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Arsenic absorption and excretion in chronically exposed developing toad *Rhinella arenarum*

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ABSTRACT

We assessed the toxicodynamics of As in developing *Rhinella arenarum* toad embryos and larvae exposed from fertilization to 0.01–10 mg As L^{-1}. We determined As content in toad embryos and larvae by X-ray fluorescence spectrometry. Toad embryos and larvae actively bioaccumulated As, reaching tissue concentrations more than one-thousand higher than control levels after 23d-exposure to 10 mg As L^{-1}. The bioconcentration factors also increased up to fifty times higher levels in toad larvae respect to media levels. Once recovered in As-free media, the larvae rapidly excreted the bioaccumulated As with a half-life of 1.6 d. By calcein transport competition assays, we infer that As is excreted through ABCC-like transporters, probably conjugated with GSH. These results are relevant for comprehending the risks posed by As exposure in this autochthonous aquatic species that develops in water courses from Argentina, that may contain As levels ranging between 10–15,000 μg L^{-1}.

1. Introduction

Arsenic contamination of the environment from both natural and anthropogenic sources is nowadays a major environmental concern in various parts of the world, due to its persistence and toxic effects on living organisms at certain concentrations (Arslan et al., 2017). It can be found in water, soil or air, existing both as inorganic as well as organic species with different oxidation states and differential toxicity. Inorganic As is the predominant form in surface and underground water reservoirs (Lu et al., 2014). Whereas humans acquire arsenic *via* food and water, aquatic animals are particularly vulnerable to As in the environment because they can also take up this toxicant through the gills or skin (Miller et al., 2007). Arsenic, like many metals and metalloids, can trigger reactive oxygen species (ROS) production, disrupt signal transduction, alter gene expression and induce lipid and DNA damage (Mardirosian et al., 2017; Sarkar et al., 2014), and many vertebrates try to counteract oxidative stress using the first-line defense system including reduced glutathione (GSH), vitamin C or E, carotenoids (Martínez-Álvarez et al., 2005) or radical-scavenging enzymes like superoxide dismutase, catalase, and glutathione peroxidase (Feidantsis et al., 2013; Valavanidis et al., 2006).

Inorganic As in water is mostly in the form of arsenate. In a pH range of 4–10, trivalent arsenic compounds have neutral charge, while pentavalent forms are negatively charged. Therefore, at physiological pH, trivalent arsenic species can more easily cross the cell membrane through their transporters than the pentavalent species (Cohen et al., 2006; Tseng, 2009). Arsenate is rapidly reduced *in vivo* to arsenite, mainly in the blood and liver (Aposhian, 1997; Gregus and Németi, 2005; Herbel et al., 2002; Vahter and Marafante, 1985). Because arsenite is taken up by cells much more rapidly than arsenate, it is preferred in many studies to evaluate the toxicity of inorganic As *in vivo* and *in vitro* (Cohen et al., 2006).

The uptake and efflux of arsenicals occur mainly through transport proteins. Transporters are critical for regulating the body burden of

Abbreviations: ABC, ATP-binding cassette; Abcb1, p-glycoprotein; Abcc, multidrug resistance-associated proteins; As, arsenic; BCF, bioconcentration factor; Bcrp, breast cancer resistance protein; BSA, bovine seroalbumine; calcein-AM, calcein acetoxymethyl ester; DW, dry weight; FW, fresh weight; GSH, reduced glutathione; GST, glutathione-S transferase; ROS, reactive oxygen species

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arsenic and potentially play both protective (through preventing cellular and tissue accumulation as well as facilitating elimination) and harmful roles (allowing the entry of arsenic across epithelial layers) (Roggenbeck et al., 2016). Different species of aquatic organisms are capable of taking up and accumulating As (Chen et al., 2009; Erickson et al., 2011; Farombi et al., 2007; Guidi et al., 2010; Heyes et al., 2014; Liu et al., 2006; Miller et al., 2007; Rahman et al., 2012; Roe et al., 2005; Suhendrayatna et al., 2002) and different members of the aquaglyceroporin family have been identified as responsible for As uptake through gills and intestine in fish (Hamdi et al., 2009).

Arsenic is excreted mainly in the form of conjugates. The enzyme glutathione S-transferase (GST) catalyzes the conjugation of As with GSH, giving rise to glutathionyl derivatives that are eliminated from the cell through ATP-binding cassette (ABC) proteins (Miller et al., 2007). However, there are still very few studies about the way in which As or its metabolites are excreted from the cells. The ABC superfamily contains membrane proteins that hydrolyze ATP to actively translocate a wide variety of substrates across plasmatic and intracellular membranes (Chan et al., 2004; Ishikawa, 1992; Klaassen and Aleksunes, 2010). The proteins ABCB1 (P-glycoprotein, Pgp), ABCC2 (multidrug resistance protein type 2, MRP2) and ABCG2 (breast cancer resistance protein, BCRP) are located in the apical membrane of polarized cells and participate in the efflux of toxic compounds. Other ABCC transporters are located in basolateral membranes, and thus are involved in absorption of toxic compounds by the organism (Chan et al., 2004). Several authors have reported the involvement of ABCC1, ABCC2 and ABCB1 in As detoxification in mammals (Gregus et al., 2000; Kala et al., 2000; Liu et al., 2002, 2001). Arsenic enhances ABCC2 expression and xenobiotic transport activity in killifish renal proximal tubules (Miller et al., 2007), leading to the acquisition of tolerance to acute toxicity. Arsenic upregulation of ABCC2 expression was also described in the liver of the same species (Shaw et al., 2007).

Amphibians are considered good bioindicators of aquatic pollution due to their susceptibility to chemicals during their freshwater cycles as embryos and larvae. Toxicants accumulated during the aquatic period and not depurated before or during metamorphosis to terrestrial juveniles may be transferred to organisms that feed on juvenile and adult amphibians (Heyes et al., 2014). *Rhinella arenarum*, the common South American toad, is widely distributed in Argentina and may be threatened by exposure to agrochemicals and other toxicants such as arsenic, which is present in concentrations between 0.01 and 15 mg L^{-1} in natural water sources throughout Argentina (Bundschuh et al., 2012). We have previously described the biochemical effects and the molecular mechanisms of action of As during the embryonic and larval development of *R. arenarum*, and reported that acute and chronic exposure to As can trigger oxidative stress in *R. arenarum* embryos and larvae (Mardirosian et al., 2016, 2015). In the present study, we focused on the uptake, accumulation and excretion of As along the embryonic and first larval stage of development of the common toad, *R. arenarum*.

2. Materials and methods

2.1. Chemicals

Sodium metaarsenite ($NaAsO_2$) was purchased from Anedra Argentina (purity > 99.95%). Calcein acetoxymethyl ester (calcein-AM, Calbiochem, San Diego, CA) was a generous gift from Dr. Amro M. Hamdoun (Scripps Institution of Oceanography, UCSD). All used chemicals and reagents were commercial products of analytical grade purity and were used as supplied. Calcein-AM was previously dissolved in DMSO. Final concentration of these solvents was always below 0.3%.

2.2. Toad embryo and larval growth

Adult *R. arenarum* males and females were collected during the breeding season (Spring) at Los Barreales Lake (S38.45344 W68.72918), a pristine environment. Animals used in this study were maintained and treated with regard for the alleviation of suffering according to recommended guidelines (Committee on Pain and Distress in Laboratory Animals et al., 2009; National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011; Schad et al., 2007). Upon collection, adults were transported to an outdoor terrarium. Ovulation was induced by intraperitoneal injection of 2500 international units (IU) of human chorionic gonadotrophin (ELEA Laboratory, Buenos Aires, Argentina). Freshly extruded eggs were fertilized *in vitro* with a testicular homogenate and depending on the assay, they were immediately exposed to As or developed in glass dishes with amphibian Ringer's solution (0.65 g L^{-1} NaCl; 0.01 g L^{-1} KCl; 0.02 g L^{-1} $CaCl_2$) and maintained at 20 ± 2 °C in a 12 h light- 12 h dark photoperiod until the exposure.

2.3. Contents of As and other elements in embryos and larvae exposed to As

The bioaccumulation of As and the effects of As exposure on Fe, Zn and Cu content in embryo and larval tissues were analyzed. For this assay, thirty minutes after fertilization, groups of 200 embryos were transferred to glass receptacles containing 200 mL of amphibian Ringer's solution either alone (control group) or with different concentrations of As solution (0.01, 0.1, 1 and 10 mg L^{-1}) (Lascano et al., 2011). The treatments were carried out in duplicate. The exposures were semi-static with solution renewals every 48 h, spanning the complete embryonic development period (9 d) and 14 d of the first larval stage, until d 23 of development, at 18–20 °C and a 12:12 h light: dark photoperiod. Embryos were fed with boiled lettuce from open mouth stage on until the end of the assay. Three independent experiments were performed. Measurements were performed by X-ray fluorescence spectrometry under total reflection conditions using synchrotron radiation (SR-TXRF) on the DO09B line at the Laboratorio Nacional de Luz Sincrotron (LNLS) of Campinas, Brazil. Samples consisting of 15 embryos from each treatment batch were collected at 5, 9, 16 and 23 d of development, washed with ultrapure water and lyophilized for transport to the LNLS. Subsequently, samples were resuspended in Milli-Q water with 0.1% Triton X100 and incubated 15 min in an ultrasonic bath for reconstitution. An aliquot of 5 µL of each sample was dispensed onto the center of a Si carrier with its mirror polished surface to achieve reflection of the incident beam. The excitation geometry of the collimated white beam (3 mm × 300 µm) was 0.1–0.2° respect to samples and the measurements were performed under conditions of total reflection of the beam. X-ray fluorescence spectra were recorded with a Si (Li) detector placed at 90° of the incident beam. The X-ray fluorescence intensities of the k-α peak of each element were recorded for quantification ($16 < Z < 42$), and the resulting spectra were analyzed with the AXIL program. The fluorescence intensity was normalized by using 10 mg L^{-1} of Ga as an internal standard and converted to element mass content using calibration parameter algorithms.

2.4. Bioconcentration of arsenic

Bioconcentration Factors (BCF) were estimated considering that larval dry weight (DW) was about 10% of the fresh weight (FW) and the exposure ratio of one larva in one mL of media. Considering an approximate fresh weight of 10 mg per larva (Lascano et al., 2011), then one g of larval dry weight would have been exposed to one L of media. Then, BCF may be expressed in L kg^{-1} from As accumulation values in mg As kg^{-1} DW divided by the corresponding media concentration in mg As L^{-1}.

2.5. Arsenic excretion assay

In order to determine the ability of *R. arenarum* larvae to excrete As,