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Histopathological and biochemical changes in the liver, kidney, and blood of amphibians intoxicated with cadmium

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Abstract: The worldwide decline in amphibian populations has made heavy metal pollution a subject of interest. The aim of this work was to analyze the effect of sublethal doses of cadmium (Cd) on the liver, kidney, and blood of *Rhinella arenarum* specimens. Serum markers indicative of hepatic injury (gamma glutamyltransferase, alkaline phosphatase, pseudo cholinesterase, and total cholesterol) and markers of renal dysfunction (blood urea nitrogen, serum creatinine, calcium, and glucose) showed variation in animals treated with a 0.5 mg/kg dose compared to controls. Histopathological images revealed alterations in the liver (hepatocyte ballooning and hyperplasia of Kupffer cells) and kidney (renal tubular lumen dilation with tubular necrosis) of animals with abnormal serum markers. The above-mentioned lesions were more evident with the 5 mg/kg dose. With the 0.5 mg/kg dose, hematological values remained normal with the exception of the leukocyte formula. Animals treated with 5 mg/kg showed a significant decrease in both white and red blood cell counts and hematocrit and hemoglobin values. Differential leukocyte counts showed neutrophilia, monocytosis, and lymphopenia. Morphological aberrations were found in white and red blood cells. Results indicated that the evaluation of morphological and functional parameters in kidney, liver, and blood is required in order to monitor amphibian populations exposed to chemical contaminants.

Key words: Amphibian, cadmium, liver, kidney, blood, toxicity

1. Introduction

One of the main reasons for the decrease in amphibian specimens in natural habitats is chemical pollution (Collins, 2010). The rapid worldwide increase in the use of electrical and electronic equipment, the disposal of which pollutes the environment, has generated the so-called E-waste (Eriksson et al., 2011). E-waste contains toxic chemical substances, among them cadmium (Cd), a heavy metal used in rechargeable batteries from computers and cell phones, switches, cathode ray tube monitors, and photovoltaic devices. Cd accumulates in the water, air, and soil, and because of its great chemical stability against biodegradation it can be incorporated into living beings and remain there for about 10–30 years (Birge et al., 2000).

Although environmental pollution studies often use physicochemical methods, bioassays are particularly important since they show the effect of contaminants on living systems and reveal functional disturbances that affect the health of the organism through all stages of life. Amphibians are good pollution indicators since their life cycle includes both water and land habitats. The embryo and larval stages take place in water, and once metamorphosis is over, they live on land and return to the water at the time of reproduction. Thus, characteristics of these sites can have important effects on their survival. With respect to the effect of Cd on amphibians, most known data come from embryos and tadpoles and show that Cd has noxious effects on their survival, growth, and behavior (James and Little, 2003; Mouchet et al., 2006; Gross et al., 2007). However, there are few studies on Cd pollution in adult specimens. In *Rana ridibunda* (Kostaropoulos et al., 2005; Sura et al., 2006; Loumbourdis et al., 2007) and *Xenopus laevis* (Woodall and Maclean, 1992) the liver and kidney are the target organs for Cd accumulation, which causes various undesirable effects. In *Bufo regularis* (Hilmy et al., 1986) and *Bufo raddei* (Zhang et al., 2007), alterations caused by Cd have been reported in several hematological parameters. However, the effect and dimensions of the alterations observed are not common to all amphibian species, since there can be interspecific (Birge et al., 2000) and intraspecific variations (Miaud et al., 2011).

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Our region, northwestern Argentina, presents a dangerous situation with respect to toxic substances as a result of, among other causes, mishandling of pesticides, inadequate treatment of urban and industrial effluents, and noncompliance with regulations on the treatment of existing hazardous residues (Sosa et al., 1999). Fernández-Turiel et al. (2001) found elevated levels of heavy metals, among them Cd (0.27–30.68 mg/kg), in urban soils located within the vicinity of a lead smelter in the province of Tucuman, northwestern Argentina. Moreover, in our region a level higher than 1 mg/L of Cd has been reported (Comisión Nacional de Energía Atómica, Informe N° 260537) in the waterways inhabited by *Rhinella arenarum* specimens. For this reason, we selected this species as a pollution indicator to analyze the effect of Cd on the vital functions of specimens and to evaluate and standardize bioassays. The aim of this work was to study variations in biochemical and morphological parameters in the kidney, liver, and blood of adult *R. arenarum* males taken from their natural habitat and treated in the laboratory with sublethal doses of Cd, since these variations reveal the physiological disturbances that may affect the health of these amphibians.

2. Materials and methods

2.1. Animals

Adult *R. arenarum* males (100–150 g body weight) were collected during September–November in the neighborhood of San Miguel de Tucumán, Argentina.

R. arenarum is a species widely distributed in South America, and its population in the Argentine northwest allows easy sampling.

2.2. Preparation of the cadmium solution

Cadmium chloride ($CdCl_2$) (Sigma-Aldrich CAS N° 10108-64-2, 99.0% pure) was dissolved in distilled water.

2.3. Intoxication treatment

The experimental conditions were previously described by Medina et al. (2012). Animals were treated for 15 days with a daily injection into the dorsal lymphatic sac of either 0.5 or 5 mg/kg $CdCl_2$ (0.9–1 mL/animal) (n = 3 per dose/year). Control animals (n = 3/year) were injected by the same route with 0.9–1 mL of distilled water during the same period. Throughout the assay both groups were kept at room temperature in plastic cages with perforated lids containing tap water, which was renewed once a day. Treatments were performed for three consecutive years.

Animal maintenance and experimental procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* (European Communities Council Directive, 1986).

Although the natural assimilation paths of environmental components are the oral and dermal routes, Cd was injected into the dorsal lymphatic sac to ensure total incorporation of the doses assayed. The $CdCl_2$ doses used in the present study were chosen according to: (a) Cd levels reported in soils (0.27 to 30.68 mg/kg) (Fernandez-Turiel et al., 2001) and water courses (1 mg/L) (Comisión Nacional de Energía Atómica, Informe N° 260537) inhabited by *R. arenarum* in our region; (b) doses used by other investigators: 0.5 mg/kg body weight in subcutaneous injection into *R. arenarum* for 10 days to study the liver (Pérez-Coll et al., 1997) and 6.2 mg/kg injected intramuscularly into *Bufo regularis* for 4 days for blood parameter studies (Hilmy et al., 1986); and (c) doses that cause alterations in the reproductive system (Medina et al., 2012) of *R. arenarum* without causing death or changes in the behavior of the specimens.

2.4. Determination of hematological parameters

At the end of the treatment, blood samples of control (n = 9) and treated animals (n = 9 per dose) were collected by cardiac puncture in the presence of EDTA (1.5 mg/mL). White blood cell (WBC) counts and red blood cell (RBC) counts were performed in a Neubauer chamber, as previously described for species with nucleated RBCs (Allender and Fry, 2008).

The hematocrit (Ht) was determined by the microhematocrit method and hemoglobin concentration (Hb) by the cyanmethemoglobin method, while differential leukocyte counts (DLC) were performed in blood smears with May–Grunwald Giemsa staining.

2.5. Micronucleus test

Micronucleus (MN) frequency was determined following Lajmanovich et al. (2005). The count of 1000 RBCs per animal was performed using a light microscope at 1000× magnification. The criteria for the identification of micronuclei were: round small-sized structure with a diameter significantly smaller than the main nucleus with similar staining, well-defined edges, and clear separation from the main nucleus (Lajmanovich et al., 2005).

2.6. Histopathological evaluation of liver and renal tissue

After treatment for 15 days, samples of liver and renal tissue of control (n = 6) and treated animals (n = 6 per dose) were taken within 15 min after euthanasia and preserved in phosphate buffered formaldehyde. The tissue was embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin–eosin (H&E) and periodic acid–Schiff (PAS) for routine diagnostics, while Masson's trichrome stain was used to detect fibrous tissue (collagen), and Perl's reaction was used for detection of ferric ions.

2.7. Serum biochemical parameters of liver and kidney function

In this set of experiments, control (n = 9) and treated animals (n = 9 per dose) were studied.

Blood samples were taken by cardiac puncture and allowed to clot in plastic tubes for 2 h at room temperature.

Serum was separated by centrifugation at 3000 × *g* for 10 min, fractionated, and stored at –20 °C until used.

2.7.1. Electrolytes

Sodium (Na^+), potassium (K^+), and chloride (Cl^-) determination was conducted with a flame photometer (Metrolab model 305). The range of concentration for Na^+ was 40–220 mmol/L, for K^+ 1–30 mmol/L, and for Cl^- 20–250 mmol/L. In all determinations deionized water was used as a blank target.

Calcium (Ca^{2+}) analyses were performed with a Jarrell Ash model 590 atomic absorption photometer. Cation standards (Riedel de Haën) gave a linear standard curve for Ca^{2+} at 4227 Å from 0.01 to 0.3 mg/dL.

2.7.2. Metabolites and enzymes

Metabolites were determined with the following techniques: albumin (colorimetric method; bromocresol green method), triglycerides (TG) (triacylglycerol) (colorimetric enzymatic method; glycerophosphate oxidase-peroxidase antiperoxidase method), total cholesterol (TC) (colorimetric enzymatic method; cholesterol esterase/peroxidase method), blood urea nitrogen (BUN) (ultraviolet kinetic method; glutamate dehydrogenase method), serum creatinine (colorimetric kinetic method; Jaffé method), uric acid (colorimetric enzymatic method; uricase method), and glucose (colorimetric enzymatic method; hexokinase method).

Enzymes: pseudocholinesterase or butyrylcholinesterases (pChE) (colorimetric enzymatic method), alanine aminotransferase (ALT) (ultraviolet kinetic method), alkaline phosphatase (ALP) (colorimetric kinetic method), gamma glutamyltransferase (GGT) (colorimetric kinetic method), and lactate dehydrogenase (LDH) (ultraviolet kinetic method).

2.8. Cd determination in liver and kidney

Cd concentrations in liver and kidney were measured with graphite furnace atomic absorption spectrometry. In order to perform the determination, 0.02–0.2 g of tissue of control (n = 3) and treated animals (n = 3 per dose) was wet-digested with concentrated nitric acid, 30% hydrogen peroxide, and concentrated sulfuric acid (10:5:1 ratio) in Teflon containers and then decontaminated and brought to volume with distilled water, as described by Medina et al. (2012).

2.9. Statistical analysis

Statistical analysis was performed using the nonparametric Kruskal–Wallis test followed by the multiple comparison method (Dunn's method). All statistical comparisons were made above the 95% confidence level.

3. Results

3.1. Hematological parameters

The hematological parameters of animals exposed to Cd are shown in Table 1. Compared to the controls, the administration of 0.5 mg/kg Cd resulted in significantly higher ($P < 0.05$) values for neutrophil percentage, although at this dose no alterations were found in the other parameters analyzed. In contrast, animals treated with 5 mg/kg Cd showed a significant decrease ($P < 0.05$) in RBC, WBC, Ht, and Hb. Differential leukocyte counts showed neutrophilia, monocytosis, and lymphopenia (Table 1).

The mature erythrocytes of control (Figure 1A) and treated animals (Figures 1B–1D) show an oblong-oval shape with a central nucleus that is clearly structured and with well-defined edges. With the 5 mg/kg dose, some erythrocytes show nuclear morphological aberrations, such as binucleated cells (Figure 1B, inset). The frequency

Table 1. Changes in the hematological parameters of male adult *Rhinella arenarum* treated with 0, 0.5, or 5 mg/kg $CdCl_2$ for 15 days.

Parameter	Control (0 mg/kg)	0.5 mg/kg	5 mg/kg
Red blood cells (T/L)	0.52 ± 0.08 [b]	0.33 ± 0.02	0.20 ± 0.01
White blood cells (G/L)	30.81 ± 1.24 [b]	30.60 ± 1.27	23.10 ± 0.05
Hematocrit (%)	41.94 ± 2.02 [b]	45.88 ± 1.41	31.85 ± 4.36
Hemoglobin (g/dL)	15.85 ± 0.94 [b]	14.06 ± 0.63	10.45 ± 1.21
Neutrophils (%)	20.83 ± 1.40 [a,b]	30.45 ± 4.87	36.50 ± 1.44
Eosinophils (%)	4.45 ± 1.08	2.40 ± 0.92	4.82 ± 0.70
Basophils (%)	2.15 ± 0.61	0.75 ± 0.33	0.65 ± 0.28
Monocytes (%)	2.23 ± 0.66 [b]	1.97 ± 0.73	8.90 ± 2.70
Lymphocytes (%)	64.43 ± 1.98 [b]	65.15 ± 5.10	50.50 ± 7.55
Red blood cell micronuclei (‰)	4.01 ± 0.65	4.21 ± 0.46	4.56 ± 0.53

Values are mean ± SEM.

a, b: control values were significantly different from values of 0.5 (a) and 5 (b) mg/kg Cd, respectively.

of MN after treatment with Cd (0.5 and 5 mg/kg) is small and not statistically significant compared to control animals ($P > 0.05$) (Table 1).

With respect to the white blood cell series, stimulated lymphocytes (Figure 1C) and toxic granulation of neutrophils (Figure 1D inset) were observed.

3.2. Histopathological study and cadmium concentrations in the kidney

The renal architecture of control animals shows preserved histological characteristics (Figure 2A). In animals treated with the 0.5 mg/kg dose, mixed-type inflammatory infiltrate with a predominance of polymorphonuclear leukocytes and glomerular congestion was observed. Some proximal tubules show hydropic tumefaction, tubular necrosis, and the presence of protein cylinders (Figure 2B). Other tubules are altered, with no cell edges, and with an amorphous substance in the cytoplasm. No glomerular or tubular basal membranes were found with the PAS technique. The above-mentioned lesions were more evident at the 5 mg/kg dose (Figures 2C and 2D).

Cd concentration increased in the kidney during treatment with both doses (Table 2). Statistically, the values determined were significantly higher than those in the control group (Table 2).

3.3. Histopathological study and cadmium concentrations in the liver

In control animals, the liver parenchyma exhibited a normal microscopic structure (Figure 3A), while in intoxicated animals histopathological alterations were found and increased with the increase in dose. At the 0.5 mg/kg dose, slight mixed portal inflammatory infiltrate with lobar compromise could be observed (Figures 3B and 3B inset) together with sinusoidal dilatation, inflammation or hepatocyte ballooning, and hyperplasia of Kupffer cells (Figure 3C). At the 5 mg/kg dose the above-mentioned lesions became more evident (Figure 3D), and there was also pericentral hepatocyte necrosis (Figure 3E).

Perl's method revealed that part of the liver parenchyma was stained pale blue/turquoise, indicating iron deposits (ferric form), and the stained area increased from the controls to the animals treated with the highest dose (Figure 3A inset and Figure 3B inset). No fibrosis was observed with Masson's technique.

Cd concentration in the liver of treated animals was significantly higher than in controls (Table 2), and the highest Cd concentration was found with the 5 mg/kg dose.

3.4. Serum biochemical modifications of liver and kidney function

In animals intoxicated with 0.5 mg/kg a significant increase ($P < 0.05$) in BUN, serum creatinine, ALP, GGT, LDH, and TC and a significant decrease ($P < 0.05$) in pChE, glucose, and Ca^{2+} were found compared to control animals (Table 2). At the 5 mg/kg dose, alteration in the parameters analyzed was found together with a significant increase ($P < 0.05$) in BUN, serum creatinine, uric acid, ALT, ALP, GGT, LDH, TC, and TG and a significant decrease ($P < 0.05$) in albumin, pChE, glucose, Na^+, Cl^-, and Ca^{2+} compared to controls (Table 2). Potassemia values were not altered during the period studied.

4. Discussion

Considering that the examination of blood samples has been widely used as an effective bioindicator in toxicology (Sancho et al., 2000), we analyzed the modifications in different hematological parameters to assess the damage caused by Cd. Under our experimental conditions we observed a marked decline in RBCs accompanied by a decrease in Ht and Hb values of animals exposed to the highest doses of Cd, suggesting anemia. The decrease in RBCs could be due to the lysis of erythrocytes, which transport oxygen and carbon dioxide to and from body tissues. Our hypothesis is supported by Kotsanis et al. (2000), who injected 0.5 mg/kg Cd, 0.2 mg/kg arsenic, or 0.5 mg/kg mercury in a single dose and separately into the yolk sacs of newly hatched rainbow trout larvae, showing that these heavy metals induce erythrocyte destruction.

The decrease in Hb content could be the result of the harmful effects of Cd at the level of parenchymal cells due to inflammation and necrosis of hepatocytes, as shown by our results in this work. These alterations would cause a decrease in easily available iron stores and, consequently, a decrease in Hb content.

Another mechanism that could explain this inhibition is oxidative stress. Enzyme systems that eliminate free radicals and their derivatives would be relatively deficient in liver cells, as reported by Sura et al. (2006). The decrease in Ht values is confirmation of the synergistic link between the two blood parameters (RBCs and Hb) in all vertebrates (Hilmy et al., 1986). This decrease, which is indicative of Cd-induced anemia, is consistent with previous studies of anemia in fish, rats, and rabbits exposed to Cd, mercury, lead, nickel, copper, and zinc (Sjobeck et al., 1984; Nanda and Behera, 1996; Bersenyi et al., 2003).

With respect to the leukocyte population, we observed leukopenia with neutrophilia and lymphopenia, which could be the response to stressors such as chemical pollutants (Srivastava and Agrawal, 1977; Barni et al., 2007; Davis et al., 2008). An increase in the circulating levels of glucocorticoids, which are hormones that act on the white blood cell series, occurs as a specific response to stress (Srivastava and Agrawal, 1977; Davis et al., 2008). In fact, Davis et al. (2008) reported a close relationship between the number of leukocytes in the blood, their differential count, and circulating levels of glucocorticoids.

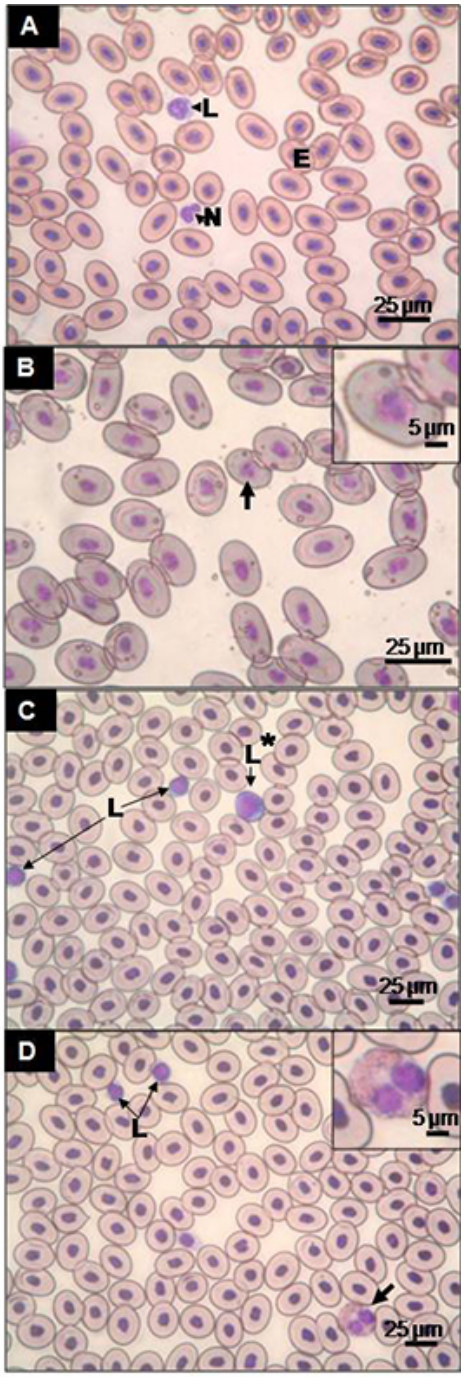

Figure 1. Light micrographs of blood cells of *R. arenarum* after treatment with Cd.

(A) Controls. Preserved cell morphology. Lymphocytes, L; neutrophils, N; erythrocytes, E. May–Grunwald Giemsa stain.

(B) Treated with 5 mg/kg. Binucleate erythrocytes (arrow). Inset: binucleate erythrocytes. May–Grunwald Giemsa stain.

(C) Treated with 5 mg/kg. Lymphocytes (L) and stimulated lymphocytes (L*) can be observed. May–Grunwald Giemsa stain.

(D) Treated with 5 mg/kg. Neutrophils with toxic granulation (arrow) can be observed. Lymphocytes, L. Inset: detail of toxic granulations. May–Grunwald Giemsa stain.

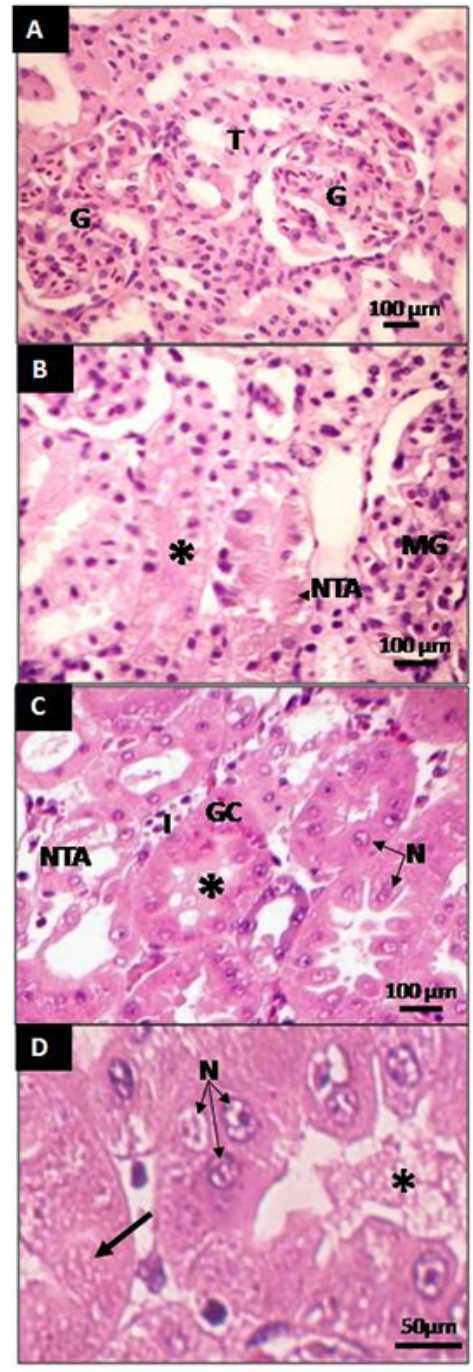

Figure 2. Light micrographs of kidney tissue of *R. arenarum* after treatment with Cd.

(A) Controls. Renal glomeruli (G) with architectural and cytological conservation can be observed. Tubules (T) with cytological preservation and absence of cylinders and inflammatory infiltrate. H&E stain.

(B) Treated with 0.5 mg/kg. Proximal convoluted tubules with acute tubular necrosis (NTA) in patches and expansion of the glomerular mesangium (MG) can be observed. Intraluminal eosinophilic amorphous material (*). H&E stain.

(C) Treated with 5 mg/kg. Acute tubular necrosis (NTA) and cytoplasmic granulations (GC) in renal tubules. Reactive and degenerative nuclear changes (N). Mixed interstitial inflammatory material (I). Intraluminal eosinophilic material (*). H&E stain.

(D) Treated with 5 mg/kg. Extensive acute tubular necrosis. Karyorrhexis and loss of cell edges (arrow). Remaining nuclei (N) with prominent nucleolus and granulations of the chromatin. Tubular lumen with eosinophilous content (*). H&E stain.

Table 2. Cadmium liver and kidney content and serum biochemical parameters of male adult *Rhinella arenarum* treated with 0, 0.5, or 5 mg/kg $CdCl_2$ for 15 days.

Parameter	Control (0 mg/kg)	0.5 mg/kg	5 mg/kg
Cd in kidney (µg/g)	9.18 ± 0.92 [a,b]	31.7 ± 1.61	214 ± 17.89
Cd in liver (µg/g)	0.262 ± 0.02 [a,b]	38.8 ± 2.42	263 ± 14.43
BUN (g/L)	0.85 ± 0.07 [a,b]	1.21 ± 0.10	2.47 ± 0.63
Serum creatinine (mg/dL)	0.23 ± 0.03 [a,b]	0.63 ± 0.08	0.89 ± 0.18
Uric acid (mg/dL)	0.50 ± 0.08 [b]	0.76 ± 0.10	1.10 ± 0.10
Albumin (mg/dL)	1.73 ± 0.10 [b]	1.70 ± 0.11	0.72 ± 0.08
ALT (UI/L)	0.63 ± 0.17 [b]	1.23 ± 0.25	2.40 ± 0.16
ALP (UI/L)	9.00 ± 6.17 [a,b]	180.00 ± 45.98	88.00 ± 22.12
GGT (UI/L)	2.26 ± 0.60 [a,b]	11.28 ± 2.03	53.75 ± 4.97
pChE (UI/L)	20997.4 ± 2701.4 [a,b]	13236.5 ± 3755.3	2803.0 ± 328.5
LDH (UI/L)	276.43 ± 94.60 [a,b]	886.00 ± 37.00	1537.33 ± 78.85
TC (mg/dL)	81.90 ± 8.60 [a,b]	166.20 ± 12.20	198.00 ± 43.00
TG (mg/dL)	40.86 ± 1.53 [b]	37.81 ± 3.85	68.24 ± 6.04
Na^+ (mmol/L)	105.62 ± 3.37 [b]	109.25 ± 2.56	91.75 ± 1.25
K^+ (mmol/L)	2.75 ± 0.19	2.95 ± 0.25	2.40 ± 0.10
Cl^- (mmol/L)	76.87 ± 3.03 [b]	79.80 ± 0.48	59.25 ± 1.65
Ca^{2+} (mg/dL)	0.43 ± 0.03 [a,b]	0.21 ± 0.04	0.19 ± 0.01
Glucose (mg/dL)	25.50 ± 0.50 [a,b]	8.02 ± 3.12	9.33 ± 2.18

Values are mean ± SEM.
a, b: control values were significantly different from values of 0.5 (a) and 5 (b) mg/kg Cd, respectively.
Cadmium (Cd), blood urea nitrogen (BUN), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), pseudocholinesterase (pChE), lactate dehydrogenase (LDH), total cholesterol (TC), and triglycerides (TG).

This phenomenon was found in *Rana esculenta* (Barni et al., 2007), *Rana catesbeiana* (Bennett and Harbottle, 1968), and *Rana pipiens* (Bennett and Alspaugh, 1964) as well as in other vertebrate taxa in response to natural stressors (Davis et al., 2008). In addition, the increase in neutrophils could also be related to the inflammatory process (Thrall, 2004) caused by Cd.

The cellular alterations observed in lymphocytes suggest that Cd would probably act either directly on the lymphocytes or on their blast cells, according to results published by Kotsanis et al. (2000). These authors demonstrated that lymphocytes were activated by a heavy metal, thereby altering their structure and influencing latent functions. The toxic granulation of neutrophils in peripheral blood could be associated with inflammatory processes related to Cd.

In the present study we demonstrated that Cd bioaccumulates in the liver and kidney of *R. arenarum.* Although Cd is deposited in both organs in a dose-dependent manner, higher doses were found in liver than in kidney. This accumulation could be due to low Cd excretion, as observed by other authors (Vogiatzis and Loumbourdis, 1998; Ezemonye and Enuneku, 2012).

Cd accumulated in the liver can alter various parameters. In fact, treatment with Cd is reflected in changes at the functional and cytological levels involving both the hepatocytes and Kupffer cells of *R. arenarum.* Our findings were consistent with those observed in *Rana esculenta*. Thus, specimens collected in areas contaminated with chromium (Fenoglio et al., 2005), similarly to those experimentally exposed to chromium and Cd, only to Cd (Loumbourdis and Vogiatzis, 2002; Loumbourdis et al., 2007), or to mercury (Loumbourdis and Danscher, 2004), showed an increase in the area of Kupffer cell clusters. These cells increase their number and/or size due to the influence of heavy metals. This proliferative and/or

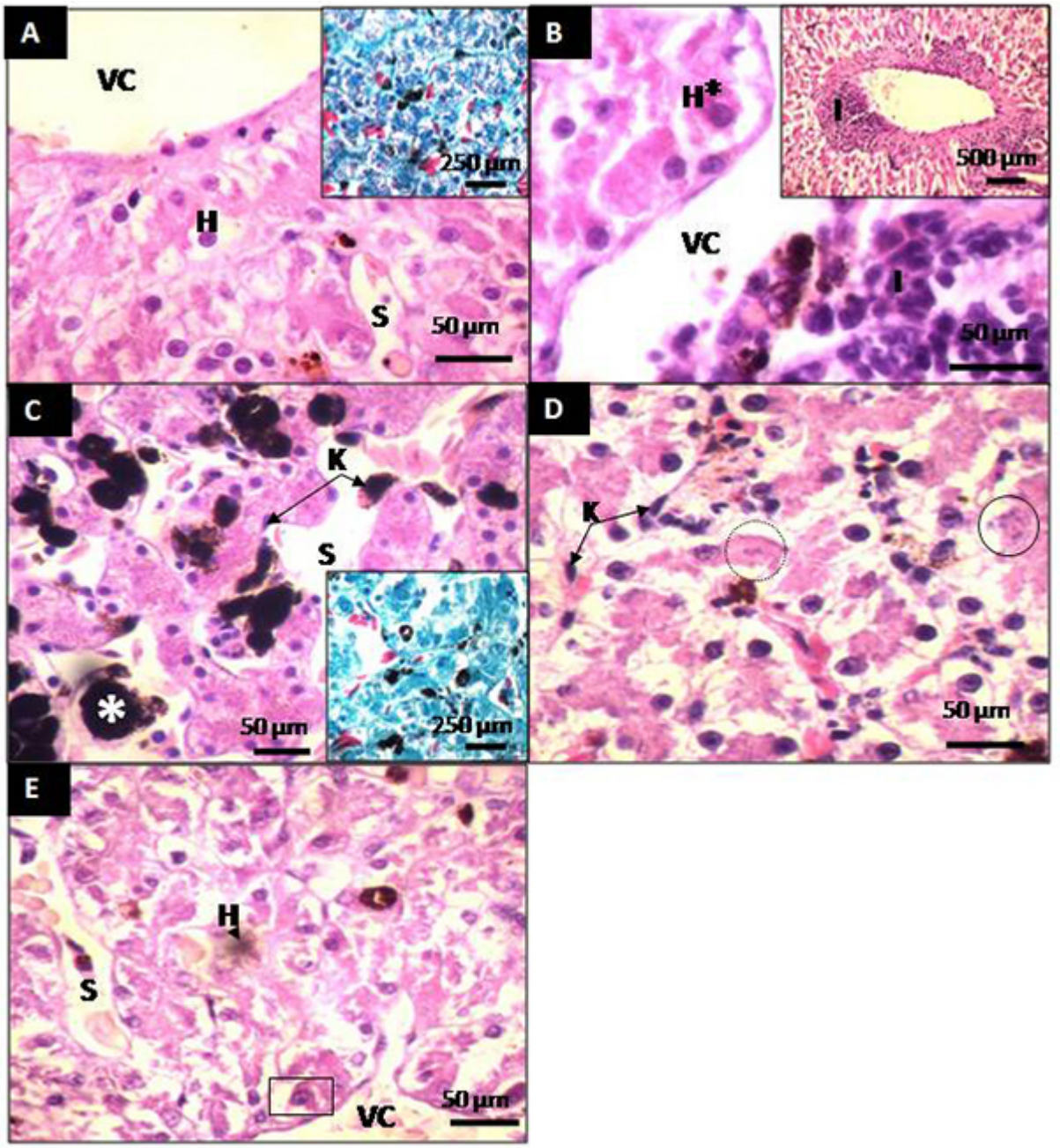

Figure 3. Light micrographs of liver tissue of *R. arenarum* after treatment with Cd.
(A) Controls. Hepatic lobule with normal histological characteristics. Hepatocytes with monomorphic nuclei (H) in sinusoidal arrangement. Central vein, VC; sinusoid, S. H&E stain. Inset: identification of ferric iron deposits (Perl's technique).
(B) Treated with 0.5 mg/kg. Centrilobular vein (VC) with adjacent necrotic hepatocytes (H*) and mixed inflammatory infiltrate (I). Inset: portal space showing arterial vessel with parietal accumulations of inflammatory cells. H&E stain.
(C) Treated with 0.5 mg/kg. Various lesions can be observed: marked sinusoidal dilatation (S), prominent iron accumulations (*), and hyperplasia of Kupffer cells (K). H&E stain. Inset: identification of ferric iron deposits (Perl's technique).
(D) Treated with 5 mg/kg. Hepatocyte with loss of trabecular arrangements can be seen as well as cytoplasmic vacuolization and necrosis (karyolysis, dotted circle; karyorrhexis, solid circle). Hyperplasia of Kupffer cells (K). H&E stain.
(E) Treated with 5 mg/kg. Necrosis of the hepatocyte (H) central perivein (VC) can be observed. Apoptotic hepatocyte (rectangle). Note important sinusoidal dilatation (S). H&E stain.

hyperplastic reaction would be the result of mechanisms that are activated before the destruction of the liver parenchyma. This is why Kupffer cells have been proposed as histopathological markers of Cd toxicity (Loumbourdis and Danscher, 2004).

The increase in the area stained blue by Perl's method in liver parenchymal cells is an indication of an increase in iron deposits (hemosiderin granules) and therefore very poorly available to supply (Loumbourdis, 2005). In mammals these deposits increase in liver pathologies, probably by phagocytosis of RBCs by Kupffer cells (Corsaro et al., 2000).

In different organs, serum levels of a particular enzyme increase in diseases leading to: (i) an increase in release by the tissue, either through greater permeability of cell membranes or necrosis; (ii) a higher amount available for release due to an increase either in cell production or in cells that produce them; or (iii) a decrease in excretion due to lack of permeability of biliary tracts (obstruction). Recognition of these mechanisms may be especially useful for identifying target organs of Cd toxicity. In our study we observed an increase in the activity of nonspecific and specific enzymes. Among the former is LDH, a cytoplasmic enzyme present in almost all tissues and a nonspecific marker of cell death (Dreiem et al., 2005) as a result of Cd-induced damage to the cell membrane. With respect to ALP (also cytoplasmic) and GGT (of microsomal localization) both indicate hepatocellular lesion and/or obstruction of the bilious tracts. Among the specific enzymes, ALT, a cytoplasmic enzyme with a higher concentration in the liver than in other tissues, reveals hepatocyte damage (Ogunkeye and Roluga, 2006). The release of these enzymes from hepatocytes into the bloodstream would result from Cd-induced histopathological alterations such as inflammation and necrosis.

Pseudocholinesterase (pChE) is a class of serine hydrolases synthetized by liver cells and immediately secreted into the bloodstream (Adham, 2002; Ogunkeye and Roluga, 2006), which is why changes in the rate of enzyme synthesis are directly reflected in serum levels. The dose-dependent decrease in the activity of pChE determined in this study would indicate a disturbance in the biosynthesis of this enzyme due to alteration of the liver parenchyma. Our results agree with those reported by Adham (2002), who found that the activity of serum pChE decreased in *Clarias gariepinus* in waters contaminated with Cd, lead, manganese, mercury, and nickel. Therefore, changes in the activity of serum pChE can be considered a specific marker of liver dysfunction, as suggested by Ogunkeye and Roluga (2006). The decrease in the capacity of liver synthesis caused by Cd also affected albumin production, although only at the highest dose.

In amphibians, as in other vertebrates, the kidneys play an important part in the maintenance of a stable inner environment that may be disturbed by the presence of xenobiotics. Our results in *R. arenarum* demonstrated that Cd produces an accumulation of metabolic waste products in serum (uric acid, BUN, and serum creatinine).

With respect to this parameter, Adham (2002) found high serum creatinine values in populations of fish (*C. gariepinus*) living in polluted environments. These values have been associated with a physiological imbalance due to renal failure and/or an increase in the catabolism of the muscle tissue. Other consequences of renal dysfunction are hypoglycemia, hyponatremia, hypochloremia, and hypocalcemia related to alterations in the proximal tubules.

The increase in TG would be the consequence of the disruption of the lipid metabolism induced by Cd. This hypothesis is consistent with the data provided by Higley et al. (2013) in *Lithobates sylvaticus* specimens exposed to the fungicide triphenyltin (TPT). These authors found that the abundance of transcripts of lipoprotein-lipase, stearoyl-coenzyme A desaturase decreased in larvae exposed to 5.0 µg TPT/L.

In conclusion, the present study shows that exposure of the frog *R. arenarum* to Cd can cause biochemical alterations that induce unfavorable physiological changes in the target organism. These alterations are considered indications of the toxic action of the metal on target organs (liver, kidney) and tissue (blood). Therefore, biochemical parameters could be used as biomarkers since their variations are warning signals of the effects of Cd at the individual or population level.

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