Bull Environ Contam Toxicol (2016) 96:591–595
DOI 10.1007/s00128-016-1774-y

Genotoxic Effects Induced by Cd^{+2}, Cr^{+6}, Cu^{+2} in the Gill and Liver of *Odontesthes bonariensis* (Piscies, Atherinopsidae)

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Received: 26 November 2015 / Accepted: 9 March 2016 / Published online: 22 March 2016

Abstract Genotoxic effects of Cd^{+2}, Cr^{+6}, and Cu^{+2} on the gill and liver of the Argentinean Silverside (*Odontesthes bonariensis*) were studied using the comet assay and in relation with the metal tissue accumulation. Fish were exposed to three waterborne concentrations of each metal for 2 and 16 days. Genotoxicity was assessed by the single cell gel electrophoresis (comet assay). After 2 days, significant increase of the genetic damage index (GDI) was only observed in the gill of fish exposed to Cr^{+6} and Cu^{+2}, and the LOECs were 2160 nM and 921.1 nM, respectively. The gill LOEC for Cd^{+2} by 16 days was 9.4 nM. In the liver, LOECs were obtained only for Cd^{+2} and Cr^{+6} and were 9.4 and 2160 nM, respectively. The three metals were able to induce genotoxic effects at environmentally relevant concentrations and the gill was the most sensitive organ.

Keywords Heavy metals · Genotoxicity · Pejerrey · "Río de la Plata Basin"

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Heavy metals are well-known water pollutants worldwide. Cadmium (Cd) and chromium (Cr) pollution are related with anthropogenic activities such as the metallurgic and chemical industries (Tchounwou et al. 2012). Copper (Cu) is an essential element, but also can be a relevant pollutant in estuary and coastal ecosystems as consequence of inputs from agricultural, mining, industrial activities, and urban sewage.

Beside other mechanisms of toxicity, Cd^{+2} has shown to be able to induce reactive oxygen species (ROS) and inhibit the DNA repair enzymes (Filipič 2012). Cadmium carcinogenicity has been demonstrated in humans and others mammals (Tchounwou et al. 2012). Genotoxic effects have been also reported in several freshwater fish species (Ahmed et al. 2010; Jindal and Verma 2015). In addition, Cr^{+6} has been classify as human carcinogen acting via oxidative stress-dependent and -independent mechanisms (Nickens et al. 2010). This metal has proved to induce DNA–protein crosslinking, micronuclei induction, and chromosomal aberrations (Velma and Tchounwou 2010; Ahmed et al. 2013). Finally, it has been shown that Cu^{+2} is able to undergoes redox cycling resulting in the production of ROS and leading to DNA damage (Bopp et al. 2008). Mild to strong genotoxic effects induced by Cu^{+2} have been reported both in vitro or in vivo (Sanchez-Galan et al. 1999; Arkhipchuk and Garanko 2005).

The alkaline single cell gel electrophoresis (Comet assay) is a method broadly used for assessing genotoxicity. It is a sensitive, reliable, and rapid methodology for detecting DNA double- and single-strand breaks and alkali-labile sites. It has been extensively used in aquatic organisms both for genotoxicity studies (Frenzilli et al. 2009) and environmental biomonitoring (Russo et al. 2004).

The "pejerrey", Argentinean silverside, *Odontesthes bonariensis* (Valenciennes, 1835), is a freshwater fish

characteristic of the southern sector of the "Río de la Plata" Basin in South America. This appreciate game fish is highly sensitive to pollutants (Somoza et al. 2008). Previous studies indicated that heavy metals are able to bioaccumulated in *O. bonariensis* gill and liver, beside other tissues (Carriquiriborde and Ronco 2008; Avigliano et al. 2015).

In the present study the genotoxicity of Cu, Cd, and Cr on the gill and liver of juvenile pejerrey *O. bonariensis* was assessed through the comet assay.

Materials and Methods

Newly born *O. bonariensis* were obtained from the Aquaculture Facility of the Buenos Aires Province Agricultural Ministry. Fish were maintained for 5 months in a recycled aquaria system supplied with dechlorinated tap water ("hard water"–ionic composition in mM: Ca^{+2} 0.85; Mg^{+2} 0.90; Na^{+} 3.13; K^{+} 0.67; Cl^{-} 0.11; hardness, 180 mg $CaCO_3$ L^{-1}; pH 7.8). Fish were fed twice a day with 24 h old nauplii of *Artemia* sp. during the first 2 months and then with a combination of live food (*Daphnia magna*) and commercial food for trout. Background levels [mean ± SEM (*n*)] of Cd, Cr, and Cu in the commercial food were 0.17 ± 0.06 (6), 0.70 ± 0.18, and 13.80 ± 0.11 (6) μg g^{-1} wet weight, respectively.

Two hundred and twenty (6-month-old) *O. bonariensis* were equally divided into 30 polypropylene aquaria of 20L at a maximum density of 0.5 fish L^{-1} filled with dechlorinated tap water filtered through activated carbon. Fish were exposed to three concentrations (high, intermediate and low) of Cd, Cr and Cu along with a control group. The high concentrations of each metal were set just below the 96-h LC_{10} of the respective metal according to previous results (Carriquiriborde and Ronco 2002). The intermediate and low concentrations were 1/2 and 1/10 of each highest concentration, respectively. The exposure periods were 2 and 16 days, except for Cu that only 2 days was tested. Test solutions were prepared from stock solutions of $CdSO_4$, $CuSO_4$, and $K_2Cr_2O_7$ and were renewed every 48 h. Testing conditions were: T = 22° C, OD ≥ 6 mg.L^{-1}; pH = 7.4 ± 0.05, total NH_4^{+} < 0.5 mg. L^{-1} and photoperiod 16L: 8D. Fish were fed only with live food (*D. magna*; 50 % body wet weight 2 h prior to solution replacement. No aeration was provided and every day the debris was removed from the bottom of the aquaria by siphoning.

At the end of each exposure period, 10 fish per group were killed by an overdose of benzocaine and blotted dry (Handy 1992). The gills and livers were immediately removed and carefully rinsed in distilled water and blotted dry. No chelating solution was used, so the accumulation in this study refers to the metal that entered the cells, as well as that which remained strongly bound to the epithelia following a rigorous wash.

Water samples for metal analysis were processed according to USEPA Method 200.2 (1991) and tissue metal analysis was conducted following Carriquiriborde and Ronco (2008). Bovine liver from the National Institute of Standards and Technology (US Department of Commerce) was used as reference material (recoveries were comprised within 95.1 % and 96.4 % interval).

Tissue cells suspensions for comet assays were prepared according to the protocol used by Deventer (1996) with some modifications. Briefly, 0.5 g of each sampled tissue was incubated in 2 ml ice-cold PBS-EDTA buffer (10 mM). Then 10 mL of trypsin (0.25 %) were added and incubated during 10 min at 37°C. Finally it was blocked with 4 mL of fetal bovine serum (10 %) in complete media. Cell suspension was centrifuged (1000 g for 10 min at 4°C), pellet was resuspended in PBS, and 25 μL were mixed with 75 μL of 0.5 % agarose (low melting point). Slides were mounted and single cell gel electrophoresis assay was performed by employing the alkaline version as described by Singh et al. (1988) with minor modifications. Slides for gill and liver Cd highest concentration were accidentally missed. Examination was performed using an Olympus BX 40 fluorescence microscope (400X). One hundred nuclei per slide were analyzed. Randomly selected nucleus were classified into five categories, 0–4, from no damage to higher relative tail length and intensity (Collins et al. 2008). Genetic damage index (GDI) was calculated as the sum of the scores of 100 cells and gives an overall score between 0 and 400 arbitrary units.

Data were analyzed using GraphPad Prism 6.0 software (Graphpad Software, Inc. San Diego, CA, USA). Results are presented as mean ± SEM. Normality of distributions was verified using ÓAgostino-Pearson omnibus normality test. ANOVA followed by Dunett post hoc test were used to compare differences between each treated group and their respective control. Differences were considered significant when $p < 0.05$. Pearson product-moment correlation was used to test correlation between metal tissue concentration and GDI. The lowest observed effect concentration (LOEC) was estimated as the lowest concentration inducing a response significantly different than the controls.

Results and Discussion

Differences between the nominal and measured waterborne concentrations were between 6 and 11 %, 4–23 %, and 7–65 % for Cd, Cr and Cu, respectively (Table 1). A significant accumulation of the three metals was observed in

Table 1 Waterborne metal concentrations in test media

Exposure level	Cd [nM]		Cr [nM]		Cu [nM]	
	Nominal	Measured	Nominal	Measured	Nominal	Measured
Control	0	nd	0	nd	0	25.5 ± 1.7
Low	9	9.4 ± 2.1	2000	2160 ± 30	150	260.6 ± 8.8
Intermediate	45	41.0 ± 7.5	10,000	7400 ± 800	750	921.1 ± 57.3
High	90	79.4 ± 7.2	20,000	18,500 ± 2100	1500	1677.3 ± 79.1

Measured concentrations are shown as mean ± SEM (n = 3), nd: below limit of detection

the gill and the liver (Table 2), showing that the metals were able to reach both tissues, as previously reported by (Carriquiriborde and Ronco 2008). Accumulation was higher and faster in the gill than in the liver in agreement with the exposure route.

Significant effects were induced by Cu exposure on GDI in the gill but not in the liver (ANOVA, $p = 0.016$ and 0.240, respectively) after 2 days. In addition, factorial ANOVA showed that GDI was significantly affected ($p < 0.05$) by the Cr and Cd concentration and the exposure time, both in the gill and the liver. Moreover, a significant interaction between these two factors was observed for Cr in the liver and Cd in both tissues. By day two (Fig. 1), significant DNA damage was induced by Cu and Cr in the gill but not in the liver. This was in agreement with the faster and higher metal accumulation measured in this organ. A similar response pattern was reported for zebrafish exposed to a reference genotoxic agent (Deventer 1996). A clear concentration–response relationship was observed for Cu exposure with the waterborne metal concentration and the tissue accumulation (Pearson $p = 0.006$). On the other hand, the response to Cr was not so easily to explain, since significant differences respect to the control group were observed only at the lowest tested concentration, at which no substantial metal accumulation was observed. An increasing trend was observed in the genetic damage induced by the highest Cd concentration after 2 days at, but differences were still not statistically significant from controls. On the other hand, by day 16 (Fig. 2), the genetic damage found in the gill and liver of fish exposed to both Cd and Cr was significantly higher than in controls. For Cd, dose–response of the GDI was evidenced in the gill, in agreement with the tissue accumulation levels (Pearson, $p = 0.002$). The response observed to this metal was less clear in the liver. The damage induced in the gill by Cr was not clearly related with the concentration neither in the water nor in the tissue. Conversely, a good concentration–response was observed for the GDI in the liver, both with waterborne concentration and tissue accumulation (Pearson, $p = 0.027$).

Copper genotoxicity is thought to be induced via free radical-induced oxidative damage (Bopp et al. 2008) and ROS induction and oxidative stress were observed in fish, either in vitro and in vivo (Lushchak 2011). In the present study a good relationship between the Cu accumulation and the genetic damage was observed in the gill after 2 days (Pearson, $p = 0.006$). DNA damage induced by Cu has been also evidenced by comet assay in trout gill cell cultures shortly exposed during 2 h to 1 and 2.5 μM of Cu (Bopp et al. 2008). In addition, 2.5 μM of Cu was capable to induce micronucleus in *Carassius auratus* and *Channa punctate* (Arkhipchuk and Garanko 2005; Yadav and

Table 2 Metal concentrations in *O. bonariensis* tissues

Tissue	Exposure level	Cd [mmol g^{-1}]		Cr [mmol g^{-1}]		Cu [mmol g^{-1}]
		2 days	16 days	2 days	16 days	2 days
Gill	Control	7.9 ± 2.0	7.7 ± 8.4	31.9 ± 4.2	23.6 ± 5.2	91.5 ± 7.7
	Low	13.2 ± 3.3	32.0 ± 3.5*	31.1 ± 3.5	23.8 ± 2.8	109.6 ± 23.3
	Intermediate	45.3 ± 7.9**	174.4 ± 8.9**	38.0 ± 7.3	51.9 ± 11.4*	149.2 ± 31.3*
	High	95.3 ± 3.3**	326.3 ± 25.1**	99.2 ± 7.2**	98.8 ± 6.2**	202.3 ± 26.7**
Liver	Control	3.3 ± 0.9	3.1 ± 0.4	1.1 ± 0.5	2.2 ± 1.0	47.1 ± 11.6
	Low	3.4 ± 0.5	3.4 ± 0.3	1.9 ± 0.6	1.7 ± 0.6	47.2 ± 14.5
	Intermediate	3.2 ± 0.2	4.5 ± 0.3*	1.6 ± 0.6	8.5 ± 3.5*	90.8 ± 25.6
	High	5.2 ± 1.0*	9.4 ± 0.4**	24.1 ± 2.5**	37.8 ± 4.9**	106.3 ± 14.0**

Mean ± SEM (n = 10); * $p < 0.05$; ** $p < 0.01$

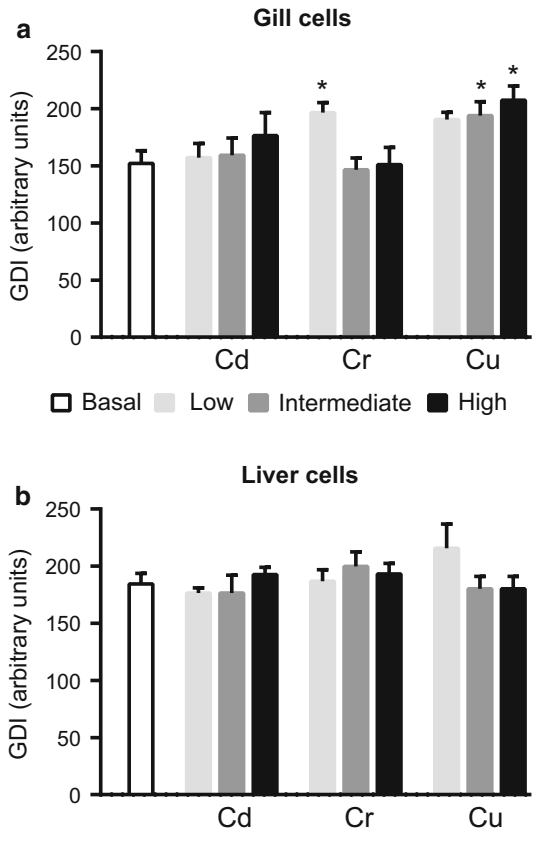

Fig. 1 Mean GDI values in gill (**a**) and liver (**b**) cells of *O. bonariensis* after 2 days exposure to basal, low, intermediate and high concentrations of Cd^{+2}, Cr^{+6} and Cu^{+2}. Error bars indicate SEM. Asterisks indicate significant differences respect to basal values (*$p < 0.05$)

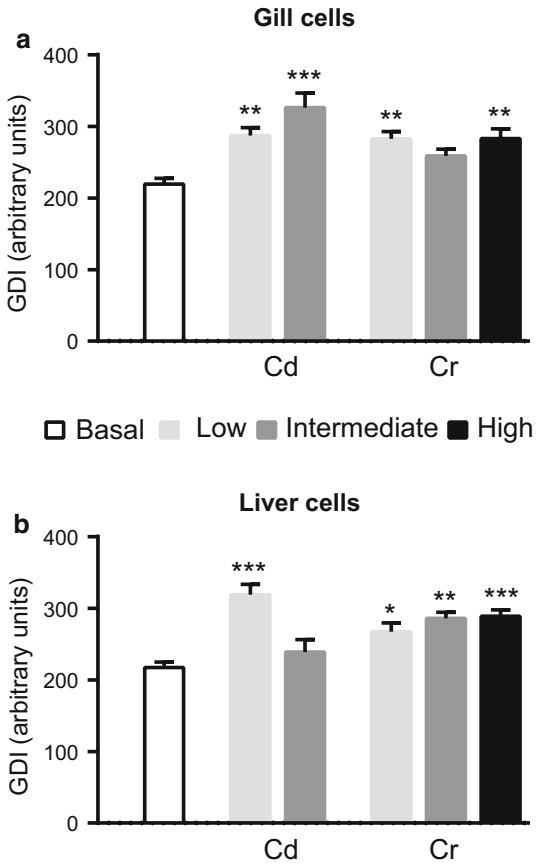

Fig. 2 Mean GDI values in gill (**a**) and liver (**b**) cells of *O. bonariensis* after 16 days exposure to basal, low, intermediate and high concentrations of Cd^{+2}, Cr^{+6} and Cu^{+2}. Error bars indicate SEM. Asterisks indicate significant differences respect to basal values (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Trivedi 2009). In the present study, the gill LOEC was 0.9 µM showing a high sensitivity of *O. bonariensisi* to Cu induced genotoxicity.

Despite significant accumulation of Cd by 2 days was observed in the gill and an initial trend of GDI was outlined by that exposure time, significant genotoxic effects were observed only after 16 days of exposure. This would be indicating that no only a specific metal level but also a given amount of time was necessary for inducing such effect. It is accepted that genotoxicity induced by Cd is the consequence of multifactorial mechanisms like ROS induction and inhibition of DNA are involved (Filipič 2012). Although, proposed mechanisms are similar to those described for Cu, process seems to be slower for Cd. Genetic damage induced in the liver by Cd after 16 days only at the lowest exposure concentration was more difficult to interpret since no significant accumulation was detected. Several hypothesis could be stated, such hormetic mechanisms involving repairing system, antioxidant response and defense mechanisms (i.e. metallothioneins) induction, at higher concentrations (Filipič 2012), but further experiments will be necessaries for testing them. In addition, it would be indicating that the method for detecting genotoxic effects seems to be more sensitive than the one for detecting accumulation. Several studies using fish (*Anabas testudineus*, *Carassius gibelio*, *Labeo rohita, Corydoras paleatus*, *Danio rerio*) have demonstrated Cd genotoxicity at concentrations ranging from 17 to 2.0 µM (Ahmed et al. 2010; Cambier et al. 2010; Jindal and Verma 2015), placing again *O. bonariensis* (gill LOEC 9.4 nM) among the most sensitive one.

Similarly to the discussed above for Cd, the genotoxic effect induced by Cr in the gill was not related neither with waterborne exposure nor with the accumulation of the metal in the tissue. In addition, the response to Cr in the liver was delayed respect to the significant accumulation. Genotoxicity of Cr^{+6} has been no only attributed to the ROS induction capacity during the intracellular reduction to Cr^{+3} but also to structural genetic lesions, including DNA adducts, DNA strand breaks, DNA–protein cross-links, oxidized bases, abasic sites, and DNA inter- and

intra-strand crosslinks (Nickens et al. 2010). DNA damage induced by Cr has also been observed in other fish species (*Carassius auratus, Heteropneustes fossilis*). However, the lowest effective concentrations were higher (10.9–800 μM) (Velma and Tchounwou 2010; Ahmed et al. 2013) than the gill LOEC (2.16 μM) observed for *O bonariensis*, placing again this specie among the most sensitive.

Maximum surface-water concentrations reported for studied metals in the "Río de la Plata" Basin, within the natural distribution area of *O. bonariensis*, were 60, 212 and 190 nM of Cd, Cr and Cu, respectively (Villar et al. 1999; Merlo et al. 2011; Rigacci et al. 2013; Avigliano et al. 2015). According to the LOECs found in the present study, and the reported metal concentrations in surface waters, it would be feasible to expect that genotoxic damage occurs in the gill and liver of *O. bonariensis* inhabiting those freshwater ecosystems, sometimes even before metal accumulation in tissues is detectable.

Acknowledgments This study was funded by CONICET and ANPCyT of Argentina. We acknowledge the Buenos Aires Province Ministry of Agriculture Affairs, particularly to the Chascomús Aquaculture Station, for providing the *O. bonariensis* larvae.

Conflict of interest The authors declare that they have no conflict of interest.

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