Effects of depuration on histopathological changes in Tilapia (*Oreochromis niloticus*) after exposure to Cylindrospermopsin

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Running title: Depuration reverses CYN-induced histopathological changes in Tilapia

Abstract

Cylindrospermopsin (CYN) is a highly water-soluble cytotoxin produced by several species of freshwater cyanobacteria and it is considered the second most studied cyanotoxin worldwide. CYN acts as a potent protein and glutathione synthesis inhibitor, as well as inducing genotoxicity, oxidative stress and histopathological alterations. Studies concerning the depuration of cyanobacterial toxins in aquatic organisms, especially in fish, are of great interest for fish economy and public health, but are scarce in the case of CYN. This is the first study reporting the ability of depuration (3 or 7 days) in reversing or ameliorating the histopathological lesions induced in liver, kidney, heart, intestines and gills of tilapia (*Oreochromis niloticus*) due to exposure by immersion to repeated doses of a CYN-containing culture of *A. ovalisporum* for 14 days. The main histopathological changes induced by CYN were glucogenic degeneration and loss of the normal hepatic cord-structure (liver), hyperemia, dilated Bowman's capsule and cellular tumefaction (kidney), myofibrolysis, hemorrhages and edema (heart), necrosis and partial loss of microvilli (gastrointestinal tract), and hyperemia and inflammatory cells infiltrates (gills). After 3 days of depuration, gills were totally recovered, while the liver, kidney and gastrointestinal tract required 7 days, and longer depuration periods may be needed for a full recovery of the heart. In addition, the morphometric study indicated that depuration managed to reverse the affectation in the hepatocytes nuclear diameters and cross sections of the proximal and distal convoluted tubules induced in CYN-exposed fish. In general, these results validate depuration as an effective practice for detoxification of fish contaminated with CYN.

Keywords: Cylindrospermopsin, Depuration, Histopathology, Morphometry, Tilapia.

1. Introduction

The occurrence and progressive proliferation of harmful cyanobacteria in freshwater and marine ecosystems have been recognized as a potential consequence of eutrophication and climate change worldwide (O'Neil et al., 2012). Most of the bloom-forming species of cyanobacteria are known to produce secondary metabolites with antiviral and antifungal properties, cytotoxic and enzyme-inhibiting activities, as well as antineoplastic and allelopathic activities (Corbel et al., 2014). Some of these secondary metabolites are cyanotoxins, which include a diversity of alkaloid and peptide toxins, being Cylindrospermopsin (CYN) one of the most emergent and important freshwater algal toxins. Structurally, CYN is a tricyclic guanidine moiety combined with hydroxymethyluracil first isolated and identified in 1992 (Ohtani et al., 1992) and highly soluble in water (Sivonen and Jones, 1999). Four naturally occurring analogues of CYN have been identified so far: 7-epicylindrospermopsin, 7-deoxy-cylindrospermopsin (Kinnear, 2010), and more recently 7-deoxy-desulfo-cylindrospermopsin and 7-deoxy-desulfo-12-acetylcylindrospermopsin (Wimmer et al., 2014).

The mechanism of CYN toxicity is mediated by inhibition of protein synthesis (Runnegar et al., 2002; 2010; Žegura et al., 2011a,b). CYN is widely cytotoxic (Hawkins et al., 1985; Harada et al., 1994; Falconer et al., 1999; Falconer and Humpage, 2006), and its ability to induce oxidative stress has been demonstrated *in vitro* (Gutiérrez-Praena et al., 2011a; 2012a,b) and *in vivo* in fish (Gutierrez-Praena et al., 2011b; Puerto et al., 2011a; Guzmán-Guillén et al., 2013a) and in several aquatic animals (Puerto et al., 2011b; Kinnear, 2010), as well as in different plant species (Prieto et al., 2011; Gutiérrez-Praena et al., 2014; Freitas et al., 2015). Moreover, CYN is known to produce histopathological changes in several tissues of tilapia (*Oreochromis niloticus*) acutely exposed to the pure toxin orally (Puerto et al., 2014; Guzmán-Guillén et al., 2015a) or by intraperitoneal injection (Gutiérrez-Praena et al., 2012c), similarly to the changes induced by other cyanotoxins such as microcystins (MCs) (Atencio et al., 2008). Those alterations has also been observed by exposure to CYN-producing cyanobacterial cells (Guzmán-Guillén et al., 2015b,c). Furthermore, some works demonstrate the ability of CYN to induce morphometric changes in the average nuclear diameter of hepatocytes, cross-sections of renal convoluted tubules in

the kidney, and in the cardiac fibers and capillaries diameters in the heart (Gutiérrez-Praena et al., 2012c; Guzmán-Guillén et al., 2015a,b,c).

Research into CYN has been performed with pure standard toxin and cyanobacterial cells containing CYN (Kinnear, 2010). Taking into account that in natural conditions fish are exposed to cyanobacterial blooms for long periods, during which toxins are produced continuously, and cell extracts are likely to contain one or more bioactive compounds other than CYN, the risk of toxic effects in this case would be increased compared with exposure to pure CYN (Hawkins et al., 1997; Falconer et al., 1999; Norris et al., 1999; Seifert, 2007).

The potential for cyanobacterial toxins to bioaccumulate, particularly in aquatic species with commercial importance for human consumption, has received some attention in the last decade (Ibelings and Chorus, 2007). High CYN levels and its persistence in water can potentiate this accumulation in a wide range of aquatic organisms (Kinnear, 2010; Gutiérrez-Praena et al., 2013; Guzmán-Guillén et al., 2015d). Moreover, despite the lack of consistent epidemiological data, consumption of contaminated water and food seems to be the major sources of chronic human exposure to CYN (Freitas et al., 2016).

Depuration is defined as the loss of a substance from an organism as a result of any active or passive process (ASTM, 2013), exploiting the natural physiological mechanisms of organisms to promote purging of the gastrointestinal (GI) tract (Jackson and Ogburn, 1999). Studies concerning cyanotoxins depuration in aquatic organisms, especially in fish, are important for fish economy and public health (Mohamed and Hussein, 2006). Several studies have demonstrated the effectiveness of depuration against oxidative stress in fish and clams exposed to various contaminants such as polychlorinated biphenyls, pesticides, polycyclic aromatic hydrocarbons and metals, among others (Ferreira et al., 2005, 2007; Özcan Oruç, 2010; Freitas et al., 2012; Gagnaire et al., 2013). However, the effect of depuration of cyanotoxins in aquatic organisms has been neglected, being more abundant with MCs or nodularin (NOD) (Sahin et al., 1996; Ozawa et al., 2003; Yokoyama and Park, 2003; Xie et al., 2004; Mohamed and Hussein, 2006; Kankaanpää et al., 2007; Tricarico et al., 2008; Galanti et al., 2013), and scarcer with CYN (Saker et al., 2004; Ríos et al., 2014; Guzmán-Guillén et al., 2014, 2015e). There is a need to further investigate the effectiveness of short-term

depuration in removing toxins from fish and thus the potential use of this process as a protective health strategy in aquaculture (Al-Ghais, 2013). Having demonstrated the usefulness of the depuration process in reversing the above-mentioned damages at different levels in fish exposed to CYN, we found it interesting to know if this process was also able to reverse or mitigate the histopathological effects of CYN in the target organs of the toxin in fish. Moreover, the way that different species respond to depuration is poorly understood, and the histopathological status of the organisms after this period is practically unknown.

The aim of the present study was to evaluate whether depuration processes (3 or 7 days) were able to reverse or ameliorate the histopathological lesions in liver, kidney, heart, intestines and gills of tilapia (*O. niloticus*) exposed by immersion to repeated doses of a CYN-containing culture of *A. ovalisporum* for 14 days. As far as we know, this is the first study concerning the reversion of these alterations by depuration in fish intoxicated with CYN.

2. Materials and Methods

2.1. Chemicals

Pure CYN (purity > 95%) was supplied by Alexis Corporation (Lausen, Switzerland). Chemicals for the different assays were provided by Sigma–Aldrich and VWR International Eurolab. HPLC-grade methanol, dichloromethane, formic acid, and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Deionized water (>18 MΩ cm^{-1} resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, USA). BOND ELUT® Carbon cartridges (500 mg, 6 mL) were supplied by Agilent Technologies (The Netherlands, Europe).

2.2. *Aphanizomenon ovalisporum* culture and determination of CYN

Aphanizomenon ovalisporum (LEGE X-001) cyanobacterial CYN-producing strain (CYN+) was isolated from Lake Kinneret (Banker et al., 1997) and kindly supplied by Dr. Vitor Vasconcelos (Marine Research Centre, Porto, Portugal). A culture of this strain was maintained in Z8 medium at 25ºC under continuous illumination with an intensity of 28 µmol photons m^{-2} s^{-1} provided by cool white fluorescent tubes. After 33 days, cultures were harvested by decantation and filtration with plankton net (20 µm

diameter). The biomass obtained was frozen at -80ºC until lyophilization (Telstar Cryodos, Madrid).

CYN extraction and purification with graphitized carbon cartridges from the lyophilized culture of *A. ovalisporum* (CYN+) was performed according to Guzmán-Guillén et al. (2012), prior to its LC-MS/MS analysis. Chromatographic separation was performed using a Perkin Elmer Series 200 HPLC system (Wellesley, USA) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, USA) consisting of an hybrid triple quadrupole linear ion trap (QqQ_{lit}) mass spectrometer equipped with an electrospray ion source. LC-MS/MS analyses were performed in the same conditions as in Guzmán-Guillén et al. (2012), detecting 0.1 µg CYN/mg and 0.012 µg deoxy-CYN/mg of culture.

2.3. Experimental setup, fish acclimation and exposure to CYN

Thirty male *O. niloticus* (Nile tilapia, average weight: 20 ± 8 g, length: 7.8 ± 2 cm) were obtained from the fish hatchery "Aquaculture Valencia", and then maintained and acclimatized in our laboratory for 15 days in the same conditions as Guzmán-Guillén et al. (2014) before the beginning of the experiment.

After the acclimation period, 6 experimental groups (5 individuals per group) were established as explained below.

- Control group: fish not exposed to the toxin and not depurated.
- Exposure group: 14-day exposed fish and not depurated.
- Depuration control groups: fish not exposed to CYN and depurated for 3 or 7 days, respectively.
- Exposure plus depuration groups: 14-day exposed fish followed by 3 or 7 days of depuration.

Intoxicated fish were subchronically exposed to CYN by immersion in aquaria added with a CYN-containing culture of *A. ovalisporum* LEGE X-001 in order to reach 10 µg CYN/L, and the same dose was added every two days, during 14 days, according to Guzmán-Guillén et al. (2014). The procedure was designed to simulate the fish diet during and after a toxic *A. ovalisporum* bloom, whereas other feeding sources (represented by 0.5 g fish food/day) were available at all times.

2.4. Light and electron microscopy

Tissue samples were taken from the liver, kidney, heart, intestines, and gills of control, exposed and depurated fish for histological examination. For light microscopy, samples were first fixed in 10% buffered formalin for 24 h at 4ºC, and then immediately dehydrated in a graded series of ethanol, immersed in xylol and embedded in paraffin wax by using an automatic processor. Sections were processed according to Guzmán-Guillén et al. (2015e).

For electron microscopy, samples were prefixed in 2% glutaraldehyde fixative (in pH 7.4 phosphate buffer for 10 h at 4ºC) and postfixed in 1% osmium tetroxide fixative (in pH 7.4 phosphate buffer for 0.5 h at 4ºC). Subsequently, they were dehydrated in a graded ethanol series and embedded in epon. Ultra thin sections (50-60 nm) were cut with a LKB microtome and were further processed according to Guzmán-Guillén et al. (2015e). Gill samples were fixed in glutaraldehyde 2.5% in 0.1 M phosphate buffer for ultrastructural study, postfixed in 2% osmium tetroxide, dehydrated in acetone, critical point dried, ion-sputter coated with gold. Tissue sections were examined in a Philips CM10 electron microscope, and gills were also viewed through a scanning electron microscope (JEOL JSM 6300).

2.5. Morphometric study

For the structural quantifications, the fixed liver, kidney and heart were cut into three sections and each portion was then histologically processed, dehydrated in a graded series of ethanol, immersed in xylol and embedded in paraffin wax. Later, the fixed liver, kidney and heart were processed according to Guzmán-Guillén et al. (2015c). To quantify any alterations on hepatocytes size, the average diameter of the nuclei was employed; the average cross sections of the proximal and distal convoluted tubules and of capillaries were estimated in the case of the kidney and the heart, respectively, according to Guzmán-Guillén et al. (2015c).

2.6. Statistical Analysis

Data were analyzed by applying bivariate comparisons considering nonparametric methods. Differences among groups, with respect to hepatocyte nuclear diameters, convoluted tubules cross-sections and capillaries diameters values were

tested using the Kruskal–Wallis test, whereas pair-wise differences were compared by the Tukey–Kramer test. The analyses were conducted using the Statistical software Statistica, version 6. Statsoft Inc. All reported *p* values were two-tailed, with $p<0.05$ considered as significant.

3. Results

No mortality or behavioural alterations were observed in any of the fish during the exposure or depuration periods. Moreover, no remarkable histopathological changes were observed in any of the studied organs of control fish (Figs. 1-5A, B).

Regarding the liver of control fish, apparently normal hepatocytes with abundant cytoplasmic organelles were observed, mainly in the rough endoplasmic reticulum (RER) (Figs. 1A, B). The microscopic examination of the HE-stained liver sections of fish exposed to CYN revealed processes of glycogenic degeneration and loss of liver cord-like structure, hepatocytes arranged in mosaic with clear nuclei and acidophilic cytoplasm with irregular content (Fig. 1C). Ultrastructurally, hepatocytes showed rounded nuclei, scant cytoplasm with very few cytoplasmic organelles and accumulation of glycogen (Fig. 1D). These lesions were scarce after 3 days of depuration, with some glycogen content (circle) (Figs. 1E, F), and almost nonexistent after 7 days of depuration, showing an apparently normal structure of hepatic cords (Figs. 1G, H).

The kidney of control fish showed apparently normal glomeruli and tubules, with normal membrane, endothelium and podocytes (Po) (Figs. 2A, B). Under light microscopy, fish kidneys exposed to CYN showed glomerulus with hyperemia (arrow), dilation of Bowman's capsule (star) and swelling of cells of the proximal and distal convoluted tubules (PCT, DCT) (Fig. 2C). Electron microscopy showed renal glomerulus with swelling of Po and cell necrosis (Fig. 2D). After 3 days of depuration, certain lipid droplets could still be appreciated (Fig. 2F), and after 7 days of depuration, the kidney displayed an apparently normal morphology (Figs. 2G, H).

Apparently normal muscle fibers and myofibrils were observed in heart of control fish (Figs. 3A, B). Fish exposed to CYN revealed a picture of myofibrolysis and haemorrhage under light microscopy (Fig. 3C), intracellular edema and certain fatty degeneration by electron microscopy (Fig. 3D). After 3 days of depuration, some

degeneration and edema were still visible (Figs. 3E, F), and after 7 days, the recovery was not complete since the edemas (arrow) still persisted (Fig. 3G).

Gastrointestinal samples from control fish displayed apparently normal villi with abundant and normal enterocytes (Figs. 4A, B). The lesions observed in the intestine of tilapias treated with CYN consisted of catarrhal necrotic enteritis (circle) (Fig. 4C), and electron microscopy showed partial loss of microvilli in the epithelial layer of the intestine (circle) (Fig. 4D). After the 3-d depuration, this degeneration of enterocytes was still persistent (circle) (Figs. 4E, F), which disappeared completely after 7 days of depuration, showing enterocytes with abundant microvilli (Figs. 4G, H).

Gills of control fish showed an apparently normal structure (Figs. 5A, B, C). Injuries observed by light microscopy in gills of fish intoxicated with CYN were characterized by a strong hyperemia in the lamellae (circle) (Fig. 5D). The ultrastructural study showed inflammatory cell infiltrate (circle) by transmission (TEM) and scanning (SEM) electron microscopy (Figs. 5E, F). From 3 days of depuration, gill structure showed to be apparently normal, exhibiting abundant mucous cells (Figs. 5G-L).

Results from the morphometric study in the liver showed a significant increase in the hepatocyte nuclear diameters in fish exposed to CYN (1.4-fold), and both depuration periods tested reduced these diameters to control levels (Fig. 6). In the kidney, the data concerning the average cross sections of the PCT and DCT showed a significant increase in fish exposed to the toxin (1.5- and 1.4-fold, respectively), in comparison to control fish (Figs. 7a, b). In the case of the PCT, after a 3-d depuration period, their cross-sections were still augmented versus its control group (1.3-fold), and this increase was restored to control values after 7 d of depuration (Fig. 7a). Regarding the cross-sections of DCT, both depuration periods managed to restore the control values (Fig. 7b). The morphometric study in the heart revealed that CYN administration did not induce any significant changes in the capillaries diameters compared to the control group (Fig. 8).

4. Discussion

Depuration is a complex process involving a number of interacting variables which affect the activity of the animals and the way that depurated material is taken and

kept away from these organisms (Lee et al., 2008). The efficiency of depuration has been discussed (Jackson and Ogburn, 1999), as some authors are of the opinion that depuration of contaminated shellfish and fish is impractical due to the associated costs and long toxin retention times found in many species (Arnott, 1998). Despite this, Wohlgeschaffen et al. (1992) had previously demonstrated the successful depuration of mussels contaminated with domoic acid (DA) under laboratory conditions, leading to further research to determine the usefulness of rapid depuration of contaminated aquatic organisms. To improve the safety of consuming shellfish potentially contaminated with toxicants, Guéguen et al. (2011) suggested that the depuration programs used to purify shellfish of microbiological contamination before commercialization should be further investigated to determine if and how these residues could be reduced, as the alternative that has been used to date, such as closing contaminated areas for long periods of time, results in significant economic losses. In this sense, Tricarico et al. (2008) observed that crayfish depurated up to 21 days showed a decrease in MCs content from day 6, demonstrating that there may be no need for longer depuration periods, which might also lead to decreased costs. These records highlight the need to investigate short periods of depuration for potentially reversing the effects of toxins in aquatic organisms.

Cyanotoxins depuration studies in aquatic organisms are scarcer compared to other contaminants, and have focused on MCs or NOD (Ozawa et al., 2003; Mohamed and Hussein, 2006; Kankaanpää et al., 2007; Osswald et al., 2008; Tricarico et al., 2008; Galanti et al., 2013). Saker et al. (2004) firstly investigated CYN accumulation and depuration in the freshwater mussel *Anodonta cygnea*. Concerning fish, short depuration periods (3 and 7 days) managed to recover from the oxidative damages induced in tilapia by exposure to *A. ovalisporum* cells containing CYN and 7-deoxy-CYN for 7 and 14 days, at biochemical and molecular levels (Guzmán-Guillén et al., 2014; Ríos et al., 2014). Moreover, the reversion of the histopathological alterations induced in the present work is generally in accordance with the one observed in brain of fish undergoing the same conditions, where after 3 days of depuration histopathological damages still persisted, but practically disappeared after 7 days (Guzmán-Guillén et al., 2015e). All this indicates that the animals are able to reprogram their cell response once transferred to an unpolluted site, thus achieving a recovery of the normal status and elimination of the toxic by themselves. CYN extreme hydrophilic nature and its relative small molecular weight (415 Da), that might facilitate passive diffusion through

epithelial cells (Chong et al., 2002), could also explain the reversibility of the lesions found in this work when fish were placed in non-contaminated waters.

In the present study, 3 and 7 days of depuration managed to slightly and completely recover, respectively, the loss of the hepatic structure induced by CYN, probably due to the loss of CYN from the tissue. In fact, in a previous study, we did not detect the toxin in liver of these fish during the depuration period (Guzmán-Guillén et al., 2015d). However, 240 µg CYN/kg dry weight was detected in liver after 14 days of exposure, which could explain that after 3 days of depuration, although not detectable, a small amount of toxin could still be present in the tissue, not allowing it to totally recover its normal structure until 7 days of depuration. Exposure to repeated doses of the toxin may also explain that the tissue takes longer to recover from the lesions. In agreement with our results, Selenium (Se) concentration was depleted to basal levels in Sacramento splittail (*Pogonichthys macrolepidotus*) after 21 weeks of depuration, observing normal liver morphology throughout the depuration period, supposing that additional liver damage due to Se toxicity would be minimum (Deng et al., 2007). On the contrary, Paris-Palacios et al. (2000) found that the hepatic histological responses induced by Copper (Cu) in zebrafish did not appear reversible even after 14 days of depuration, since Cu was still remnant in liver in this period.

Persistence of CYN toxicity in kidney after a 3-day depuration in this work, as seen by the existing fatty infiltrate, could be due to the continuous input of the toxin from liver and other organs during the early phase of depuration. This is in accordance with Deng et al. (2007), who observed that after a 13- and 21-week depuration, Se-induced kidney lesions decreased compared to a 3- and 7-week depuration, and this is likely due to a significant decrease in the input of Se from liver and muscle. Lefebvre et al. (2007) investigated in salmon tissue uptake, distribution and depuration patterns of DA, a highly hydrophilic toxin like CYN, revealing that the highest toxin levels accumulated in kidney and bile, and that the majority of accumulated DA depurated quickly from fish, but a portion of the toxin remained for several days.

The reversion of histopathological lesions found in the present work in liver and kidney is in accordance with the one observed of the biochemical changes in the same fish organs in a previous study (Guzmán-Guillén et al., 2014). After 3 days of depuration, tilapia liver and kidney still underwent oxidative stress, as marked by the

lipid and protein oxidation and alterations in catalase activity and glutathione (GSH) content, but their overall response was of adaptive nature. After 7 days of depuration, proteins were still undergoing oxidation only in liver, which could explain that some histopathological lesions were still evidenced in 3 day-depurated fish liver compared to kidney. This tendency agrees with the results of Jiang et al. (2009), who observed that malachite green was highly and widely distributed in the excretory tissues of liver and kidney from three common freshwater fish, and its depuration occurred quickly, being faster in kidney than in liver in all fish. This suggests the possibility of a more active degradation of the toxicant in excretory tissues for kidney.

According to Yokoyama and Park (2003), a decline in the production of detoxification enzymes might decrease the depuration rate constant for MC-LR in *U. douglasiae.* In the case of our fish, the opposite process might be occurring, with an increased metabolism of CYN in the cleaning period, as shown in a previous study by glutathione peroxidase and glutathione S-transferase (GST) relative gene expressions and GST activity and relative abundance, all still developed in liver and kidney after 7 days of depuration (Ríos et al., 2014). Thus, this fact may increase the depuration rate in these organs of fish, as these detoxifying systems are efficient allowing the degradation of CYN to other less or non-toxic products by metabolic processes. This increased metabolism/excretion is consistent with the recovery of the histopathological lesions observed in the present study in liver and kidney of the exposed fish, being higher with the longer depuration time.

In this work, even after the 7-day depuration period, the potential of tilapia heart to cope with the toxin effects was still impaired, as the heart was the only organ that did not recover totally from the edema after this process. This organ showed to be the most reluctant also toward prevention of CYN-induced histopathological lesions in fish by administration of different antioxidants (N-acetylcysteine, vitamin E and L-carnitine -LC-) (Gutiérrez-Praena et al., 2014; Guzmán-Guillén et al., 2015a, c). Similarly to our results, Milinkovitch et al. (2013) found that after a 14-day depuration of golden grey mullet (*Liza aurata*), the toxicity induced by oil dispersants in heart was still present. Kinnear (2010) reported the likely affinity of CYN for blood or lymph, in contrast to other cyanotoxins such as MCs and NOD. Thus, being highly water-soluble, CYN is easily distributed in blood and go through the heart for its later distribution to the rest of

the body. This could explain the later recovery of the heart in the present work, needing longer depuration periods than the studied for a total recovery.

In this study, the reversion of GI tract pathologies follows a time-dependent pattern (which may be related to a dose-response pattern too), in which some lesions are prevalent after the 3-day depuration and absent at the end of the 7-day depuration. In agreement with our results, Yuen et al. (2007) found that intestinal morphofunctional changes in the fish *Epinephelus coioides* exposed to benzo[*a*]pyrene were reversible from 1-week depuration. They suggested that the alterations observed could be a mechanism employed to excrete the toxicant accumulated in the enterocytes, and continued during depuration, being essential to ensure a complete elimination and replacement of damaged epithelial cells. Moreover, Álvarez-Muñoz et al. (2009) found that depuration for 3 days did not manage to reverse the epithelial desquamation in intestines of fish exposed to a surfactant for 5 days, even observing an increase in lipid droplets in the depuration period. Elimination of MCs from the digestive gland of *Lymnaea stagnalis* observed by Lance et al. (2010) after a 3-week depuration may occur by degradation and consequent elimination of damaged cells, as suggested by the presence of regenerating lobules after depuration and/or detoxification pathways, such as conjugation to GSH. According to the latter hypothesis, in our work during the first days of depuration, the intestinal alterations are still present because GST and GSH are working in conjugation with CYN for its elimination. Indeed, this is what happened in liver of the same fish in previous works, evidenced by decreases in GST activity, protein and gene expression and GSH content, that did not recover until 7 days of depuration (Ríos et al., 2014; Guzmán-Guillén et al., 2014), as well as the normal intestinal histopathology in the present work. So we could assume these parameters followed the same tendency in intestines.

In this work, gills were the first organ to completely recover from the lesions induced by CYN from 3 days of depuration. In agreement with our results, Álvarez-Muñoz et al. (2009) also found a general decrease in all the histopathological responses in gills of fish exposed to a surfactant after being depurated for 3 days, with values returning to the control situation. Considerably less biotransformation of CYN takes place in gills and therefore less reactive oxygen species are generated, allowing this organ to better support the exposure to the toxin, compared to the organism's internal tissues. This, together with the high water-solubility of CYN, may explain the faster

depuration in gills compared to other organs of fish, being this organ constantly and directly in contact with the aquatic medium. Moreover, toxicant-induced changes in the gills tend to be largely non-specific, suggesting that they might simply reflect a physiological adaptation to stress (Mallatt, 1985). According to this, the alterations observed in gills in the present study might be viewed as a protective mechanism, because this may slow down CYN uptake (Saravana Bhavan and Geraldine, 2000), more than a progressive loss of gill biological functions, and this is supported by the quick recuperation of the tissue after the 3-day depuration.

Concerning morphometric studies, the reversion of the increased nuclear diameters of hepatocytes found in the present work from 3-day depuration is in agreement with the prevention of this alteration obtained by vitamin E in fish exposed to CYN (Guzmán-Guillén et al., 2015a). These results also revealed a higher susceptibility of the kidney by exposure to CYN, obtaining a reversion of the increased PCT and DCT cross-sections in fish depurated for 7 and 3 days, respectively. This high susceptibility, especially of the PCT, is in accordance with Guzmán-Guillén et al. (2015a, c), where a protection was observed by administration of vitamin E and LC. The kidney has a great blood supply and a large tubular epithelial surface, which makes CYN, highly hydrophilic, have high affinity for this organ, and this fact may difficult its recuperation. The higher susceptibility of the PCT could be explained because this is where secretion and reabsorption of toxicants take place, with a high rate of toxification processes.

In general, the histopathological changes detected in this study could be considered as a stress response against CYN exposure and a general adaptive mechanism, as the results demonstrated that the organism can recover from them by a depuration process. These damages decreased after depuration following a different pattern in each organ, explaining the longer time of recovery for some of them. Previous investigations performed with antioxidants demonstrated that pretreatments with vitamin E (700 mg/kg fish bw/day) and LC (400 or 880 mg/kg bw fish/day) for 7 and 21 days, respectively, were able to prevent similar histopathological alterations induced by CYN in all organs of tilapia (Guzmán-Guillén et al., 2015a, c). Therefore, we consider that although depuration is a more natural and economical procedure compared to pretreatment with antioxidants, it did not manage to totally prevent the same alterations

in all organs of fish intoxicated with CYN, as the heart still presented damages after 7 days of depuration.

5. Conclusions

This work demonstrates for the first time the effectiveness of short depuration periods (3 or 7 days) in the recovery of different organs of tilapia (*O. niloticus*) from the histopathological damages induced by repeated exposure to *A. ovalisporum* cells containing CYN and deoxy-CYN for 14 days. After 3 days of depuration only the gills achieved a recovery of their normal structure, while the liver, kidney and GI tract required 7 days. However, longer periods of depuration than the studied in this work may be needed for a full recovery of the alterations observed in the heart. In general, these results validate the depuration process as an effective practice for detoxification in fish contaminated with CYN.

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Figure legends

Fig. 1. Histopathological changes in liver of Tilapia (*Oreochromis niloticus*) subchronically exposed to repeated doses 10 µg CYN/L by immersion in an *A. ovalisporum* culture for 14 days and submitted to two depuration periods (3 and 7 days). (**A, C, E, G)**: HE-stained liver sections. Bars: 100 µm. (**B, D, F, H)**: Ultrastructural observations. Bars: 10 µm. **(A, B)** Control fish: **(A)** normal hepatic cords, polyhedral morphology with central nucleus and clear cytoplasm; **(B)** detail of apparently normal hepatocyte, with abundant cytoplasmic organelles, reticulum and mitochondria. Apparently normal bile ducts (Bd). **(C, D)** Tilapia exposed to CYN for 14 days: **(C)** hepatic parenchyma containing hepatocytes with glycogen content (circle); **(D)** hepatocyte with clear cytoplasm due to the presence of glycogen (circle). **(E, F)** Tilapia exposed to CYN for 14 days and depurated for 3 days: **(E)** hepatocytes with an apparently normal cord-like structure and low presence of glycogen (circle); **(F)** hepatocyte with glycogen content (circle) and abundant rough endoplasmic reticulum (RER). **(G, H)** Tilapia exposed to CYN for 14 days and depurated for 7 days: **(G)** Detail of liver parenchyma with apparently normal structure of hepatic cords; **(H)** Hepatocyte with central nuclei (Nu) surrounded by low glycogen content (circle) and abundant rough endoplasmic reticulum (RER).

Fig. 2. Histopathological changes in kidney of Tilapia (*Oreochromis niloticus*) subchronically exposed to repeated doses 10 µg CYN/L by immersion in an *A. ovalisporum* culture for 14 days and submitted to two depuration periods (3 and 7 days). (**A, C, E, G)**: HE-stained kidney sections. Bars: 100 µm. (**B, D, F, H)**: Ultrastructural observations. Bars: 10 µm. **(A, B)** Control fish: **(A)** apparently normal renal parenchyma; **(B)** detail of glomerulus (Gl) with apparently normal membrane, endothelium and podocytes (Po). **(C, D)** Tilapia exposed to CYN for 14 days: **(C)** detail of renal parenchyma where the glomerulus shows hyperemia (arrow), dilation of Bowman's capsule (star) and cell tumefaction from the proximal (PCT) and distal (DCT) convoluted tubules; **(D)** renal glomerulus with podocytes (Po) tumefaction and cell necrosis (circle). **(E, F)** Tilapia exposed to CYN for 14 days and depurated for 3 days: **(E)** apparently normal glomeruli (circle) and proximal (PCT) and distal (DCT)

convoluted tubules; **(F)** detail of renal glomeruli with some lipid droplets (circle). **(G, H)** Tilapia exposed to CYN for 14 days and depurated for 7 days: **(G)** apparently normal glomeruli (circle) and proximal (PCT) and distal (DCT) convoluted tubules; **(H)** detail of glomerulus with apparently normal podocytes (Po).

Fig. 3. Histopathological changes in heart of Tilapia (*Oreochromis niloticus*) subchronically exposed to repeated doses 10 µg CYN/L by immersion in an *A. ovalisporum* culture for 14 days and submitted to two depuration periods (3 and 7 days). (**A, C, E, G)**: HE-stained heart sections. Bars: 100 µm. (**B, D, F, H)**: Ultrastructural observations. Bars: 10 µm. **(A, B)** Control fish: **(A)** apparently normal muscle fibers; **(B)** apparently normal myofibrils and abundant mitochondria (circle). **(C, D)** Tilapia exposed to CYN for 14 days: **(C)** detail of myocardium with myofibrolysis (circle) and haemorrhage (arrow); **(D)** detail of degenerated myofibrils (arrow) and presence of intracellular edema (circle). **(E, F)** Tilapia exposed to CYN for 14 days and depurated for 3 days: **(E)** parenchyma with little degeneration (arrow) and some lipid content (circle); **(F)** cardiac fiber with apparently normal myofibrils and some signs of degeneration (circle). **(G, H)** Tilapia exposed to CYN for 14 days and depurated for 7 days: **(G)** Detail of apparently normal cardiac parenchyma with slight edema (arrow); **(H)** detail of apparently normal myofibrils and desmosomes bands (circle).

Fig. 4. Histopathological changes in gastrointestinal tract of Tilapia (*Oreochromis niloticus*) subchronically exposed to repeated doses 10 µg CYN/L by immersion in an *A. ovalisporum* culture for 14 days and submitted to two depuration periods (3 and 7 days). (**A, C, E, G)**: HE-stained intestine sections. Bars: 100 µm. (**B, D, F, H)**: Ultrastructural observations. Bars: 10 µm. **(A, B)** Control fish: **(A)** apparently normal villi with abundant and normal enterocytes; **(B)** Enterocytes with highly developed microvilli and apparently normal. **(C, D)** Tilapia exposed to CYN for 14 days: **(C)** intestinal mucosa with abundant necrotic enterocytes (circle) and degenerated mucosa in

the apical area (arrow); **(D)** areas from the epithelial layer of the intestines with partial loss of microvilli (circle). **(E, F)** Tilapia exposed to CYN for 14 days and depurated for 3 days: **(E)** Detail of intestine with scarce degeneration of the intestinal cells (circle); **(F)** Well-structured microvilli with scarce degeneration (circle). **(G, H)** Tilapia exposed to CYN for 14 days and depurated for 7 days: **(G)** detail of apparently normal intestinal microvilli; **(H)** detail of enterocytes with abundant microvilli.

Fig. 5. Histopathological changes in gills of Tilapia (*Oreochromis niloticus*) subchronically exposed to repeated doses 10 µg CYN/L by immersion in an *A. ovalisporum* culture for 14 days and submitted to two depuration periods (3 and 7 days). (**A, D, G, J)**: HE-stained gill sections. Bars: 100 µm. (**B, E, H, K)**: Ultrastructural observations, transmission electron microscope (TEM). Bars: 10 µm. (**C, F, I, L)**: Ultrastructural observations, scanning electron microscope (SEM). Bars: 10 µm. **(A, B, C)** Control fish: **(A)** detail of apparently normal gill; **(B)** typical lamella structure with apparently normal capillaries; **(C)** lamellae with apparently normal structure. **(D, E, F)** Tilapia exposed to CYN for 14 days: **(D)** lamellae of the gill arch great developed, but a strong hyperemia is highlighted in their capillaries (circle); **(E)** lamellae with presence of inflammatory infiltrate (circle); **(F)** Presence of inflammatory cell infiltrate (circle). **(G, H, I)** Tilapia exposed to CYN for 14 days and depurated for 3 days: **(G)** apparently normal lamellae with abundant mucous cells (Cc); **(H)** apparently typical lamellae with highly developed mucous cells (Cc); **(I)** apparently normal lamella structure. **(J, K, L)** Tilapia exposed to CYN for 14 days and depurated for 7 days: **(J, K)** detail of apparently normal lamellae; **(L)** apparently typical lamella structure.

Fig. 6. Hepatocyte nuclear diameters values (µm) of Tilapia (*Oreochromis niloticus*) subchronically exposed to repeated doses 10 µg CYN/L by immersion in an *A. ovalisporum* culture for 14 days and submitted to two depuration periods (3 and 7 days). The values are expressed as mean ± sd (n=5). The significance levels observed

are $**p < 0.01$ in comparison to the respective control group, and $##p < 0.01$ when comparing depurated to non-depurated fish.

Fig. 7. Proximal (a) and Distal (b) convoluted tubules cross sections (µm) of Tilapia (*Oreochromis niloticus*) subchronically exposed to repeated doses 10 µg CYN/L by immersion in an *A. ovalisporum* culture for 14 days and submitted to two depuration periods (3 and 7 days). The values are expressed as mean ± sd (n=5). The significance levels observed are $*p < 0.05$ or $***p < 0.001$ in comparison to the respective control group, $\#p < 0.05$ or $\#\#\#p < 0.001$ when comparing depurated to non-depurated fish, and ‡‡$p < 0.01$ when comparing both depuration periods.

Fig. 8. Capillaries diameters (µm) of Tilapia (*Oreochromis niloticus*) subchronically exposed to repeated doses 10 µg CYN/L by immersion in an *A. ovalisporum* culture for 14 days and submitted to two depuration periods (3 and 7 days). The values are expressed as mean ± sd (n=5).

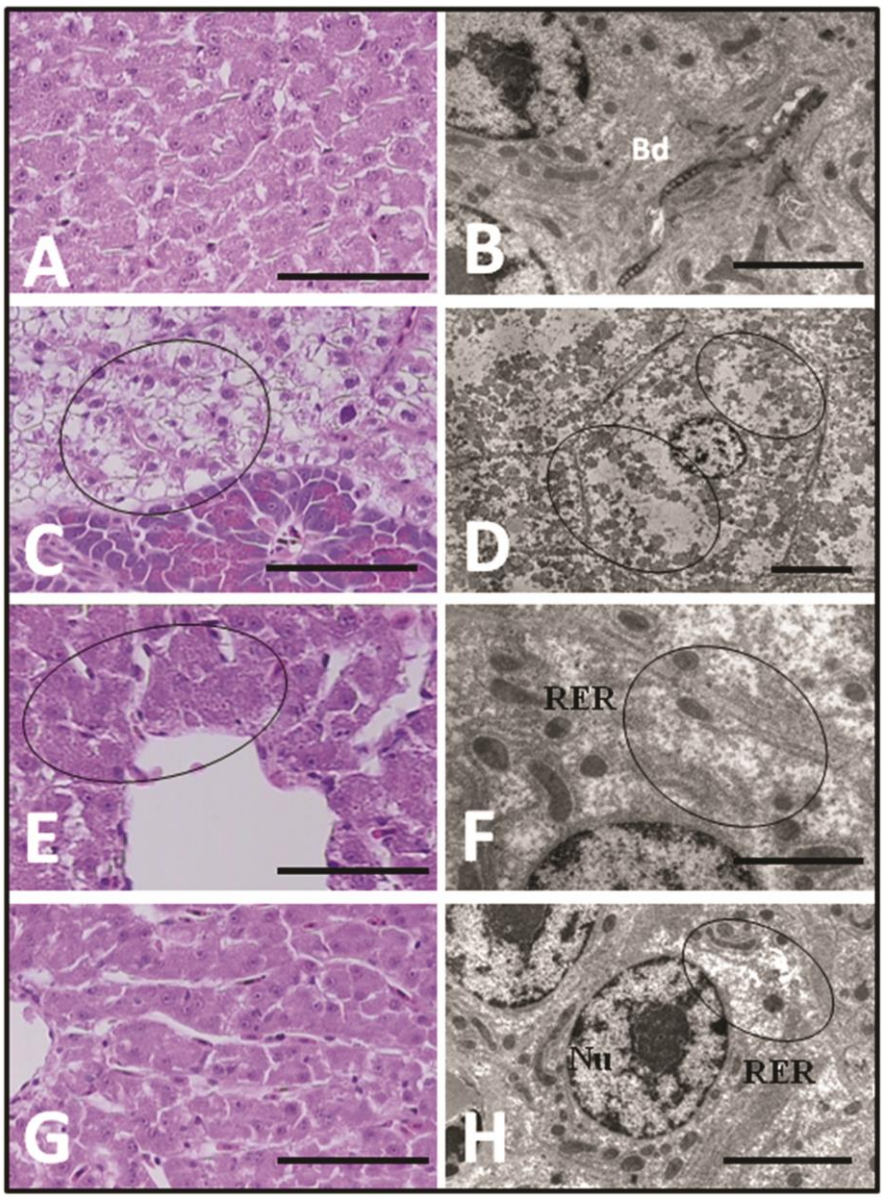

Figure 1

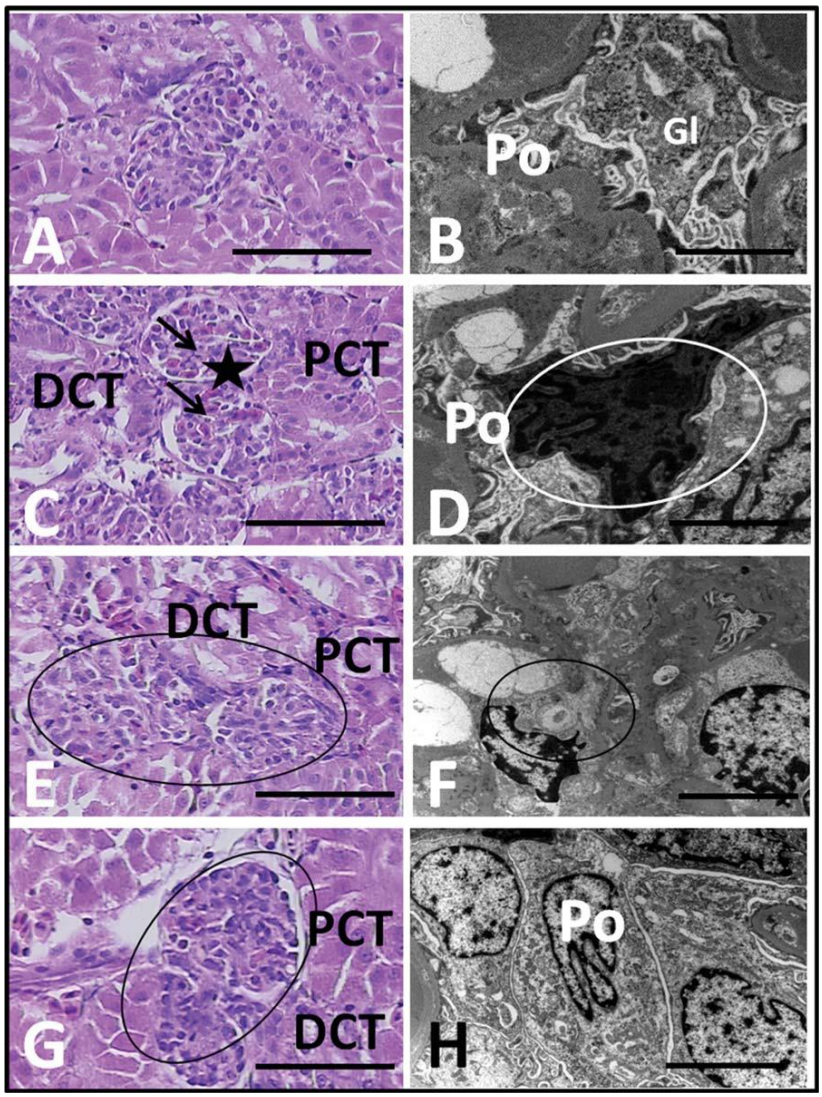

Figure 2

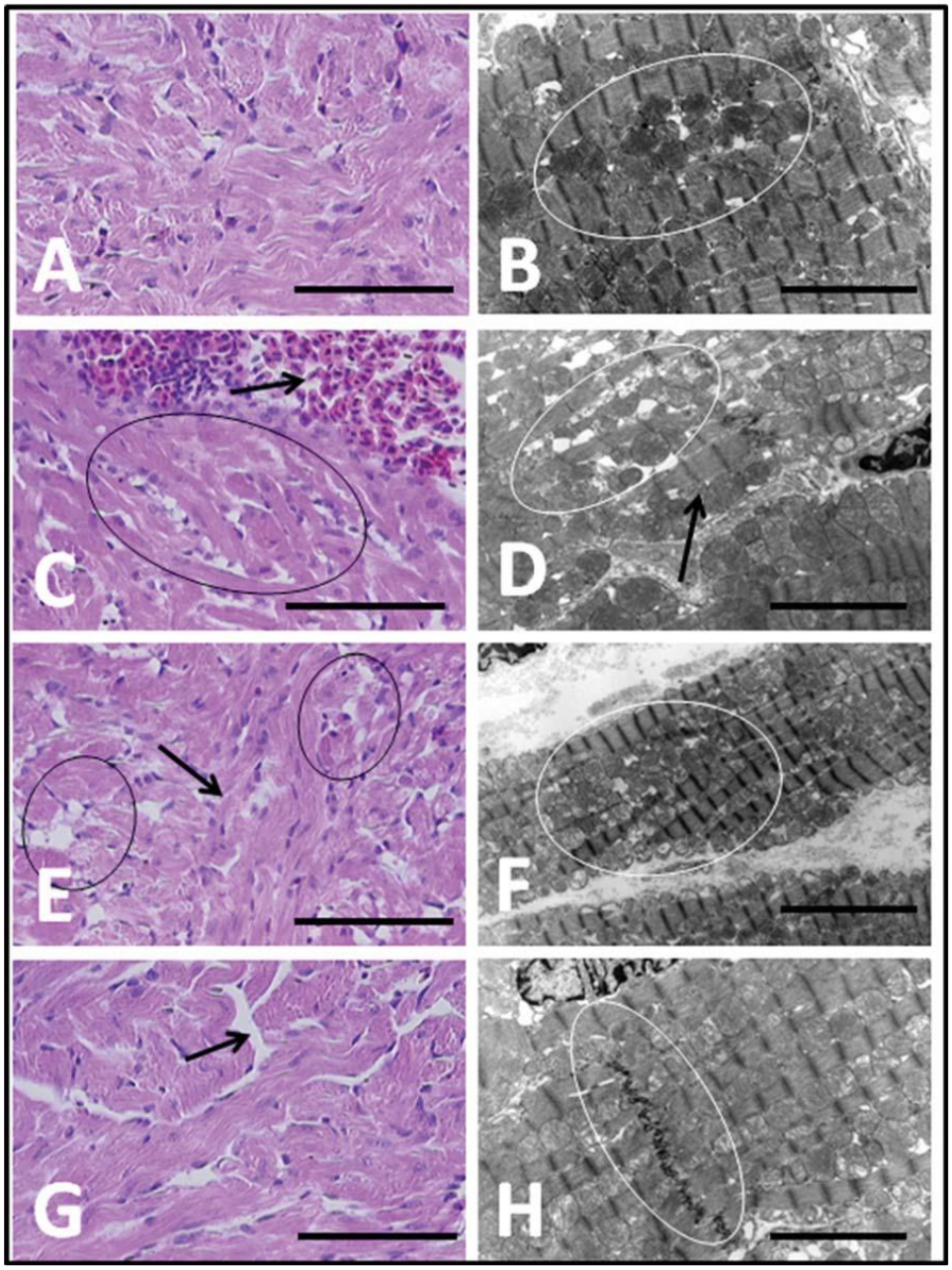

Figure 3

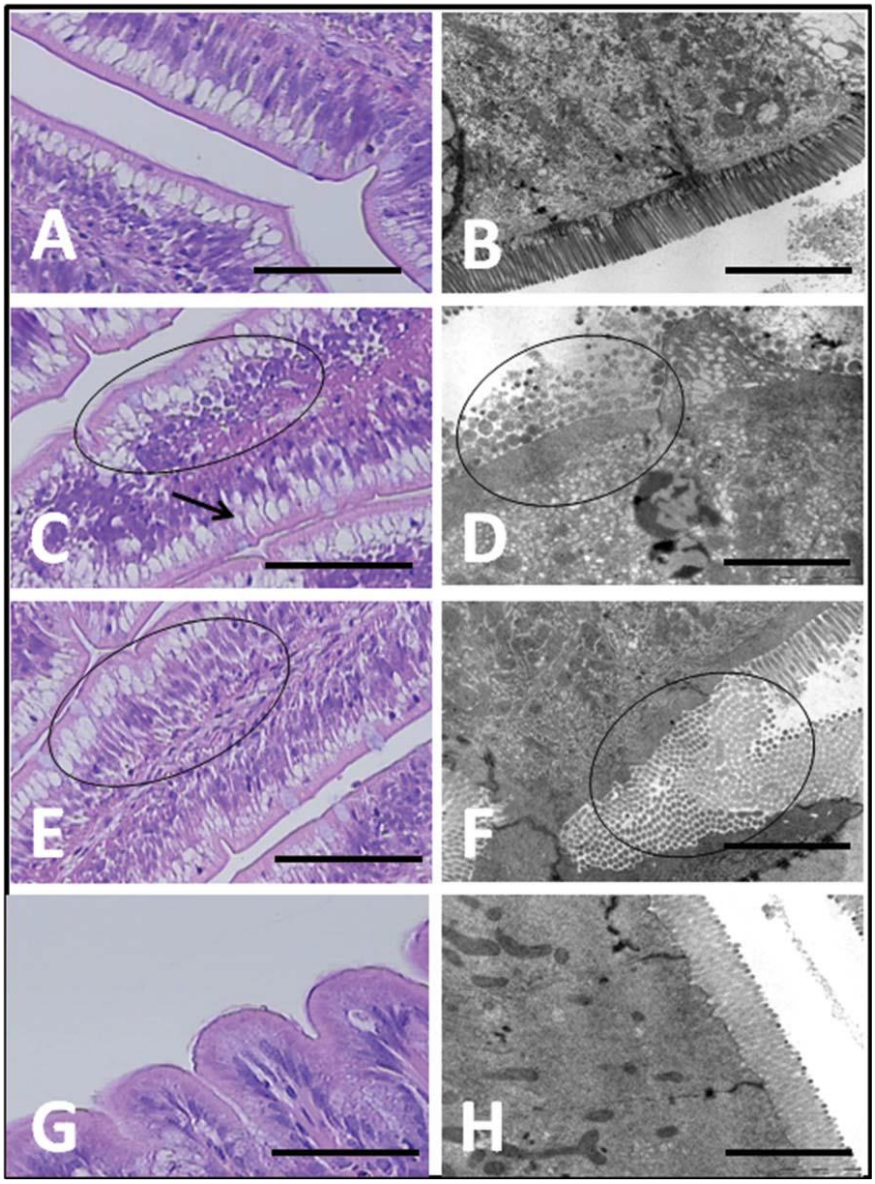

Figure 4

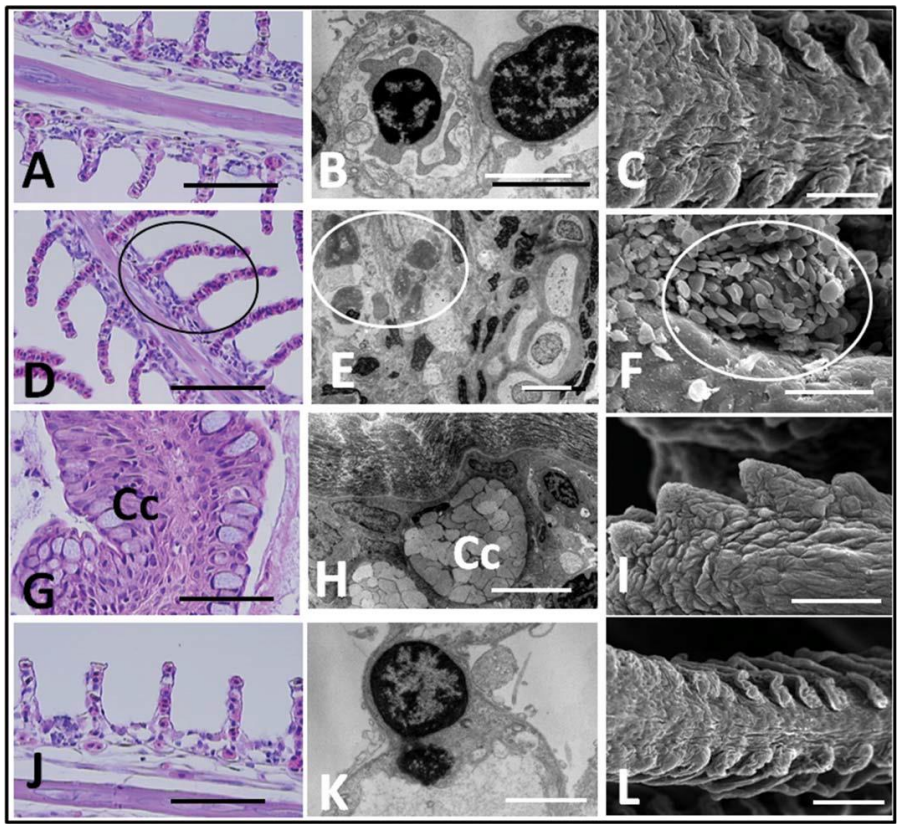

Figure 5

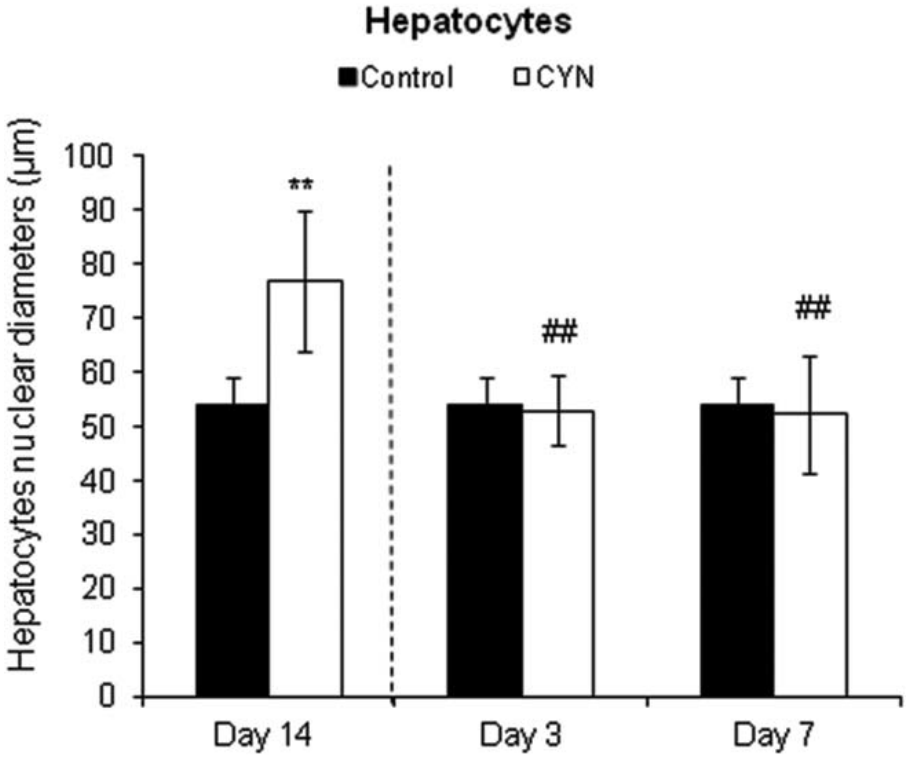

Figure 6

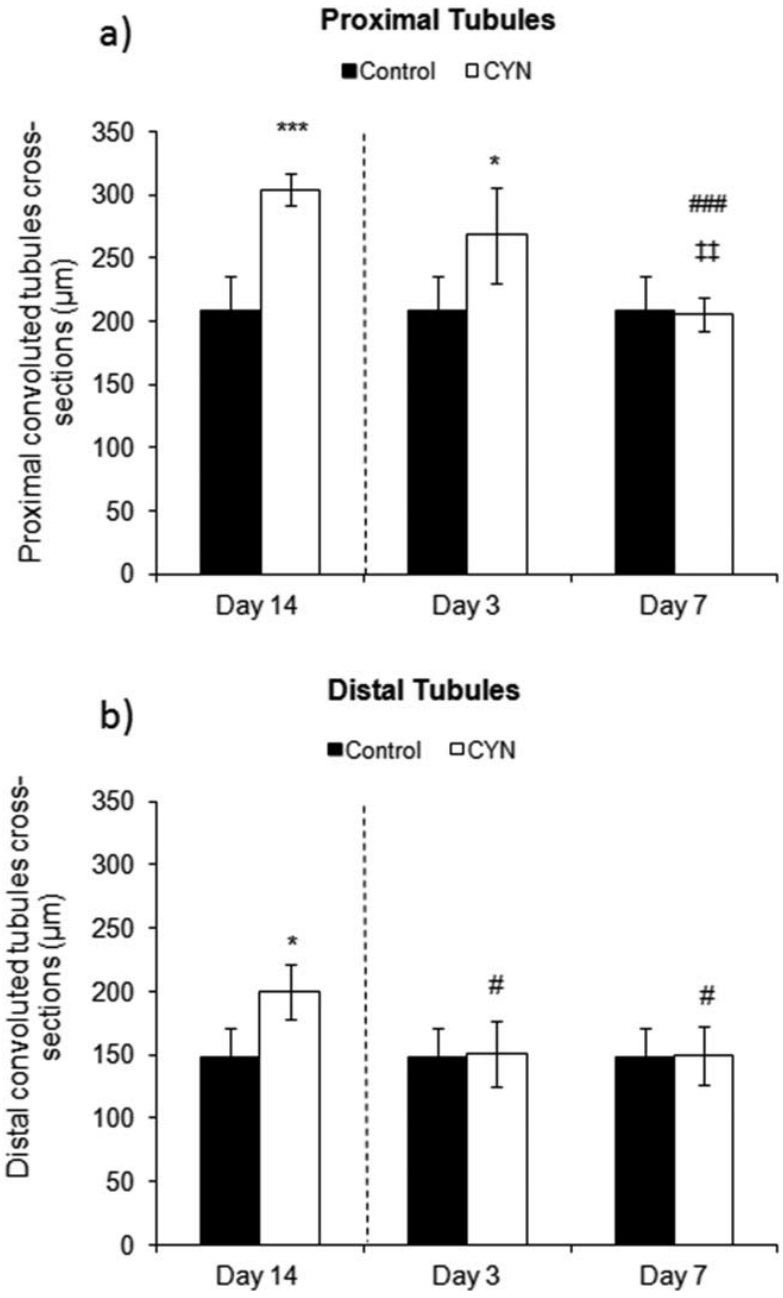
a)
Proximal Tubules
Control
CYN
Proximal convoluted tubules cross-sections (µm)
350
300
250
200
150
100
50
0
***
*
###
‡‡
Day 14
Day 3
Day 7
b)
Distal Tubules
Control
CYN
Distal convoluted tubules cross-sections (µm)
350
300
250
200
150
100
50
0
*
#
#
Day 14
Day 3
Day 7

Figure 7

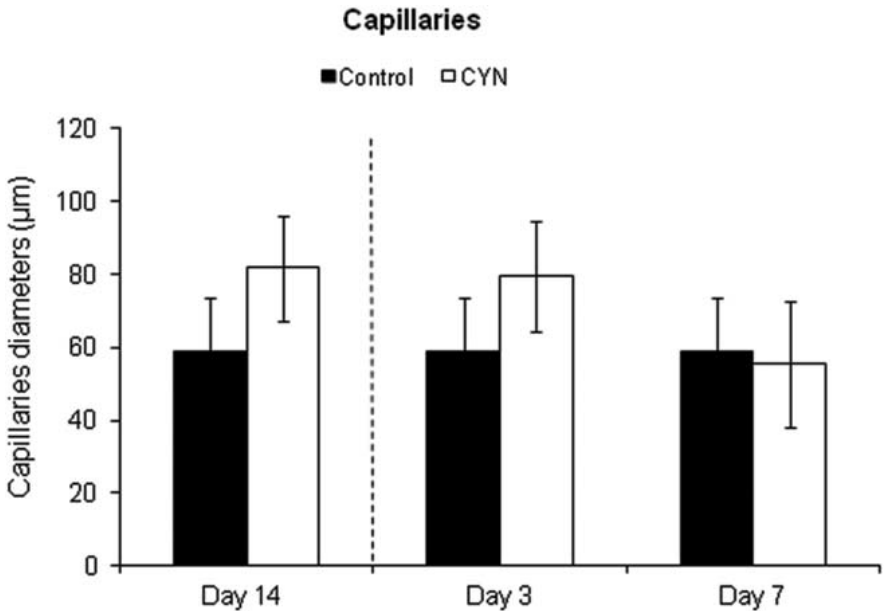
Capillaries
Control
CYN
Capillaries diameters (µm)
120
100
80
60
40
20
0
Day 14
Day 3
Day 7

Figure 8