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Cadmium effects on the retina of adult *Danio rerio*

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HSP, Heat Shock Protein
IGF-I, Insulin-like Growth Factor I
PBS, Phosphate Buffered Saline
TEM, Transmission Electron Microscopy

ABSTRACT

The aim of this work is to describe the effects of cadmium pollution on the vision of adult zebrafish, *Danio rerio*. Retinal morpho-cytological alterations were investigated by light and electron microscopy, while the functionality of cadmium-exposed retinae was assessed by re-illumination behavioral tests with white or colored light. Our results demonstrate that cadmium toxicity causes significant degeneration and loss of organization at both macro and microscopic levels. These alterations impair functional responses particularly through an increase in light sensitivity. Metallothioneins were not seen to be up-regulated, while the recovery of visual acuity is due to a regenerative process by Müller cells.

1. Introduction

Cadmium has become a common pollutant in aquatic environments, with high concentrations often seen in industrialized areas [1]. Once dissolved, it is readily absorbed and bio-accumulated with toxic effects, at all levels of the food chain [2]. In addition, cadmium accumulates in sediments from which it is gradually released with resultant short- and long-term contamination [3].

Exposure to cadmium causes a plethora of negative effects in aquatic organisms. In invertebrates, it generates oxidative stress and time- and dose-dependent deregulation of transcription. For example, it up-regulates the expression of antioxidant enzymes [4], metallothioneins (MTs) and heat shock proteins (HSPs) [5], whereas it down-regulates the expression of digestive enzymes [6], esterases and phospholipase A2 [7]. Cadmium also interferes with immune responses [8,9], tissue organization and histology [10,11], and cell cycles by inducing apoptosis [12].

In teleosts, multiple effects of cadmium contamination have been described for major sites of metal absorption (gills and guts), and in predominant detoxification sites (liver and kidney). In all these organs, the metal causes morphological alterations [13], oxidative stress [14], and induces variations in the expression of several genes (MT, HSP, IGF-I) implicated in stress response [15,16] or in housekeeping activities [17]. Cadmium also decreases

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growth [18], delays reproduction [19], and reduces swimming performance [20]. Embryo development is markedly affected by cadmium as well. Malformations, increased mortality [21–23], induced oxidative stress [24] and behavioral defects [21] are also reported. Many of these effects are species-specific [25,26].

Cadmium exposure causes significant ocular malformations in fish embryos. Microphthalmia, reduction in the number of retinal ganglion cells, loss of photoreceptors and optic nerve growth failure have been reported. Visual abilities after cadmium exposure are obviously reduced and embryos are behaviorally blind [27]. In contrast, cadmium toxicity in the retina of adult teleosts is poorly known, even though, in these animals, most environmental interactions are based on visual information. For example, prey capture, escape from predators [28], timing of the reproductive cycle [29,30], and camouflage [27] are vision-dependent activities.

This work describes the effects of environmentally realistic concentrations of cadmium on the vision of adult zebrafish (*Danio rerio*), a preeminent experimental model for vertebrate vision research [31]. Here, we characterize cadmium-induced retinal morpho-cytological alterations by light and electron microscopy and assess visual performance by studying the sensitivity of cadmium-exposed animals to white or colored light by re-illumination tests.

Our results show that cadmium induces significant toxic effects in adult zebrafish with degeneration, loss of retinal organization, and consequent impairment of the functional response, particularly through increased light sensitivity.

2. Materials and methods

2.1. Animal care and treatment

Adult zebrafish (*Danio rerio*) were acclimatized in 50-L tanks at a temperature of 26 °C with natural photoperiod and fed twice daily. Under our experimental conditions, fish were maintained in 10-L test tanks, placed in a temperature-controlled water bath, aerated and provisioned with a mechanical filter to remove food debris and maintain water circulation. Animals were randomly allotted to three groups and treated as follows: group I received no treatment (control); group II was transferred to water containing $CdCl_2$ at a concentration of 0.3 mg/L (environmental concentration; [32]); group III was transferred into water containing $CdCl_2$ at a concentration of 3.0 mg/L. Treatment was static (solutions remained unchanged throughout the duration of the test) and lasted for 30 days. The experiments were carried out in compliance with ethical provisions established by the EU directive 2010/63/EU for animal experiment and authorized by the National Committee of the Italian Ministry of Health for in vivo experimentation (Dept. for Veterinary Public Health, Nutrition and Food Safety). Animal welfare was maximized by reducing and refining animal use in accord with the 3Rs principle [33].

2.2. Light microscopy

At sampling, animals were anesthetized with MS222 (tricaine methanesulfonate, 1:15000 w/v) and sacrificed by decapitation. Retinae were rapidly dissected, fixed in Bouin's solution and processed for paraffin wax embedding according to routine protocols. Sections were stained with haematoxylin and/or eosin to show general morphology.

2.3. Electron microscopy

For electron microscopy analysis, the retinae were dissected and fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M PBS at pH 7.4 for 24 h at 4 °C, washed in the same buffer and post-fixed in 1% osmium tetroxide in 0.1 M PBS, pH 7.4, at 4 °C for 1 h. They were then dehydrated in ascending series of ethanol and embedded in Epon. Ultra-thin sections were stained with 3% uranyl acetate in 50% ethyl alcohol and with 2.6% lead citrate and observed under a Philips EM 208S transmission electron microscope at 100 kV.

2.4. Behavioral tests

A modification of the behavioral test of Li and Dowling (1997) [34] based on a visually mediated escape response, by dark-adapted adult zebrafish was used in these experiments. The sensitivity to light of different wavelengths was compared in control and cadmium-treated animals.

An undisturbed, environmentally controlled room, with artificial illumination mimicking the natural photoperiod, held test tanks in which single animals were acclimatized for 24 h. The tank, in which the fish could swim freely in a circular motion, consisted of a cylinder (25-cm diameter) containing a smaller (5-cm diameter) opaque cylinder in the middle serving as a light shelter. A small lamp equipped with an aquarium light bulb and a web camera to capture fish behavior responses were mounted above the tank.

At 11 a.m. in the morning following acclimatization, fish were exposed to a 60-min period of dark followed by 1 min of illumination with a white, or red, or yellow, or blue or green light. These were obtained by placing a photographic filter in front of the light bulb. The light was placed very close to the water to create a bright sector in the tank on one side and a relatively "dark" sector, at the opposite side behind the small opaque central cylinder.

The escape response evoked by the light was determined by counting the number of times the fish passed through the bright sector of the tank per minute of observation. Tests at each filtered wavelength were carried out on six animals in each of the three treatment groups. Fifteen untreated animals, exposed to unfiltered diffuse white or colored light were used as blank controls. Results obtained were pooled and examined for significance at $P < 0.05$ by ANOVA.

2.5. Statistical analysis

The apoptotic cells on TEM sections were quantified on 10 non-overlapping areas of 1080 μm^2, considering at