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# Cadmium-induced ovarian pathophysiology is mediated by change in gene expression pattern of zinc transporters in zebrafish (*Danio rerio*)

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ABSTRACT

This study explored the potential for expression pattern of genes encoding zinc (Zn) transporters to be involved in the cadmium (Cd)-induced reproductive toxicity in female of zebrafish. For this purpose, oocytes maturity and ovarian histology as well as Cd, Zn and metallothioneins (MTs) accumulation and expression of genes encoding Zrt-,Irt-related protein 10 (ZIP10), Zn transporter 1 (ZnT1) and zebrafish metallothionein (zMT) were examined in ovaries of adult zebrafish exposed to 0.4 mg/L Cd in water and supplemented with Zn (5 mg $kg^{-1}$) in their diet for 21 days. Cd-exposure decreased the expression of ZnT1 and caused up-regulation of ZIP10 and zMT gene expression. These changes were accompanied by increased Cd and MTs accumulation, decreased Zn contents as well as by histopathological damages in ovarian tissues. The co-exposure of fish to Cd and Zn abolished ZnT1 down-regulation and rendered a persistently increased ZIP10 mRNA level. This treatment also decreased Cd and MTs accumulation, reversed Cd-induced Zn depletion and partially restored Cd-induced histological changes in ovarian tissues. These results imply that the downregulation of ZnT1 as well as the overexpression of ZIP10, in responses to the ovarian Zn depletion induced by Cd, play a major role in Cd accumulation and consequently in its toxicity. The protective effect of dietary Zn supplementation against Cd-induced toxicity is mediated, at least in part, by the increase of Zn availability and subsequently the induction of ZnT1 gene expression.



## 1. Introduction

Cadmium (Cd) is a widely spread toxicant with endocrine disrupting properties. Various effects of Cd on reproductive endocrinology have been described, but the precise mechanisms underlying its toxicity remained unclear. In the female of both humans and experimental mammals, Cd affects progesterone and estradiol synthesis [1,2], whereas in the male, Cd exposure can lead to testosterone suppression [3,4], reduced sperm motility [5,6] and, at high doses, testicular damage [7]. Several studies have also investigated the effects of Cd exposure on the reproductive function of fish. Studies have shown that exposure to Cd damages gonads and impairs gametogenesis. Das [8] has reported that the ovaries of Cd-exposed Asian cyprinids (*Labeo bata*) lacked mature oocytes and contained a significantly higher proportion of atretic follicles compared to the ovaries from unexposed females. Also, in Japanese medaka (*Oryzias latipes*), the decrease of ovarian steroid release after Cd exposure was described [9].

A number of mechanisms of Cd toxicity have been suggested, including oxidative stress, ionic and molecular mimicry [10], interference with cell adhesion and signaling [11], inhibition of energy metabolism [12], apoptosis [13], genotoxicity and cell-cycle disturbance [14]. The interaction between Cd and essential trace elements, such as zinc (Zn) could be also one of the reasons for Cd-induced reproductive toxicity. Zn is ubiquitously distributed in hundreds of cellular proteins and it is required for a variety of biological activities such as growth, development, and reproduction. It has been reported that gonadic and plasmatic depletion of Zn, under Cd influence, may be a causal factor in Cd-induced adverse effects on reproductive processes [15,16].

The ability of Cd to imitate Zn in many biological pathways and processes is a very probable explanation for its toxicity. Experimental evidence indicates that Cd may interact with membrane transporters involved in the uptake of Zn through a process of "ionic mimicry" [10]. Several studies indicate that Cd gains access to testicular cells by mimicking Zn at the site of Zn-transporters [17–19] which are transmembrane proteins controlling the movement of Zn across cellular and intracellular membranes. Transport of Zn into the cytosol is mediated by members of the

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Zrt-, Irt-related proteins (ZIP) transporter family. Flux of Zn away from the cytosol, either into organelles or out of the cell, is mediated by members of the Zn transporter family (ZnT). At least eight ZnTs and eleven ZIPs are present in the zebrafish genome and expressed in a tissue- specific manner. Several of these transporters are expressed in the zebrafish organs, such as gill, intestine and ovary [20]. Intracellular Zn is strictly regulated by binding to metallothionein (MTs) and glutathione and by compartmentalization through the activities of Zn transporters [21]. MTs have high binding affinity for Zn and play a central role in maintaining stable intracellular zinc availability through sequestration or release of Zn [22]. Using zebrafish metallothionein gene (zMT), the same isoform tested in the present study, Cd and Zn were found to be the most efficient inducers of zMT mRNA (up to 250- and 100-fold, respectively) [23,24].

Since Cd toxicity is closely associated with Zn homeostasis, it raised the question whether the co-treatment by Cd and Zn influences the Zn transporters and zMT genes expression in gonad to protect it from Cd toxicity. Up to date, there is no information regarding the effects of combined subchronic exposure to Cd and Zn on these gene expressions in fish ovaries. Therefore, the purpose of this study is to test the hypothesis that Cd-induced reproductive toxicity in zebrafish ovary as well as the ameliorative effect of Zn against this toxicity could result from changes of the mRNA expression pattern of Zn transporters.

## 2. Materials and methods

### 2.1. Test animals

Sexually mature female zebrafish (*Danio rerio*) were purchased from a local dealer and acclimatized for 1 week in well aerated holding glass aquaria (100 L), supplied with tap water (pH=7.02; dissolved oxygen = 11 mg/L; salinity = 0.09%), under a photoperiod 14 h:10 h (light:dark) cycle and temperature of (25 ± 1) °C. Fish were fed twice daily with commercially balanced fish food (Tetramin, Hagen, France). The medium was renewed every day. During the acclimatization period, inspections were conducted twice a day to discard wounded, diseased and dead fish.

### 2.2. Experimental design

The experiment was conducted over a period of 3 weeks. It was performed on 75 females whose weight reaches (0.89 ± 0.18) g and length (4.20 ± 0.21) cm. Fish were equally divided into three groups and transferred in separate glass aquaria. Animals in the first group were maintained in Cd-free water and were fed with Tetramin (Table 1) to serve as control. In the second group, Cd was added in water at a concentration of 0.4 mg $L^{-1}$, as $CdCl_2$, and animals were fed with Tetramin. In the third group, fish were exposed to Cd and fed with Tetramin supplemented with 5 mg $kg^{-1}$ Zn as $ZnCl_2$. In each aquarium, water was pumped continuously over a biofilter column at the rate of 4 L/min. The water was continuously aerated throughout the experiment. During the experimental period, the fish from all groups were fed once a day at a rate of 0.5% of fish biomass. This low level was chosen to avoid Cd contamination of feed remaining in the aquarium. No mortalities occurred during the experiment and no weight loss was observed. The experimental water was changed every week to keep the water quality in acceptable limit according to APHA [25], and the proven exposure concentration for Cd was verified. The Cd tested concentration represents 1/10 of the acute toxicity $LC_{50}$ (for 96 h) [26]. Previous studies showed that Zn supplementation lower than 20 mg $kg^{-1}$ diet had positive effects on animal growth and feed conversion rate and did not produce adverse effects in fish [27]. Thus, in the present study, the chosen Zn level (5 mg $kg^{-1}$diet) would not cause toxic effects.

**Table 1**
Composition of the Tetramin diet (Hagen, France).

| Ingredients | Contents |
|---|---|
| Crude protein (%) | 44 |
| Crude fat (%) | 5 |
| Natural fibers (%) | 2 |
| Moisture (%) | 8 |
| Ash (%) | 9 |
| Phosphorus (%) | 0.9 |
| Vitamins (UI/kg) | |
| A | 15,000 |
| D2 | 2100 |
| E | 100 |
| Zinc[a,b] (g/kg dry weight) | $2.1 \times 10^{-4}$ |
| Cadmium[a] (g/kg dry weight) | 0[c] |

[a] Cadmium and zinc contents in the diet was assessed in our laboratory. All the remaining data are given according to the producer.
[b] For fish exposed to Cd and Zn, the final Zn concentration in the diet is 4.86 ± 0.44 ($10^{-3}$ g/kg dry weight).
[c] Undetectable.

On the last day of experimentation, fish were anesthetized on ice and ovaries were excised and weighed for gonadosomatic index (GSI) determination. The GSI was expressed in grams per 100 g body weight. For histological studies, gonads were immediately fixed in Bouin's fluid for 24 h. Ovaries destined for molecular analysis were kept in RNA later solution (Sigma–Aldrich, St. Louis, USA) at −80 °C and the remaining ovaries were flash frozen in liquid nitrogen and stored at −80 °C for MTs, Cd and Zn estimation.

### 2.3. Analytical procedures

#### 2.3.1. Histological analysis

Fixed ovaries were dehydrated through a graded series of ethanol and embedded in paraffin. Serial sections were cut at 5 μm and collected onto glass slides, stained with haematoxylin and eosin solution, and then examined under a light microscope. Gonads from six females were examined from each treatment, and three dorso-ventral sections from different levels were examined per individual. The center of each ovary was subjected to histological analysis, considering that oocyte development in zebrafish ovaries does not vary with position [28]. Gonads were examined for oocytes maturity as well as for histological abnormalities. Maturity was evaluated with respect to oocytes in the following stages: (1) previtellogenic oocytes, (2) vitellogenic oocytes, (3) mature oocytes and (4) post-ovulatory follicles. The number of oocytes at different developmental stages per microscopic light field was counted and included normal and affected oocytes, e.g. atretic follicles. A qualitative evaluation of the histopathological findings in the ovaries was done by indicating the number of animals affected by each kind of histological alteration.

#### 2.3.2. Chemical analysis

Gonads destined for Cd and Zn analyzes were digested with concentrated nitric acid (Merck, 65%) at 120 °C. When fumes were white and the solution was completely clear, the samples were cooled to room temperature and the tubes were filled to 5 mL with ultra pure water. All samples were analyzed to determine Zn and Cd concentrations using flame atomic absorption spectrometry. These measures were implemented using a ZEEnit 700-Analytik-Jena, Germany equipped with deuterium and Zeeman background correction, respectively, as recommended by the manufacturer. Samples were analyzed in triplicate and the variation coefficient