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RESEARCH ARTICLE

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Analysis of vitellogenin by histochemical method as an indicator of estrogenic effect in male *Danio rerio* exposed to metals

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Abstract
Several chemicals present in the aquatic environment have the ability to alter the endocrine system of aquatic organisms, including the metals cadmium (Cd) and zinc (Zn). In males, a tool to analyze this effect is the vitellogenin (VTG) detection, a protein with defined function in the yolk production and subsequent aid to embryo-larval development. This protein is produced just by females and can be detected by simple and cheap methods such as histochemical method. Thus, this study aimed to analyze the capacity of Cd and Zn to induce VTG production in *Danio rerio* males and determine whether the histochemical labeling method is efficient to study estrogenic effects in this species. For this, *D. rerio* adult males were exposed chronically (21 days) to 0.25 and 1 μg/L of Cd and 120 and 180 μg/L of Zn, values allowed by the Brazilian and American legislation to aquatic life protection. After which, the organisms were submitted to a histological process as preparation to liver VTG marking by histochemical method and subsequent qualitative analysis of this protein in zebrafish's liver. After analyzes, it was possible observed that Cd and Zn are capable of inducing VTG production in *D. rerio* males and that the histochemistry method is efficient for detection of estrogenic effects in this species.

Keywords Cadmium · Zinc · Histochemistry · Endocrine disruptor · Vitellogenin

Introduction

Recently, the chemical group of essential and non-essential metals such as zinc (Zn) and cadmium (Cd), respectively, have been identified as potential pollutants to act as endocrine disruptors (ED) (Iavicoli et al. 2009) in aquatic animals, supported by some studies, such as, Vergílio et al. (2015) that detected alterations in *Gymnotus carapo* male gonads exposed to Cd and a decrease in female egg production in *Mysidopsis juniae* exposed to Zn by Figuerêdo et al. (2016), e.g., once these alterations, and other, are included in possible ones provoked by ED compounds, thus, highlighting the possibility of these metals acting as ED.

These group of chemicals – ED – (Hiramatsu et al. 2005) can act on organisms in different ways: (I) mimicking the effects of endogenous hormones, (II) antagonizing the effects of endogenous hormones, (III) disrupting the synthesis and metabolism of endogenous hormones, and/or (IV) disrupting the synthesis of specific hormone receptors, leading to disruption of the endocrine system at multiple levels in exposed organisms (Aris et al. 2014; Dzieweczynski and Hebert 2013).

In the literature, the ED damage in fish (i.e.) has as one of the main endpoints the vitellogenin (VTG) production, which is a phospholipoglycoprotein produced mainly in ovaries under development (Jones et al. 2000). VTG is the yolk precursor (energy reserves in eggs) synthesized in all egg-laying vertebrates and released from the liver into the bloodstream to assist eggs development (eggs/oocytes) and later in the embryo-larval development (Wallace 1985). Thus, by presenting these functions, males and juvenile fish, despite having the gene for VTG production, it is inactive, and this protein is not synthesized (Denslow et al. 1999). Therefore, VTG detection in the juveniles and/or male organisms is considered a biomarker for estrogenic exposure (OECD 2012c; Örn et al. 2003).

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Despite just detect of the presence or absence, take into account the ecological relevance, the histochemistry method is an important one and can be recommended, since, the quantity of VTG produced is not the most important result, but whether or not it is being expressed in males, and any signs of its production (in males) is an indicative of estrogenic compounds exposure and, consequently may cause endocrine interference. Therefore, performing the VTG analysis by histochemistry becomes a viable alternative for the detection of this physiological change, because it has low cost and ease of realization, a possibility that other methods do not provide, being more complex and expansive techniques to be performed.

Finally, the hypothesis of this study is that Cd and Zn are capable to act as ED, even in allowed concentrations by legislation; therefore, this research seeks to answer two main questions:

a) Are Cd and Zn concentrations allowed in aquatic environmentals by Brazilian and north American legislation capable to demonstrate potential endocrine interference considering the production of VTG in *D. rerio* male?
b) Is histochemical labeling an efficient method to study estrogenic effects in this species?

Materials and methods

Chemicals

For the preparation of stock solutions, Cd (II) and Zn (II) used Cd sulfate ($CdSO_4.8/3H_2O$, Dinâmica®, purity 99–102%) and zinc sulfate ($ZnSO_4.7H_2O$, Dinâmica®, purity 99–103%), respectively, studying only the metal concentration in each salt.

Fish maintenance

D. rerio (zebrafish) adults (~ 3/4 months), obtained commercially in Pet Shop Águia de Ouro (Sorocaba/SP), were acclimated for at least 15 days and kept under controlled conditions (ABNT 2015). Water from public supply previously filtered, using activated carbon filter and type silk screen (mesh aperture of 25 μm), unchlorinated by 24 h of aeration, and physical-chemical characteristics (pH – 7.5 ± 0.2, hardness – 44 ± 1 mg/L of $CaCO_3$, and ~ 95% of dissolved oxygen saturation) following ABNT (2015) recommendations was used. The animals were fed once a day with Tetramim® (97% crude protein) "*add libitum*" and maintained at controlled temperature and photoperiod (26 °C ± 1 °C and 12 h:12 h, light:dark, respectively).

Exposure

Male fishes (0.237 ± 0.048 g) were subjected to a chronic exposure (21 days; OECD 2010 – adapted to the exposure time) to nominal concentrations of 1 μg/L and 0.25 μg/L of Cd and 180 μg/L and 120 μg/L of Zn based on Brazilian and American legislation to aquatic wildlife protection, respectively, (BRASIL 2005; USEPA 1995, 2001) plus control (water without contaminant). All tested conditions (control and contaminated ones) were performed with the same water used and described in the maintenance section (ABNT 2015). A semistatic regime was adopted, and the test solutions were completed, replaced every 72 h. For each experimental condition, glass aquariums (2 L) covered with plastic sacks with 2 L of test solution were used and 3 replicates containing 3 organism-test each were performed, and the tests were conducted in the same culture conditions described above. Besides that a positive control was performed with female (0.289 ± 0.027 g) to ensure the VTG presence in this animal species.

Histochemical analysis

After exposure, six males from each experimental condition and six female from positive control were randomly selected, sacrificed by direct introduction in alcohol PA; the head and tail fin were cut and removed and the remaining were fixed in formalin 10% for 18 h, following to water washing, dehydration (alcohol and xylene), and paraffin embedding. The sections (4 μm) were obtained with steel razor in microtome (micron HM340E) and underwent histochemistry labeling for VTG according to the methodology proposed by Van Der Ven et al. (2003), in two steps: (1) sections were deparaffinized in graded series of ethanol and xylene and washed in distilled water. Tissues were then incubated in 10 mM/L ferric chloride hexahydrate, for 1 h (room temperature) so that the VTG phosphoproteins present in the molecules are complexed with iron (III). (2) Coloring with the Pearl's Prussian blue method, which stains the iron (III) complex formed in step 1 gives blue shades VTG present in the liver. The tissues were qualitatively analyzed by optical microscope Zeiss – Scope A1.

Results

Male fishes usually do not produce VTG, and it can be seen in the Fig. 1a. According the absence of extracellular blue shade regions, the blue color is presented just in the nucleus and membrane characterized by the phosphate group in these cells part and not in extracellular one (VTG). On the other hand, VTG production is intense in female, observed by the dominance of the blue color (according to the used technique) in

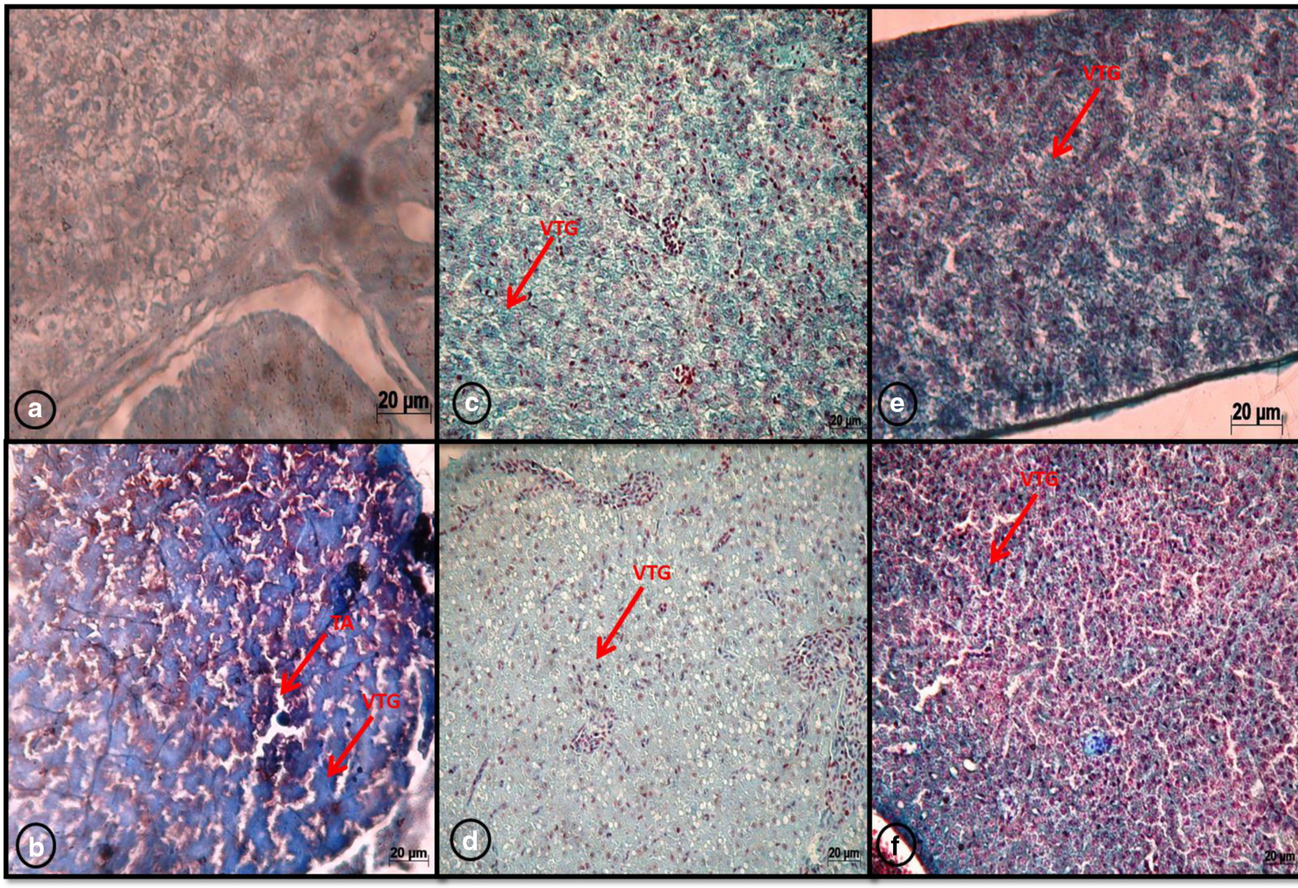

Fig. 1 Histochemical section of 21 days *Danio rerio* control (male and female) and metal exposed males to cadmium and zinc concentrations allowed by north American and Brazilian legislation. **a** Male control liver. **b** Female control liver. **c** Male liver exposed to 0.25 µg/L of cadmium. **d** Male liver exposed to 1 µg/L of cadmium. **e** male liver exposed to 120 µg/L zinc. **f** Male liver exposed to 180 µg/L of zinc. A – technical artifact caused by steel razor, VTG – vitellogenin

Fig. 1b. In the Fig. 1 (c–f), it is possible to see the presence of blue areas, indicating the VTG presence in male *D. rerio* liver caused by chronic exposure to the both tested metals (Cd and Zn) in all here studied concentrations.

Discussion

A characteristic of EDs compounds is the cause, in males, of VTG production; after Zn exposure, this phospholipoglycoprotein was detected in this study and in high levels in frogs (Falfushynska et al. 2016) and fishes (Falfushynska et al. 2014) followed by reproduction impairment. These alterations can be related with the VTG physiology production, since vitellogenesis is linked with Zn homeostasis in vertebrates. In summary, VTG can carry Zn into the oocytes for the formation of metalloproteins involved in embryo development. Thus, Zn plasma levels can too provide a simple and accurate marker for VTG production and reproductive status in oviparous vertebrates (Falchuk and Montorzi 2001). However, Falfushynska et al. (2015) show that a VTG level increase in male frogs is not associated with higher levels of Zn sequestration in the liver.

Therefore, it is well-known that the VTG is a Zn-binding protein (Falchuk and Montorzi 2001), and in some fish species, like *Holocentrus adscensionis*, VTG is a potential vehicle, in female, to Zn transport between liver and ovaries, and as expected, plasma VTG concentrations are elevated during the egg-producing periods in the reproductive cycle in this species (Thompson et al. 2003). That is, it can be assumed that, in male fish, the Zn concentration increase can follow a trigger for VTG production in the liver.

On the other hand, Valenzuela et al. (2015) show that Zn is responsible, in a fish (*Cyprinus carpio*), to increase the gene expression of metallothionein but not alter VTG expression. Taking into account the results obtained in this study, in which Zn was able to cause the production of VTG in male zebrafish and other works here presented, are possible hypothesize, that the ED action of this metal can be attributed to species-specific interaction. As previously mentioned, in some fish spicies, Zn levels can be a trigger to male VTG production, justifying the results obtained in this work. However, to a better interpretation and conclusion of results in ED studies, it is necessary to take into account the time and doses of exposure and animal size, factors that can influence the animal physiology as the metal uptake and directly interfere in the obtained results and action of metals as ED.

The chronic Cd exposure also was able to induce VTG production in zebrafish males even in concentrations allowed by the legislation. This result can be corroborated as long as Cd has been proposed to be able to alter the normal endocrinology of organisms, mainly in humans and rodents (Henson and Chedrese 2004; Iavicoli et al. 2009; Takiguchi and Yoshihara 2006). However, it is possible to see endocrine effects in another animals like copepods (Hwang et al. 2010), terrestrial (Laskowski 2001) and aquatic (Park and Kwak 2012), insects, and another fish species (Gerbron et al. 2015), i.e., after Cd exposure.

In vertebrates, the exposure to Cd can affect the release of pituitary hormones and the gonad production of steroid hormones (Lafuente et al. 2003; Smida et al. 2004), acting as steroidal estrogens (Johnson et al. 2003; Henson and Chedrese 2004) and, in invertebrates, can act as ED too (Cervera et al. 2005). In general, Cd exposure can cause estrogenic and androgenic effects according to the organism, time, and exposure concentration (Byrne et al. 2009), corroborated by Martin-Diaz et al. (2005) that suggest that the bioaccumulation is one of the most important factor to Cd-induced effects. Besides that, a justification for this effect is the possibility of Cd-VTG interaction occurring (Lee et al. 2008), also justifying the results here obtained.

In summary, the potential mechanism by which Cd may act as an ED is the interference with the DNA-binding Zn finger motif through the substitution of Cd for Zn (Henson and Chedrese 2004). It could mimic or inhibit the actions of endogenous estrogens (Henson and Chedrese 2004), and their capacity to increase the transcription and expression of estrogen regulated genes such as the progesterone receptor (Garcia-Morales et al. 1994) possibly triggering a male VTG production.

Furthermore, ecotoxicology faces a challenge in order to evaluate how chemical compounds, i.e., metals, can affect the endocrine system in aquatic organism and determine their toxicity, since this system has a complex network of interactions within the hormonal regulatory axis (León-Olea et al. 2014), and in this case, this complexity is associated with the metal specificity.

However, this study was capable to indicate the possibility of Zn and Cd act as an estrogen, inducing VTG production in *D. rerio* males when exposed to low concentrations. On the other hand, studies that report VTG detection by histochemistry method are scare in the literature. This affirmation can be evidenced by the absence of this technique in the above-mentioned studies along this discussion.

Furthermore, it is possible to associate this technique in evaluating histological effects, allowing a unique technique to check the physiological and morphological responses against exposure to contaminants, reinforcing the possibility of its use in studies seeking safe chemical concentrations to environmental and to aquatic life protection. Besides that, the here obtained results can assist government agencies in making decisions and restructuring the legislation to permit more stringent contaminants concentrations in the environment, ensuring endocrine integrity of aquatic organisms.

Finally, it is possible to conclude that, even at allowed concentrations in legislation for aquatic life protection, Cd and Zn are capable of causing physiological effects in *D. rerio* male liver by inducing VTG production, indicating that these metals are able to act as endocrine disruptors. In that way, it is clear that the histochemistry method is efficient for detection of estrogenic effects in this species and can be used as an endpoint in environmental and (eco) toxicological researches. Also, it is important to say that studies using metal as potential EDs are a scare, and this topic needs more attention.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest in this research.

The authors ensure that the research was conducted within the ethical standards protecting animal welfare.

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