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Bioaccumulation of cadmium and its cytotoxic effect on zebrafish brain

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In order to study the accumulation and cytotoxicity of cadmium (Cd) in the brain of zebrafish (*Danio rerio*), specimens were exposed for 16 days to $1.0\,mg \cdot L^{-1}$ of $CdCl_2$ and the brains were analysed at 2, 7 and 16 days of treatment. The concentration of Cd in the brains was detected by atomic absorption spectrometry; tissue alterations were revealed under light and electron microscopes. Cadmium concentrations in the brain of treated fish appeared to increase exponentially over time, reaching a peak at 16 days. As regards brain histomorphology, we observed evident tissue disorganisation in treated fish compared with control specimens. This disorganisation appeared particularly at 16 days in the optic tectum, in the ventricle area where the ependymal cells appeared sparsely distributed and in the medulla oblongata. Moderate ultrastructural damage was observed in the fish at 7 days of treatment. In the brain at 16 days of treatment, we observed considerable mitochondrial swelling with a loss of cristae, and several cells showed large autophagic vacuoles. This is indicative of a defence mechanism which occurs over time and in parallel with the accumulation of metal in the organ.

Keywords: cadmium; zebrafish; brain

1. Introduction

Water contamination by heavy metals such as cadmium is a major environmental problem worldwide. Such metals form toxic compounds which can enter the aquatic environment naturally from rocks and soils directly exposed to surface water. However, they are mainly released by industrial activities, leading to substantial concentrations in water. In recent years, metal concentrations have exceeded natural background levels in many aquatic systems. Cadmium (Cd) is a highly toxic metal that is involved in a variety of pathological conditions at low levels of exposure [1]. Although a non-essential element, it is unfortunately increasingly abundant in the environment as the result of industrial and agricultural practices [2], thereby entering the food chain [3].

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As a non-degradable cumulative pollutant, Cd presents a major ecological problem due to its ability to accumulate in living organisms [4,5]. Fish accumulate heavy metals at high concentrations in their tissues by absorption through the gut and the gills. Ingestion of Cd induces histopathological changes in the liver and kidney of fish [6]. In the gills, Cd is taken up passively through calcium (Ca) channels and is then transferred actively into the blood from where it is transported to storage in organs, as for zinc (Zn) [7]. In this case, the gills are the first target for this metal and they appear much altered after exposure to waterborne Cd [8,9]. Cd can pass the blood–brain barrier and exert neurotoxic activity. In mammals, studies have shown the neurotoxicity and damage to cerebral tissue induced by Cd [10]. Cd also has several properties that are toxic for the central nervous system (CNS) [11]. It affects the structure of nucleic acids, the activity of certain enzymes, the uptake of catecholamines and the levels of various neurotransmitters [12].

Although the toxicity of Cd to freshwater fish has been studied extensively in various organs such as the liver, gills, gut and kidney [13], little has been reported for the brain. Cd accumulation in different brain regions under acute and chronic conditions was found to be much higher than the levels recommended in *Labeo rohita* fish [14]. In fish, several studies have also indicated that toxic agents may affect behavioural traits, such as aggressiveness, which are good indicators of damage to the CNS [15,16]. The brain of fish has been shown to contain low levels of antioxidants and higher levels of peroxidisable unsaturated lipids [17]. Hence the fish brain may be mainly vulnerable to pollutants like metals. *Danio rerio*, commonly known as the zebrafish, is an excellent model for toxicological and neurobiological studies. The fact that its natural habitat is water provides easy exposure through this medium. Because correct brain structure is intimately connected with normal brain functionality, we studied the accumulation of Cd in the brain of zebrafish exposed for 16 days to $1.0\,\mathrm{mg}\cdot\mathrm{L}^{-1}$ of $CdCl_2$, a representative dose potentially found in water near industrial areas, and the possible correlative histomorphological alterations of nervous tissue.

2. Materials and methods

Fifty-four adult zebrafish were acclimatised for 2 weeks in well-aerated holding tank, under a natural photoperiod 12 h:12 h light/dark cycle, at temperature of 26 °C and pH 7.6. They were fed twice daily with commercial food. The water parameters and quality were daily measured from the beginning and to the end of the experiment. The experiments were performed under the approval of institutional committees; all efforts were made to avoid suffering and to minimise the number of animals used. The fish were divided into two groups: 27 were kept in uncontaminated water and constituted control specimens; another 27 fish were exposed to contaminated water with $1.0\,\mathrm{mg}\cdot\mathrm{L}^{-1}$ of $CdCl_2$. This dose was chosen according to what was observed at sites where very high Cd levels were found [18]. Nine fish from each aquarium were sampled at 2, 7 and 16 days post exposure. The fish were anaesthetised with ice and rapidly killed with a cervical cut. The brains were then removed and processed.

2.1. *Atomic absorption*

To determine the total Cd content in zebrafish brains from control and Cd-treated groups, organs were weighed and rapidly frozen at −80 °C. The method used is reported in 'APAT IRSA-CNR Sez. 3000'-'Metals and metal species'- division 3010 'Preliminary treatment of samples for heavy-metal analysis through acid mineralisation'. Tissue samples were digested in 65% HNO_3 at 80 °C. HNO_3 was evaporated and the mineral residue was solubilised in acidified water (37% HCl). Accumulated Cd was quantified by graphite furnace atomic absorption spectrometry (Varian AA280FS). The results are expressed as average metal concentrations accumulated in brains (three replicates per exposure condition) in $\mu\mathrm{g\ Cd}\cdot\mathrm{g}^{-1}$ wet weight ± SD.

2.2. *Light microscopy*

For histomorphological investigation, the isolated brains were fixed in Bouin's solution for 24 h, dehydrated in graded ethanol for a total of 2 h and embedded in paraffin. Serial sections of 6 μm were processed by hemalum–eosin for general morphology and 0.2% Cresyl violet for the specific stain of glial and nervous cells. All stained sections were dehydrated and mounted with DPX (Carlo Erba, Italy). The images were examined and acquired by a Kontron Electronic Imaging System KS300 (Zeiss, Germany).

2.3. *Transmission electron microscopy*

For ultrastructural study, brains were immediately taken and fixed in Karnovsky's liquid for 1 h 30 min, post-fixed in 1% osmium tetroxide for 1 h and dehydrated in graded ethanols for a total of 1 h 30 min. After 5 min of propylene oxide, the brains were then enclosed in Epon 812. Ultrathin sections were collected on nickel grids and stained with 2% uranyl acetate for 1 min and 0.65% lead citrate for 1 min. The TEM observations were carried out at the Anton Dohrn Zoological Station of Naples with a Leo 912 AB electron microscope.

3. Results

None of the fish died during the course of the experiment. The control fish swam normally without signs of any other abnormality. After 7 days of exposure to Cd the fish exhibited erratic swimming and aggressiveness. Moreover, hyperventilation was particularly evident in fish at 16 days.

3.1. *Cd accumulation in the brain*

Determination of the Cd concentration in the brain by atomic absorption spectrometry showed traces of Cd also in control fish. Over time, the Cd concentration in the brain of treated fish appeared to increase exponentially and peaked at 16 days. After only 2 days of treatment its concentration was $20.23 \pm 1.0\,\mu g\,Cd \cdot g^{-1}$ wet weight and at 16 days it was $39.04 \pm 1.02\,\mu g\,Cd \cdot g^{-1}$ wet weight. The data are reported in Table 1 and represented in Figure 1.

3.2. *Light microscopy observations*

Under the light microscope the brain of control specimens showed the typical appearance of teleost brains in which a homogeneous tissue with well-defined structures was observed in all the regions of the organ where the neurons and the glial cells showed regular shape and well-defined edges (Figure 2a–c). This was also observed in the fish brains at 2 days of treatment in which a normal histological architecture without any indication of deformities was revealed. By contrast, the fish

Table 1. Cadmium accumulation in *Danio rerio* brains after 2, 7 and 16 days of treatment.

	Treatment time (days)		
Control	2	7	16
0.4 ± 0.1	20.23 ± 1.0	30.16 ± 0.82	39.04 ± 1.02

Note: Values are reported as $\mu g\,Cd \cdot g^{-1}$ wet weight and expressed as mean ± SD.

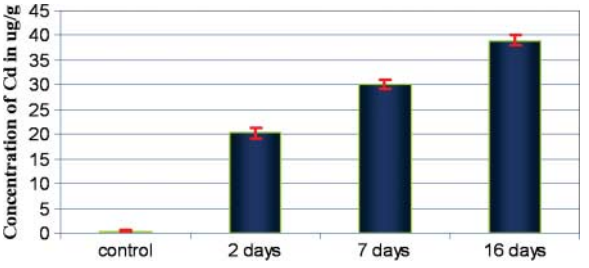

Figure 1. Cadmium concentration (mean ± SD) in the brain of zebrafish during treatment, determined by atomic absorption spectrometry.

at 7 days showed tissue disorganisation in several areas. This occurred especially at the level of the ventricles where the ependymal cells appeared sparsely distributed and with a scarce affinity to Cresyl violet staining (Figure 2d), the optic tectum (Figure 2e) and the medulla oblongata (Figure 2f) where the cells appeared well spaced due to intercellular spaces of different width. This disorganisation of the tissue appeared more highly expressed at 16 days in the ventricles where the ependymal cells showed greater alteration of the whole ependymal layer compared with those at 7 days, with an extension to the subependymal layer (Figure 2g). Again at 16 days of treatment, also in the optic tectum and the medulla oblongata, we noted the presence of wide intercellular spaces of a probable oedematous nature (Figure 2h,i).

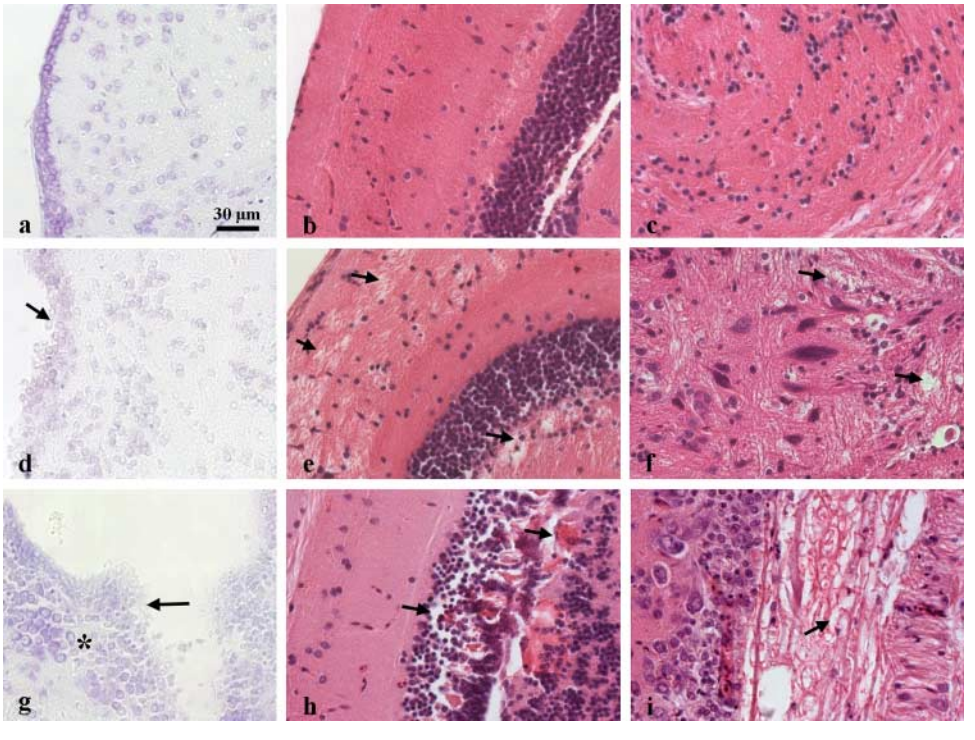

Figure 2. (a–c) Control brains. The nervous tissue appears homogeneous with well-defined structures. The neurons and glial cells are a regular shape with well-defined edges. (d–f) Brains of fish at 7 days of treatment. (d) Note the ependymal cells (→) appear sparsely distributed and with a scarce affinity to Cresyl violet staining. (e,f) Cells (→) appear well spaced due to intercellular spaces of different width. (g–i) Brain of fish at 16 days of treatment. (g) Alterations in the ependymal layer (→) are also extended to the subependymal layer (*). Scale bar reported in (a) applies for all images. Stains: (a, d, g) Cresyl violet, ventricle; (b, e, h) hemalum–eosin, optic tectum; (c, f, i) hemalum–eosin, medulla oblongata.

3.3. *Transmission electron microscopy observation*

Transmission electron microscopy (TEM) ultrastructural studies showed in control specimens the typical organisation of the nervous tissue of fish brain with a dense tangle of well-conserved axon terminals, dendrites, glial cell processes and myelin fibres (Figure 3a). The cell membranes and nerve terminals appeared intact and the mitochondria showed good morphology (Figure 3b). At 2 days of treatment, no significant ultrastructural alteration was detected. Indeed, in the specimens at 7 days of exposure to Cd, in the corresponding regions in which we observed the presence of dilated spaces between cells under light microscope, we detected the occurrence of vacuolisations (Figure 3c), lamellar inclusions in some terminals of which the membrane was ruptured in many places, and a few mitochondria with irregular structures (Figure 3d). Myelin bodies were also found. In the brain of fish at 16 days of treatment, we observed considerable mitochondrial swelling with a loss of cristae (Figure 3e). A slight increase in glycogen granules was observed in several areas and different terminals showed evident alteration of the membrane (Figure 3e). Large and autophagic vacuoles, with a mean diameter of 1.3μm, were revealed in different cells (Figure 3f).

4. Discussion

Cd is known to accumulate mainly in the metabolically active tissues of fish such as the gills, liver, kidney and gastrointestinal tract [6,8]. Accumulation sites vary with the uptake route and among fish species [19]. Further, waterborne Cd accumulates more efficiently in this vertebrate than dietary Cd [20]. In this study we showed that Cd also accumulates in the brain of zebrafish exposed for 16 days to $1.0\,\mathrm{mg}\cdot\mathrm{L}^{-1}$ of $CdCl_2$, and that this accumulation is time-dependent because the values of the metal in the brain increase exponentially during treatment. In fish, Cd captured by the gills is evidently transferred actively into the blood from which it can pass the blood–brain barrier and accumulate in the brain. Although the gills are the first storage organs for heavy metals as already reported [7], the brain is also attacked and Cd levels in this organ reflect prolonged exposure as in the kidney and liver of rainbow trout [21]. In addition, a comparative study on the effects of direct Cd contamination on gene expression in zebrafish reports that the highest bioaccumulation after Cd exposure was observed in the gills, then in the liver followed by the brain [22].

The metal accumulation that we detected is also clearly the cause of the behavioural response observed in fish after 7 days of exposure and more evident at 16 days, when the fish showed hyperventilation in addition to erratic swimming and aggressiveness. Similar observations in *Paralichthys olivaceus* also showed excessive mucus production on their opercular surface at high Cd exposure, concluding that Cd in seawater affects the fish brain directly [16]. Furthermore, Cd exposure affected antioxidant enzymes and oxidative stress inducing changes in the social status in Nile tilapia (*Oreochromis niloticus*) [23]. In our study, the neurotoxic effects of Cd in fish brain were also revealed by evident histomorphological damage under the light and electron microscope in treated animals compared with controls. Such changes become more marked with increasing Cd accumulation in the brain, thus indicating that this organ is sensitive to Cd toxicity. At 2 days of treatment, the absence of morphological changes is explained by considering that toxicity in the cells starts when loading with Cd ions exceeds the buffering capacity of intracellular metallothioneins [24]. In our previous studies, we reported tissue alterations induced by Cd in lizard pituitary gland exposed to acute and chronic treatments [25,26]. In zebrafish, we reported that the main effects appear in the optic tectum, the ventricles and the medulla oblongata. Mitochondrial swelling with the disappearance of cristae is indicative of cellular damage, just as the autophagic vacuoles indicate a defence mechanism. This evidence has been reported in the kidney and liver of the fish *Puntius gonionotus* in which Cd intake via feed causes histopathological alterations and

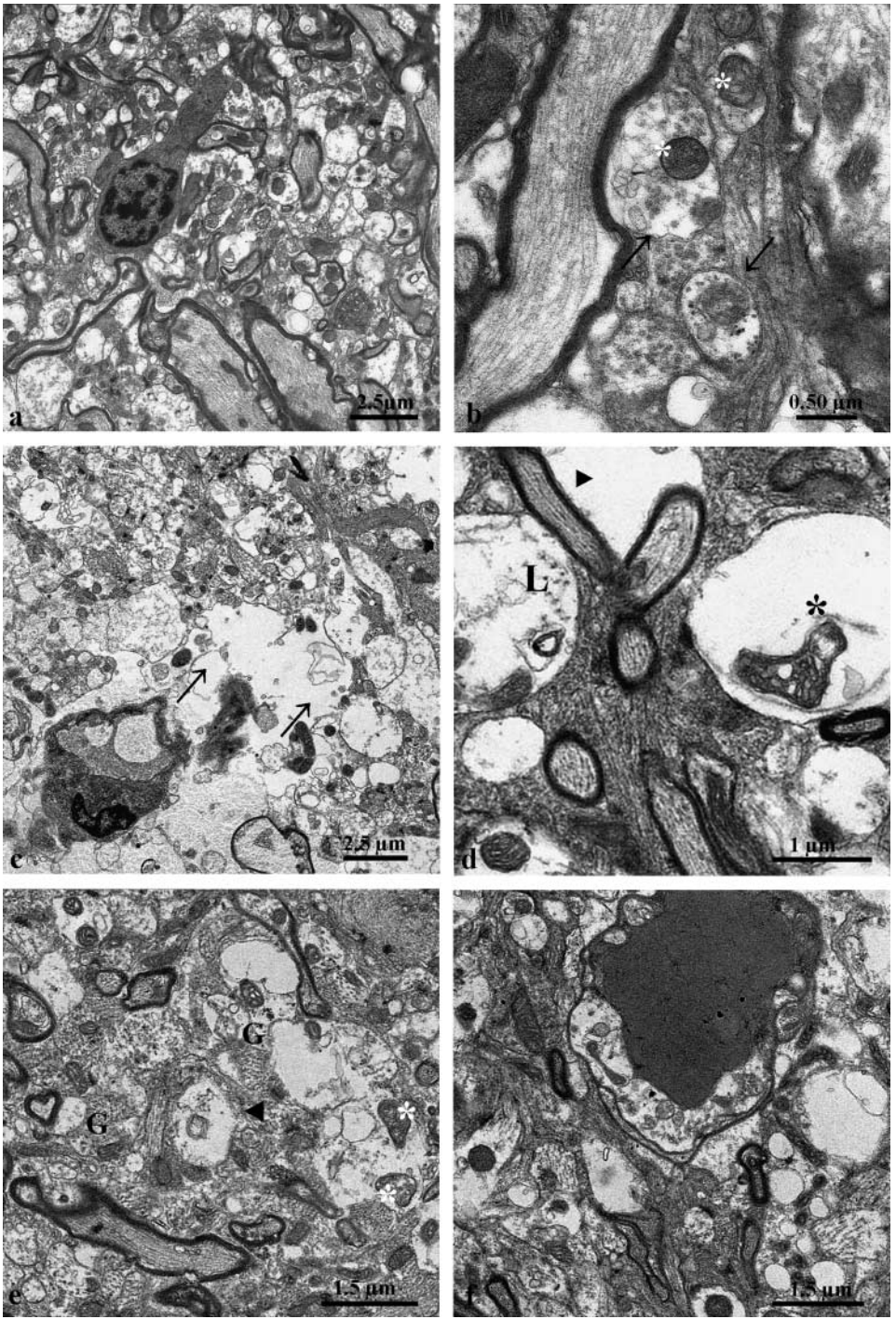

Figure 3. Transmission electron micrographs of fish brains. (a, b) Control brains. (a) Note the well-conserved nervous tissue. (b) Cell membranes and nerve terminals are intact (→) and the mitochondria (*) show good morphology. (c, d) Brain of fish at 7 days of treatment. (c) Some terminals whose membrane is ruptured in many places (→). (d) A mitochondrion with irregular structure (*), vacuolisations (►) and lamellar inclusions (L). (e, f) Brain of fish at 16 days of treatment. (e) Note mitochondrial swelling with a loss of cristae (*), the glycogen granules (G) and different terminals with evident alteration of the membrane (►). (f) Note large and autophagic vacuoles.

ultrastructural changes like proliferation of vacuoles and lysosomes, formation of myelin bodies and swelling of mitochondria with loss of cristae [6]. All these alterations are a typical cellular response to heavy metals and may represent an important parameter for the study of environmental pollutants. In the light of our findings, the zebrafish *Danio rerio* may be considered a good model system to further investigate the action of heavy metals on the CNS and the various mechanisms involved.

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