium.

However, even the above-mentioned authors acknowledge that the fact that marine mammals have an apparently greater capacity of metallothionein synthesis would be insufficient as explanation to how these animals can cope with such high Cd concentrations. Such insufficiency was reinforced by investigation involving in vitro perfusion of rabbit proximal tubules, which demonstrated that the cadmium-metallothionein (Cd-MT) complex exerts an inhibitory effect on the protein responsible for glucose reabsorption [70]. This protein is a co-transporter located on the apical membrane of proximal tubule cells and enterocytes [71]. Thus, the metallothionein synthesized by the renal cells would be unable to prevent this toxic effect in particular. For this effect to occur, the absorption of the Cd-MT complex by the renal proximal tubule cell would not be necessary. The knowledge generated from the study of Tsuruoka et al. [70] is consistent with the symptoms observed among Itai-itai disease patients, since glycosuria was one of the signs found [21].

The transporter inhibited by the Cd-MT

complex [70] is called SGLT (Sodium GLucose Transporter). It is a group of proteins with which two sodium ions combine in intestine (absorption) and proximal tubule (reabsorption), causing a conformational modification that amplifies the affinity of the transporter (T) for glucose (GL). Thus, glucose rapidly combines with the $2Na^+$-T complex, forming the ternary complex $2Na^+$-T-GL, which redirects itself in the apical membrane towards the cytoplasm, exposing the glucose and Na^+ binding sites to the intracellular medium. Subsequently, sodium ions and glucose dissociate from the carrier [20]. In addition to the role of the Na^+/glucose co-transporter in the absorption of dietary monosaccharides, it plays a key role in the absorption of salt and water. While entering the cell from the intestinal lumen, through this protein, Na^+ is pumped into the intercellular spaces by Na^+-K^+-ATPase, resulting in a local osmotic gradient, which, in turn, directs water absorption. Therefore, the presence of luminal sugar stimulates the absorption of sodium and, consequently, water. This phenomenon is the scientific basis for the oral rehydration therapy (ORT, widely cited in Brazil as "homemade serum" therapy) used with great advantages in the treatment of cholera [72]. According to the Victora et al. [73], about four million children succumb to acute diarrhea every year, with ORT saving more than one million children annually. It is argued that no child or adult would die from diarrhea if ORT was readily available [73]. This relevance for human health explains the large amount of information about the SGLT protein group in humans and laboratory animals [74]. It also explains the effort made to fully map the gene of this protein [75].

The energetic impairment generated by SGLT deactivation can be evaluated when the treatment for type 2 diabetes mellitus is considered, since SGLT antagonists have been used in patients of this disease as hypoglycemic drugs with great efficiency [76]. Tsuruoka et al. [70] describe an interruption effect of the Cd-MT complex on amino acid reabsorption as measured by alanine uptake. The presence of proteins and amino acids in urine is a symptom commonly associated with renal dysfunction [77]. Such evidence demonstrates a nephrotoxic profile of the Cd-MT complex, which was 100 times more potent than $CdCl_2$ in inhibiting these pathways. In fact, studies have shown endocytosis of the transport molecules of the brush-bordered cells of the proximal tubule in the presence of this complex [78].

4. Conclusions

Therefore, the simple increase in metallothionein (MT) synthesis is not sufficient to attenuate cadmium toxicity. In other words, neither an enhanced production of MTs by hepatocytes nor an augmented synthesis of MTs by the proximal tubule cells would constitute an acceptable explanation to the absence of Cd nephrotoxicity in highly exposed mammals, as the only explanation. An increased MT synthesis by hepatocytes would enhance the input of Cd-MT complex into the glomerular filtrate. This enhanced input would amplify the toxic effects of this complex on SGLT, which constitute effects that occur in the extracellular medium and therefore could not be counteracted by MTs produced by the proximal tubule cell and present inside the cell. It is undeniable that an enhanced MT synthesis would help to attenuate several effects generated by Cd. Nevertheless, the abovementioned studies on effects of the Cd-MT complex on the transport of vital molecules across the apical membrane of the proximal tubule cells have made clear that an increased MT synthesis could not constitute the only adaptation presented by cetaceans for tolerating an extremely high exposure to cadmium.

Considering the high toxicity of cadmium, the more than one hundred deaths as a result of the Itai-itai disease, as well as the increase in environmental levels of this metal as a consequence of anthropogenic activities, it can be concluded that the higher tolerance to cadmium presented by certain mammal species in comparison to humans constitutes an issue of increasing importance. Studies on amino acid sequencing of the renal SGLT (SGLT2) would constitute an important step to amplify the knowledge on cetacean adaptations to their high exposure to Cd and should shed further light on the possibility of a conformational different SGLT2 in cetaceans as one of the adaptations.

Due to the probable adaptations not yet understood, cadmium is not generally considered as a threat to cetaceans. However, it is important

to keep in mind that any detoxification process has a cost to the cell or organism involved and that there must be a threshold above which the detoxification mechanism is no longer an effective tool to prevent a certain toxic effect. Additionally, if cetaceans have been exposed to metals during the evolution process, the same cannot be said about other contaminants such as organohalogen compounds for example, so that possible synergistic effects should be evaluated when considering the conservation of these marine mammals.

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