

ORIGINAL ARTICLE

## Histopathology of fathead minnow (*Pimephales promelas*) exposed to hydroxylated fullerenes

Boris Jovanović<sup>1,3</sup>, Elizabeth M. Whitley<sup>2</sup>, & Dušan Palić<sup>1</sup>

<sup>1</sup>Chair for Fish Diseases and Fisheries Biology, Faculty of Veterinary Medicine, Ludwig Maximilians University of Munich (LMU), Munich, Germany, <sup>2</sup>Department of Veterinary Pathology, Iowa State University, The College of Veterinary Medicine, Ames, IA, USA and <sup>3</sup>Center for Nanoscience (CeNS), LMU, Munich, Germany

### Abstract

Hydroxylated fullerenes are reported to be very strong antioxidants, acting to quench reactive oxygen species, thus having strong potential for important and widespread applications in innovative therapies for a variety of disease processes. However, their potential for toxicological side effects is still largely controversial and unknown. Effects of hydroxylated fullerenes C<sub>60</sub>(OH)<sub>24</sub> on the fathead minnow (*Pimephales promelas*) were investigated microscopically after a 72-hour (acute) exposure by intraperitoneal injection of 20 ppm of hydroxylated fullerenes per gram of body mass. Cumulative, semi-quantitative histopathologic evaluation of brain, liver, anterior kidney, posterior kidney, skin, coelom, gills and the vestibuloauditory system revealed significant differences between control and hydroxylated fullerene-treated fish. Fullerene-treated fish had much higher cumulative histopathology scores. Histopathologic changes included loss of cellularity in the interstitium of the kidney, a primary site of haematopoiesis in fish, and loss of intracytoplasmic glycogen in liver. In the coelom, variable numbers of leukocytes, including many macrophages and fewer heterophils and rodlet cells, were admixed with the nanomaterial. These findings raise concern about *in vivo* administration of hydroxylated fullerenes in experimental drugs and procedures in human medicine, and should be investigated in more detail.

**Keywords:** nanoparticles, liver, kidney, fish, pathology, hydroxylated fullerenes

### Introduction

Hydroxylated fullerenes (fullerenols) are *de facto* nanoparticles with primary particle size of 1 nm, made soluble in water by the introduction of hydroxyl groups to C<sub>60</sub> molecule during their preparation (Kokubo 2012). Although readily soluble in water due to hydroxyl groups, the large fullerene core and  $\pi$ - $\pi$  interactions are responsible for

formation of nanoparticle aggregates with a size range of 20–450 nm (Kokubo 2012). Due to their intrinsic properties, hydroxylated fullerenes act as potent, non-selective antioxidant agents and can intercept and quench all of the physiologically relevant reactive oxygen species (ROS) (Ueng et al. 1997; Markovic & Trajkovic 2008; Yin et al. 2009). Owing to these abilities, C<sub>60</sub>(OH)<sub>24</sub> hydroxylated fullerenes are being investigated as experimental drugs in the treatment of Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Cai et al. 2008; Cataldo & da Ros 2008; Dugan et al. 2001), as well as in other therapeutic and diagnostic purposes such as anti-cancer/tumour/proliferative/metastatic/bacterial and antiviral agents (Bogdanovic et al. 2004; Cataldo & da Ros 2008, Kokubo 2012). The safety profile of C<sub>60</sub>(OH)<sub>24</sub> is still controversial, with studies demonstrating a range of results. Certain studies claim that C<sub>60</sub>(OH)<sub>24</sub> are non-toxic, well tolerated by mammals and therefore safe for therapeutic use (Monteiro-Riviere et al. 2012), while others are reporting substantial adverse effects in human cell lines, rats and fish (Gelderman et al. 2008; Yamada et al. 2010; Nakagawa et al. 2011; Jovanović et al. 2011). Differences in surface modification, i.e., hydroxylation, are likely to contribute to differences in results obtained from various studies. These recent studies have raised concerns about the potential toxicity of C<sub>60</sub>(OH)<sub>24</sub> as, to a certain extent, the properties which benefit some biomedical functions may damage others as side effects. For example, the ability of C<sub>60</sub>(OH)<sub>24</sub> to prevent mitochondrial dysfunction and oxidative damage in an MPP<sup>+</sup>-induced cellular model of Parkinson's disease (Cai et al. 2008) can also cause mitochondrial arrest and depletion of ATP (Johnson-Lyles et al. 2010) with serious consequences.

A frequent method of fullerene delivery to target organisms for potential medicinal use is by injection. This may include intraperitoneal (Mori et al. 2007a, b; Cai et al. 2010; Vapa et al. 2012), subcutaneous (Wang et al. 2006) and intravenous routes (Monteiro-Riviere et al. 2012). The usual

concentrations of various fullerene species and its derivatives administered for exploration of therapeutic use with a desired protective effect against ROS production are in the range of 1–100 ppm (Monteiro-Riviere et al. 2012; Mori et al. 2007a, b; Lin et al. 2002; Cai et al. 2008, 2010; Chen et al. 2004; Vapa et al. 2012). The safety profiles of these concentrations of fullerenes and fullerene derivatives have not been fully explored.

Recently, *in vivo* and *in vitro* studies, we showed, using therapeutically relevant concentrations and exposure routes, that nanoparticles of hydroxylated fullerenes are immunotoxic to mature and developing fathead minnows (*Pimephales promelas* Rafinesque, 1820). Hydroxylated fullerenes (0.2–200 ppm *in vitro*) caused concentration-dependent inhibition of neutrophil function and suppressed oxidative burst, release of Neutrophil Extracellular Traps and degranulation of primary granules (up to 70%, 40% and 50%, respectively). Administration of 2 ppm of hydroxylated fullerenes *in vivo* by intraperitoneal injection suppressed these neutrophil functions by 10%, 10% and 25%, respectively (Jovanović et al. 2011). With only 48 h of topical exposure, experimental administration of 0.01 ppm hydroxylated fullerenes in water resulted in development of severe pericardial oedema and yolk coagulation in up to 25% of *P. promelas* embryos (Jovanović et al. 2011). Intraperitoneal injection of 20 ppm per gram body mass of hydroxylated fullerenes caused 12% mortality in adult fish within the first 36 h of exposure (Jovanović et al. 2011).

Here, we build on our previous work to characterise morphologic changes associated with hydroxylated fullerene exposure, using a well-established fish model in toxicology – *P. promelas* (Ankley & Villeneuve 2006).

## Methodology

### Fish housing and experimental treatment

Adult fathead minnows (average weight 4.5 g) were maintained in the Iowa State University College of Veterinary Medicine Laboratory Animal Resources Facility, Ames, IA, USA, under conditions approved by the Institutional Animal Care and Use Committee. Fish were housed in a water recirculation system supplied with dechlorinated tap water at 23.5 °C and fed twice daily to satiation with dried flake food (2:1 w/w mixture of Aquatox<sup>®</sup> and Plankton/Krill/Spirulina flake food, Zeigler Bros Inc., PA, USA). Control and hydroxylated fullerene-treated fish were housed under identical daily lighting conditions – 14 h of daylight and 10 h in the dark.

Minnows were anaesthetised with 100 ppm of aerated and buffered (sodium bicarbonate, pH 8.0) solution of tricaine methane sulphonate (MS-222, Argent Laboratories, Redmond, WA, USA). Anaesthetised fish, from the treatment group ( $n = 32$ ), were weighed and injected intraperitoneally to achieve final concentration of 20 ppm of hydroxylated fullerenes ( $C_{60}(OH)_{24}$  (MER Corporation, Tuscon, AZ, USA, cat# MR16, 99.8% purity)), per gram of body mass. Concentration of 20 ppm was chosen because it was the lowest concentration to cause mortality (12% mortality) in our previous study (Jovanović et al. 2011). Fish in the control

group ( $n = 30$ ) were injected with Hank's balanced salt solution (HBSS) without Ca, Mg and phenol red. Injected fish were transferred to 40-L flow-through tanks (pH 8 and water temperature 23.5 °C) and fed twice daily to satiation. After 72 h, 10 of the surviving fish were randomly selected from the control and experimental groups and euthanised using an overdose of MS-222. These groups consisted of five males and five females in the experimental group, while the control group had four males and six females. A small incision was made into the coelomic cavity through the vent, and fish were immediately fixed by submersion in 10% neutral buffered formalin.

### Hydroxylated fullerene characterisation

Fullerenes were dissolved in sterile, non-pyrogenic, HBSS without Ca, Mg and phenol red. Some characterisation of fullerenes in such medium has been performed earlier by us (Jovanović et al. 2011). Here, additional DLS characterisation at the concentration of 2000 ppm was performed (the exact concentration in the syringe with which were the fish injected) using Malvern Zetasizer instrument. Size distribution was multimodal, similar to the earlier observation (Jovanović et al. 2011). Mean polydispersity index was  $0.46 \pm 0.06$  (standard error of the mean). Average diameter of the particles (Z-average d.nm) was  $153 \pm 18$  nm; intensity mean  $438 \pm 47$  d.nm; number mean  $3.6 \pm 0.5$  d.nm; and volume mean  $5.4 \pm 0.5$  d.nm.

### Histopathology

Fish were sectioned along the sagittal plane and placed in processing cassettes, demineralised for 12 h in 25% formic acid solution and processed routinely for embedding in paraffin. Tissues were sectioned at 5 µm and stained with haematoxylin and eosin. Selected sections were stained with Gram, Ziehl-Neelsen and Periodic acid-Schiff reagents for bacteria, acid-fast bacteria, and carbohydrates and fungi, respectively.

At least two representative sections of each fish were evaluated microscopically. Tissue architecture and cytomorphologic features of the brain, vestibular system, liver, anterior kidney (head kidney), posterior kidney (trunk kidney), skin, coelom, gills and skeletal muscle were evaluated by a board-certified veterinary pathologist. External factors that might have affected the morphology or interpretation of the tissue sections were considered and, to avoid effects of non-treatment factors on data, samples were processed and stained in parallel and evaluated by a single observer (EMW).

### Statistics

A semi-quantitative grading scale was used to evaluate microscopic features of tissues and cells (Table I). Significance of differences between histopathology scores was evaluated using a two-tailed Mann-Whitney U test or Wilcoxon signed-rank Test.

## Results

Within 72 h of exposure, 3 of 32 fish (9.5%) died in experimental hydroxylated fullerene treatment. There was no

Table 1. Semi-quantitative histopathology scoring system used to evaluate tissues and organs of fish. Each pathological feature is given a score of 0-3. If the evaluated organ has more than one pathological feature then the score of each pathological feature is summed to give a histopathology score for the organ. Histopathology score of the organ may therefore be greater than 3.

Organ	Pathologic feature	Score			
		0	1	2	3
Brain	Congestion	None	Mildly distended vessels	Moderately distended vessels	Vessels markedly distended
	Haemorrhage	None	Mild or focal	Moderate focal or mild multifocal	Multifocal or severe focal
	Inflammation in meninges	None	Mild	Moderate	Severe
	Rodlet cells in meninges	None	1-3/40 × field	4-8/40 × field	>8/40 × field
	Rodlet cells in neuropil	None	1/40 × field	2-4/40 × field	>4/40 × field
Liver	Congestion	None	Mildly distended vessels	Moderately distended vessels	Vessels markedly distended
	Hepatocyte glycogen accumulation	Normal, finely vacuolated, pale hepatocytes	Mildly reduced	Moderately reduced	Densely eosinophilic hepatocyte cytoplasm
	Lipid accumulation	None	<4 lipid-laden cells/40 × field	4-10 lipid-laden cells/40 × field	>10 lipid-laden cells/40 × field
	Hepatocyte degeneration, necrosis or apoptosis	0/40 × field	<4/40 × field	4-10/40 × field	>10/40 × field
	Mononuclear cell infiltration	0/40 × field	<2/40 × field	2-5/40 × field	>5/40 × field
Anterior kidney	Sinusoidal hematopoietic cells	4-6 cells between sinusoids	3-4 cells between sinusoids	1-2 cells between sinusoids	Absence of cells
	Necrotic or apoptotic cells	None	Rare pyknotic nuclei or swollen cells	Few to scattered pyknotic nuclei	Abundant apoptotic or amorphous debris
	Congestion	None	Mildly distended vessels	Moderately distended vessels	Vessels markedly distended
	Tubular epithelial degeneration or necrosis	None	Mild cytoplasmic hyper eosinophilia of tubular epithelial cells	Moderate eosinophilia or rare pyknosis	Epithelial sloughing, pyknosis, karyorrhexis
	Interstitial pigment (melanomacrophages)	None	1/40 × field	2-3/40 × field	>4/40 × field
Posterior kidney	Presence of interstitial hematopoietic tissue	Groups of 4-10 cells between tubules	Mildly reduced	Moderately reduced	Markedly reduced
	Alarm cells	Dense, uniform distribution	Loss of 1 alarm cell/20 × field	Loss of 2-3 alarm cells/20 × field	Loss of >3 alarm cells/40 × field
	Erosion or Ulcer	Intact epithelium	Small focal erosion	Small, focal ulcer	Multiple or large ulcers
	Peritoneal inflammation	None	Small numbers of leukocytes	Scattered groups of leukocytes	Large groups of leukocytes
	Peritoneal pigment distribution	Evenly distributed along body wall	Small, focal area of pigment clumping	Large clumps of pigment and phagocytes	Pigment absent
Coelom	Epithelial oedema or necrosis	None	Mild	Moderate	Severe
	Epithelial lamellar hypertrophy/hyperplasia	None	Mild	Moderate	Severe
	Lamellar haemorrhage or fibrin exudation	None	Mild	Moderate	Severe
Gills	Disorganisation or apoptosis of ciliated sensory or ampullary epithelial cells	None	Mild disorganisation or rare apoptosis	Moderate	Frequent apoptosis or loss of many cells

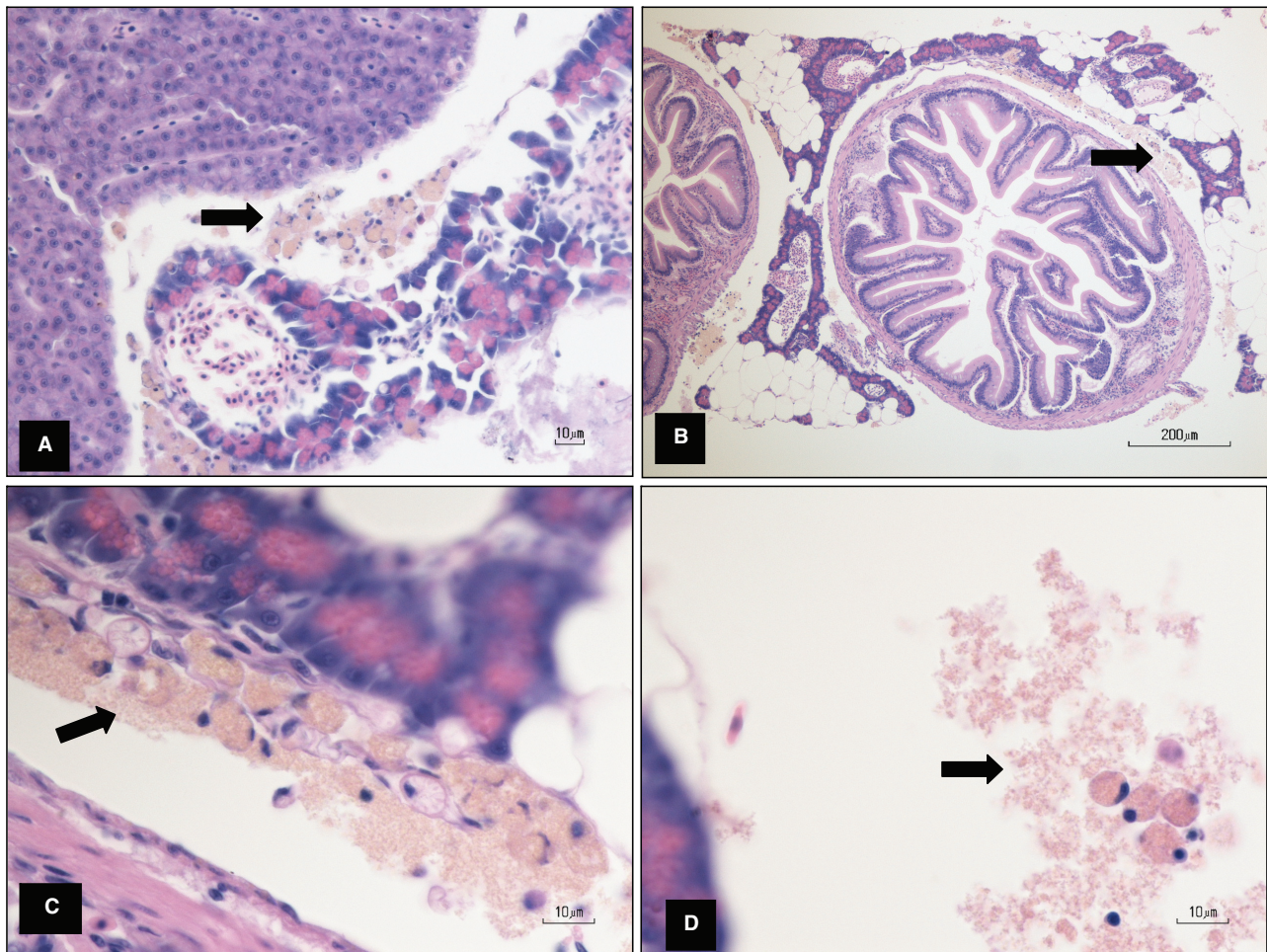


Figure 1. Presence of hydroxylated fullerenes in the coelomic cavity. Amorphous to finely particulate, yellow to golden brown fullerene nanomaterial (black arrows) is present in the coelomic cavity and is loosely associated with serosal surfaces of organs, liver (A), pancreas (A, B, C), small intestine (B). Variable numbers of leukocytes with the morphology of macrophages and fewer heterophils and rodlet cells are present, with some phagocytes ingesting abundant nanomaterial (C, D). Nuclear morphology of cells with abundant intracytoplasmic nanomaterial closely resembles normal, non-phagocytic cells of the monocytic/macrophage lineage, indicating lack of activation.

mortality in the control (HBSS-injected group,  $n = 30$ ) during this time. There was no observed difference in the feeding behaviour of fish between the groups and all of the fish consumed food *ad libitum* during the experiment. Personal observation, however, indicated that the hydroxylated fullerene-injected fish were more lethargic and tended to spend more time on the aquarium bottom, clustered in a school, as opposed to HBSS-injected fish which exhibited normal activity levels and swimming behaviour.

Large aggregates of hydroxylated fullerenes were visualised in the coelomic cavity of treated fish and were often infiltrated and surrounded by epithelioid macrophages. In haematoxylin-and-eosin-stained sections, the nanomaterial was finely particulate to amorphous and golden brown. The nanomaterial was distributed throughout the ventral portion of the coelomic cavity, on the serosal surfaces and the mesentery between organs (liver and pancreas in Figure 1A, small intestine and pancreas in Figure 1B). In fish treated with hydroxylated fullerenes, serosal pigment of the coelomic wall was clumped by phagocytes. Variable numbers of leukocytes, including macrophages and fewer heterophils and rodlet cells, were admixed with the nanomaterials (Figures 1C and D). Some phagocytes were

distended by abundant intracytoplasmic nanomaterial (Figure 1D). Examination of representative sections stained individually with Gram, Periodic acid Schiff or Ziehl-Neelsen stains did not reveal any infectious agents.

Cumulative semi-quantitative histopathologic scoring of the brain, liver, anterior kidney, posterior kidney, skin, coelom, gills and the vestibuloauditory system revealed significant differences between control and fullerene-treated fish ( $p < 0.001$ , Figure 2A). Individual histopathology scores of the coelomic cavity and the anterior kidney (Figure 2B and C) were significantly higher in fish treated with hydroxylated fullerenes ( $p < 0.001$ ). Fish treated with hydroxylated fullerenes also had significantly higher posterior kidney histopathology scores (Figure 2D,  $p < 0.05$ ). Histopathology score for liver morphology (Figure 2E) was significantly higher in hydroxylated fullerene-treated fish, compared with control ( $p < 0.001$ ).

In the liver of fullerene-treated fish, hepatocytes were relatively small, with condensed cytoplasm and finely crystalline intracytoplasmic proteins (Figure 3A). Hepatocyte morphology was not related to gender. Staining with Periodic acid-Schiff reagent (Figure 3B) revealed scant carbohydrate in hepatocytes of fullerene-treated fish, followed by

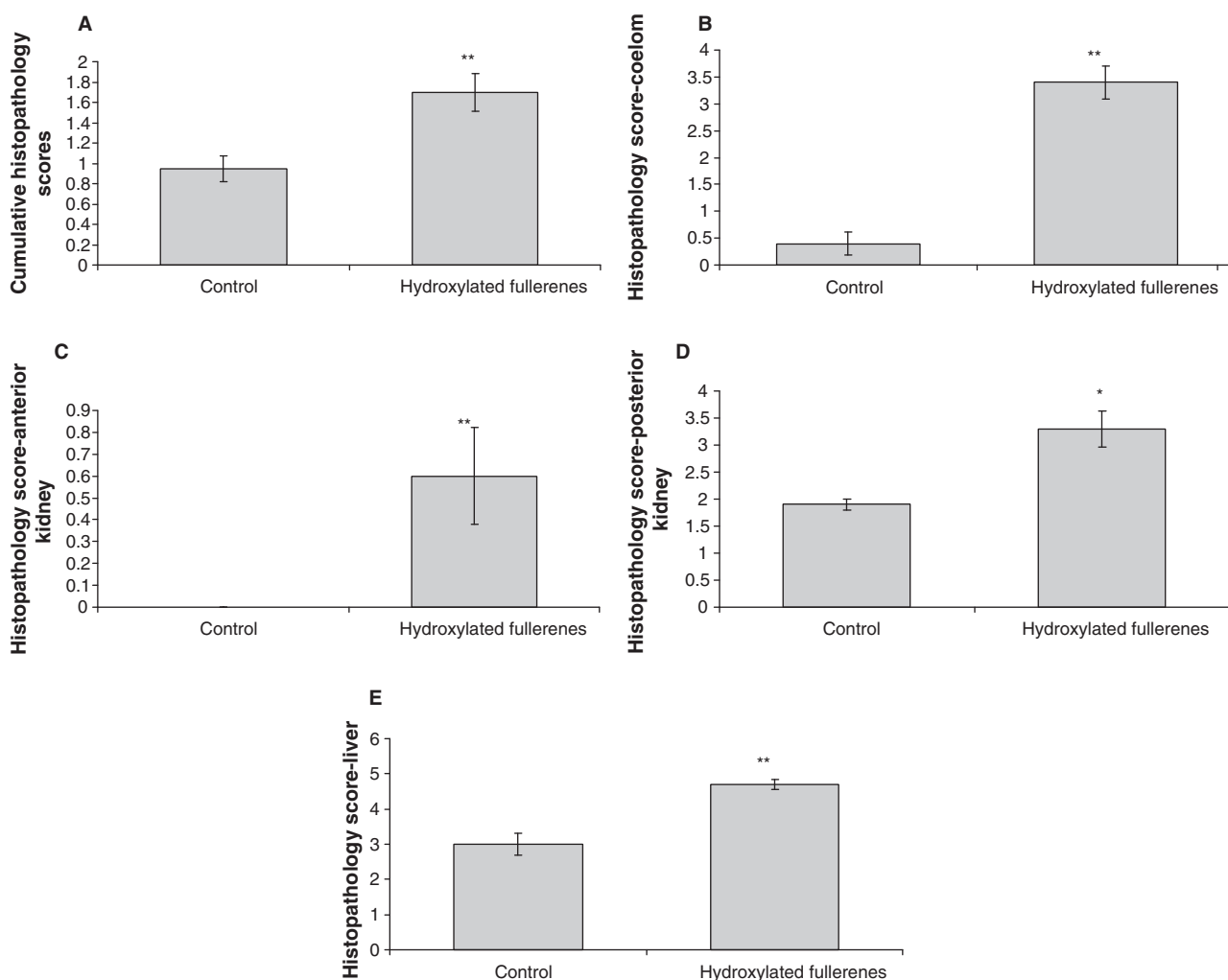


Figure 2. Histopathology scores. Semi-quantitative scores representative of histopathologic features in control and fullerene-treated fish. In A, the mean cumulative histopathology scores for all organs evaluated (brain, liver, anterior kidney, posterior kidney, skin, coelom, gills and vestibuloauditory system) demonstrate a significant difference ( $p = 0.001$ ) between the control ( $n = 10$ ) and treated ( $n = 10$ ) groups. Comparison of mean histopathology scores for the coelomic cavity (B), anterior kidney (C), posterior kidney (D) and liver (E) in these same populations of fish similarly reveal significant differences in morphology associated with fullerene treatment. \*Indicates that the effect is statistically significant at  $p < 0.05$ ; and \*\*indicates that the effect is statistically significant at  $p < 0.001$ . Whiskers indicate standard error of the mean.

loss of pink staining after amylase treatment, consistent with the presence of minimal intracytoplasmic glycogen (Figure 3C). The cytoplasm of hepatocytes of control fish, in contrast, were vacuolated and pale, with condensed to crystalline, intracytoplasmic proteins with haematoxylin and eosin staining and have moderate to abundant intracytoplasmic glycogen as identified by Periodic acid-Schiff staining (Figure 3D - F). Similar reduction in amylase-sensitive carbohydrate was observed in skeletal muscle in hydroxylated fullerene-treated fish, consistent with reduced glycogen storage (data not shown). Accumulation of pigments in hepatic melanomacrophage centres was not assessed, because of the difficulty of differentiating lipofuscin and nanoparticles and the non-homogenous distribution of hepatic melanomacrophage centres in livers of control fish. Compensatory hepatic hematopoietic tissue was not observed in control or treated fish.

Reduced numbers of hematopoietic cells, both myeloid and lymphoid lineages, were present in the interstitium of the anterior and posterior kidneys (Figure 4A - D). Melanomacrophage centers in fullerene-treated fish often were

expanded by amorphous, golden brown material, consistent with nanomaterial and/or lipofuscin. Morphologic changes in the nephron or increased numbers of mitotic figures or apoptotic bodies among interstitial hematopoietic cells between treatment groups were not observed.

The heart was not present in enough tissue sections, even with repeated sectioning of the paraffin blocks, to provide accurate statistical data. Golden brown intracytoplasmic pigment was present in many fixed atrial phagocytes, critical members of the teleost mononuclear phagocyte system, in 3 of 7 fullerene-treated fish for which heart was present on the slides but were not present in the atria of control fish (data not shown). Significant differences in morphology of the gills, skin, brain or vestibuloauditory system between treatment groups were not observed. Splenic tissue was rarely present in examined sections because of orientation of tissue sections.

## Discussion

Administration of 20 ppm of hydroxylated fullerenes per gram body mass caused 9.5% mortality in experimental

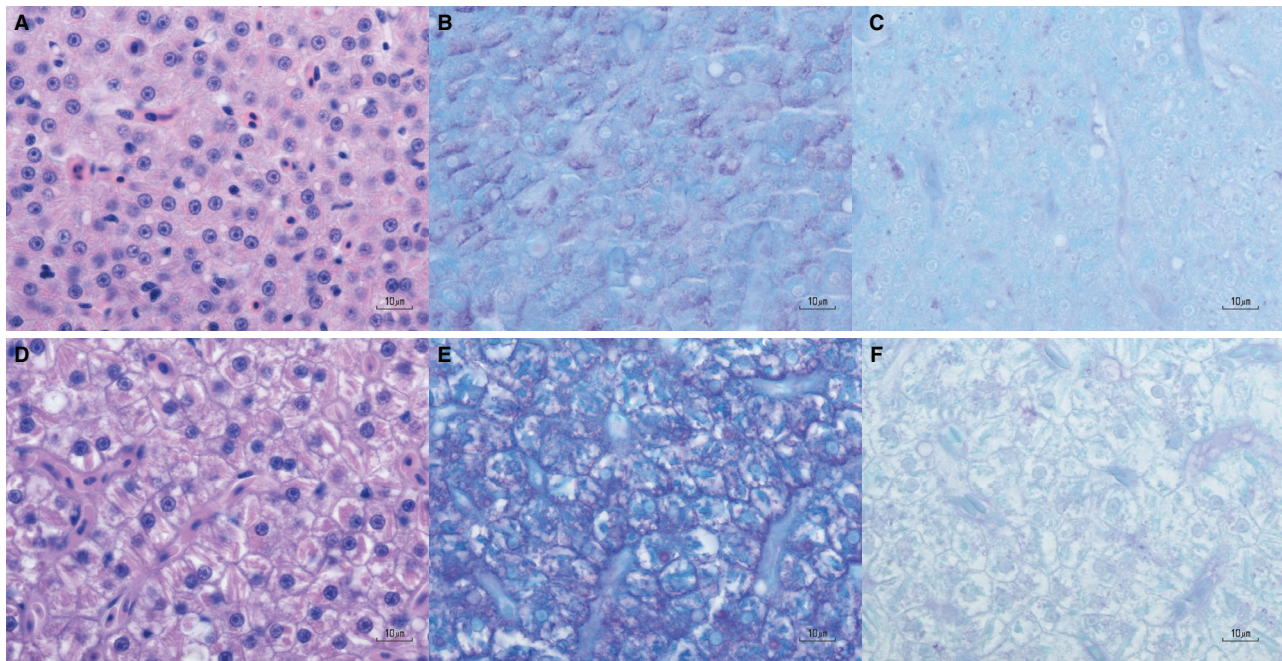


Figure 3. Liver of *P. promelas* treated with hydroxylated fullerenes showing hepatocyte morphology (A–C); liver of *P. promelas* from the control group showing normal hepatocyte morphology (D–F). Hepatocyte cytoplasm in hydroxylated fullerene-treated fish contains scant carbohydrate. Some of the intracytoplasmic material is glycogen, indicated by pink staining with periodic acid-Schiff reagent (B) and loss of this colouration after amylase treatment (C). Hepatocyte cytoplasm is pale with crystalline to clumped intracytoplasmic proteins in this female fish from the control group (D) with abundant intracytoplasmic glycogen (E, F). Haematoxylin and eosin (A, D), periodic acid-Schiff reagent (B, E) and periodic acid-Schiff with amylase pre-treatment (C, F). 1000× magnification.

fish population which is congruent with previously reported mortality of 12% (Jovanović et al. 2011) and contrasts to 0% mortality among control fish.  $C_{60}(OH)_{24}$  also caused pathologic changes in several vital organ systems in adult, tank-raised fathead minnows. Morphologic lesions include reduced numbers of renal interstitial hematopoietic cells and depletion of hepatic and skeletal glycogen stores. These changes are expected to reflect functional alterations in the innate immune response and energy metabolism, respectively, and will be the focus of future investigations.

The results of this study complement our previous findings (Jovanović et al. 2011) which demonstrated that hydroxylated fullerenes have a direct effect on the teleost immune system. This work is also congruent with the work of others that demonstrate that various species of hydroxylated fullerenes can interact (either benevolently or malevolently) with cells of the murine or human immune system, in particular, natural killer cells (Bunz et al. 2012), dendritic cells (Yang et al. 2010), mast cells and basophils (Ryan et al. 2007), and macrophages (Pirutin et al. 2012). Here, we demonstrate a phagocytic response to hydroxylated fullerenes in the coelomic cavity (Figure 1C and D) and in renal melanomacrophage centres. Furthermore, both the anterior and posterior kidneys of hydroxylated fullerene-treated fish contained reduced numbers of myeloid and lymphoid cells, suggesting the probability of impaired innate and adaptive immune responses. One of the critical functions of the fish kidney is to serve as a major myelopoietic and lymphopoietic site (Kobayashi et al. 2006; Zapata 1979), as teleost fish have no intraosseous medullary hematopoiesis. Possible mechanisms for the loss of hematopoietic cells includes cell death,

perhaps by calcium flux-mediated apoptosis, as has been described in endothelial cells (Gelderman et al. 2008) or disruption of cell membranes (Tramer et al. 2012). Less likely, to account for this loss of cells would be massive mobilisation of phagocytes in response to foreign nanoparticles, because this mechanism would not account for the observed loss of multiple cell lineages and the absence of compensatory proliferation. The loss of hematopoietic cells was more pronounced in the anterior kidney, probably because this organ is the central organ for immune regulation in teleosts. Compensatory hepatic hematopoiesis was not observed, perhaps due to the short duration of the study or treatment-associated toxicity or a precursor population. The loss of hematopoietic potential is expected to have serious repercussions for homeostasis and would reduce immunocompetency during a concurrent infectious disease.

Alterations in energy metabolism secondary to exposure to hydroxylated fullerenes are suggested by loss of glycogen stores in the liver and skeletal muscle. The liver of fishes is an important storage site for large amounts of glycogen or lipid, depending on fish species (Hinton et al. 2008). The main difference between teleost and mammalian hepatic architecture is the absence of functional metabolic zonation, and thus glycogen storage in the teleost liver is not heterotopically distributed in the parenchyma as in the mammalian liver (Hinton et al. 2008), where it can serve as an indicator of mild hepatocyte damage. The cytoplasm of hepatocytes of control fish in this study was vacuolated and pale, with condensed to crystalline, intracytoplasmic proteins and moderate to abundant intracytoplasmic glycogen. Treatment with hydroxylated fullerenes caused marked loss of

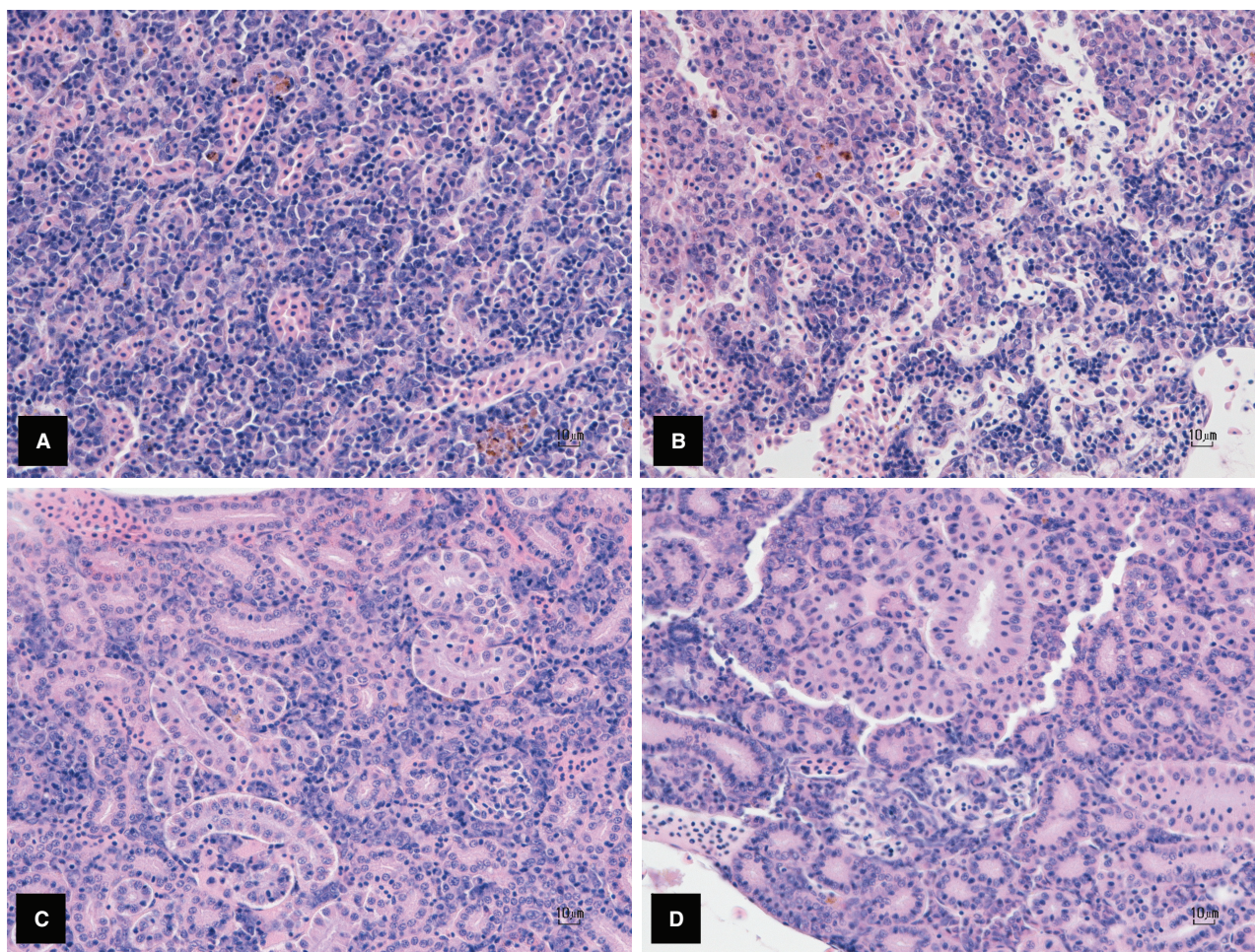


Figure 4. Histopathology of anterior and posterior kidneys of *P. promelas* exposed to hydroxylated fullerenes. The anterior kidney of hydroxylated fullerene-treated fish (B) often contained reduced numbers of lymphoid and myeloid cells compared to control fish (A). 400 $\times$  magnification. The interstitium of the posterior kidney in this representative hydroxylated fullerene-treated fish (D) contains reduced numbers of lymphoid and myeloid cells compared to control (C). 400 $\times$  magnification.

intracytoplasmic glycogen in hepatocytes. One of the main functions of the liver is to catabolise stored glycogen during starvation or stress, via the process of glycogenolysis. Glycogenolysis in the liver and skeletal muscle is particularly important in stressful situations, such as fight-or-flight responses as it provides a ready supply of glucose to be used in glycolysis as precursor for ATP. A previous study has demonstrated that treatment of animals with hydroxylated fullerenes can cause mitochondrial arrest and depletion of ATP (Johnson-Lyles et al. 2010). Such toxic effects can be attributed to the mode of action of hydroxylated fullerenes, being potent antioxidants with the ability to quench ROS (Markovic & Trajkovic 2008; Jovanović et al. 2011). In animals, mitochondrial compartments are the largest producers of ROS within cells, and ROS are essential for the process of oxidative phosphorylation. When ROS are quenched, ATP is depleted and intensive glycogenolysis must take place to replenish ATP stores. This ability is likely met with limited success when animals are treated with hydroxylated fullerenes, explaining the loss of glycogen from the liver and skeletal muscle that we observed in fish treated with hydroxylated fullerenes. Alternatively, fullerene-treated fish may have had less hepatic glycogen due to reduced feeding although no difference in feeding patterns was observed

between experimental and control group in this study. Fullerene-treated fish exhibited diminished swimming performance, were lethargic and were observed to spend a large amount of time lying on the aquarium floor. However, we did not conduct extensive behavioural analyses.

This manuscript identifies pathology in target organs caused by hydroxylated fullerenes that have not been observed in several other studies (Monteiro-Riviere et al. 2012; Vapa et al. 2012). In particular, our study evaluated several sites of hematopoiesis in fish. Histopathologic evaluation of bone marrow, a site of hematopoiesis in mammals, has not been reported in previous similar studies (Chen et al. 1998; Monteiro-Riviere et al. 2012). Differences in results across studies may also be associated with other factors, such as variances in fullerene chemistry and conjugation, environmental lighting conditions, and dis-similarities in physiology and oxidative stress responses among animal species. In contrast to studies using small numbers of rodents (Chen et al. 1998; Monteiro-Riviere et al. 2012), this study had the benefit of higher animal numbers per group, allowing robust statistical analysis.

The semi-quantitative histologic scoring system we describe was developed for this project to allow evaluation of the cellular and architectural changes associated with

fullerene exposure. Although it is a time-consuming process, the application of semi-quantitative techniques to evaluate histologic sections provides information otherwise inaccessible through qualitative, nominal descriptions. The scoring system was developed using a combination of specific and arbitrary categories reflecting organ-specific features associated with cell injury, with arbitrary categories assigned to features for which numerical parameters were not well identified or for features that were represented by a continuum (accumulation of hepatic glycogen, for example). The ordinal data generated from this scoring system allowed summarisation of several morphologic features from a single organ and from multiple organs, with data reported as the means and standard deviations of the population in each group. Our laboratory also performs computer-aided image analysis of histologic sections for lesion-treatment correlations, which allow numerical quantification of various features, but the small size of this animal model and difficulties in sample orientation during tissue processing do not routinely provide broad-scale comparable sections of individual organs that are most amenable to computer-aided image analysis.

## Conclusion

In conclusion, treatment of fish with hydroxylated fullerenes caused clear histopathological changes with main features being loss of renal interstitial hematopoietic cells and loss of glycogen from the liver and skeletal muscle, which was consistent with previous research and apparent mode of action of hydroxylated fullerenes. The morphologic changes associated with hydroxylated fullerenes raise concern about their use in experimental drugs and procedures in human medicine, and the potential for adverse effects of hydroxylated fullerenes should be further investigated.

## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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