Morphological alterations and acetylcholinesterase and monoamine oxidase inhibition in liver of zebrafish exposed to Aphanizomenon flos-aquae DC-1 aphantoxins

De Lu Zhang\textsuperscript{a}, Jing Zhang\textsuperscript{b}, Chun Xiang Hu\textsuperscript{c,∗}, Gao Hong Wang\textsuperscript{c}, Dun Hai Li\textsuperscript{c}, Yong Ding Liu\textsuperscript{c}

\textsuperscript{a} Department of Life Science and Biotechnology, School of Chemistry, Chemical Engineering and Life Science, Wuhan University of Technology, Wuhan 430070, PR China
\textsuperscript{b} College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, PR China
\textsuperscript{c} Key Laboratory of Algal Biology, Institute of Hydrobiology, The Chinese Academy of Sciences, Wuhan 430072, PR China

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\textit{Aphanizomenon flos-aquae} is a cyanobacterium that produces neurotoxins or paralytic shellfish poisons (PSPs) called aphantoxins, which present threats to environmental safety and human health via eutrophication of water bodies worldwide. Although the molecular mechanisms of this neurotoxin have been studied, many questions remain unsolved, including those relating to in vivo hepatic neurotransmitter inactivation, physiological detoxification and histological and ultrastructural alterations. Aphantoxins extracted from the natural strain of \textit{A. flos-aquae} DC-1 were analyzed by high-performance liquid chromatography. The main components were gonyautoxins 1 and 5 (GTX1, GTX5) and neosaxitoxin (neoSTX), which comprised 34.04%, 21.28%, and 12.77% respectively. Zebrafish (\textit{Danio rerio}) were exposed intraperitoneally to 5.3 or 7.61 μg STX equivalents (eq)/kg (low and high doses, respectively) of \textit{A. flos-aquae} DC-1 aphantoxins. Morphological alterations and changes in neurotransmitter conduction functions of acetylcholinesterase (AChE) and monoamine oxidase (MAO) in zebrafish liver were detected at different time points 1–24 h post-exposure. Aphantoxins significantly enhanced hepatic alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities and histological and ultrastructural damage in zebrafish liver at 3–12 h post-exposure. Toxin exposure increased the reactive oxygen species content and reduced total antioxidative capacity in zebrafish liver, suggesting oxidative stress. ACHE and MAO activities were significantly inhibited, suggesting neurotransmitter inactivation/conduction function abnormalities in zebrafish liver. All alterations were dose- and time-dependent. Overall, the results indicate that aphantoxins/PSPs induce oxidative stress through inhibition of AChE and MAO activities, leading to neurotoxicity in zebrafish liver. The above parameters may be useful as bioindicators for investigating aphantoxins/PSPs and cyanobacterial blooms in nature.

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1. Introduction

Toxicogenic cyanobacterial blooms dominated by \textit{Aphanizomenon flos-aquae} secrete cyanobacterial neurotoxins or paralytic shellfish poisons (PSPs). They occur worldwide and represent a global problem (Ballot et al., 2010; Gkelis and Zaoutsos, 2014). These blooms and the harmful secondary metabolites produced (cyanotoxins) have a deleterious effect on water ecosystems (Gkelis and Zaoutsos, 2014). Blooms dominated by \textit{A. flos-aquae} DC-1 strains have occurred in Dianchi Lake, which has provided fresh water for agriculture, drinking, entertainment, and tourism for several million people in the vicinity of Kunming city, Yunnan Province, China in recent decades (Liu et al., 2006). Water blooms and their associated toxins have been responsible for severe environmental safety and human health problems for people living around the lake (Liu et al., 2006; Zhang et al., 2011, 2013a,b).

PSPs are potent alkalioid neurotoxins that mainly occur in the ocean where they are produced by dinoflagellates, but also in freshwater, where they are secreted by freshwater cyanobacteria and bacteria (Prot et al., 2009; Ballot et al., 2010; Hackett et al., 2013). PSPs may accumulate at high levels in aquatic animals with no immediate affect; however, consumption of these contaminated foodstuffs by humans or other animals leads to
intoxication (Ferrão-Filho and Kozlowsky-Suzuk, 2011). Furthermore, aphanotoxins/PSPs may not be completely removed using normal treatment methods such as cooking because of their resistance to heat, acids and alkalis (Gastro et al., 2004).

Fish are generally considered as important organisms at the top of the food chain, and thus representative indicators of the overall health of the aquatic ecosystem (Oliva et al., 2013). Zebrasfish provide a crucial model organism in the freshwater ecosystem. Interest in this species has recently increased because of its suitability for monitoring environmental pollutants and their toxicity. Zebrafish synthesize most typical vertebrate neurotransmitters and its neuroendocrine system demonstrates vigorous physiological responses to stress. It also has the classic advantages of small size, low cost, easy reproduction, a short breeding cycle, high egg production, external fertilization and development, transparent embryos and a well-documented genetic background (Panula et al., 2006; Alsop and Vijayan, 2008; Dai et al., 2014). Previous investigations showed PSPs may enter fish through the consumption of PSP-contaminated foodstuffs (Jiang et al., 2007). PSPs may then accumulate in fish muscles and influence development, leading to impaired swimming abilities, and deformities and death in larval and adult fish (Samson et al., 2008; Bakke et al., 2010; Zhang et al., 2013c).

The liver is important for the detoxification and metabolism of toxins (Gavrilović et al., 2014). Histological and/or ultrastructural alterations, and changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST), reactive oxygen species (ROS), and total antioxidative capacity (T-AOC) represent valuable parameters that can be used to assess the effects of exposure to a variety of anthropogenic or natural pollutants in fish, which demonstrate representative physiological, biochemical and histopathological responses to stress (Abdel-Moneim, 2014; Bacchetta et al., 2014; Jia et al., 2014). Monoamine oxidase (MAO) and acetylcholinesterase (AChE) are crucial enzymes involved in the decomposition of monoamine neurotransmitters such as norepinephrine/epinephrine and others from sympathetic nerves, and cholinergic neurotransmitters from vagus nerves in animal liver (Askar et al., 2011; Hussien et al., 2013). Previous studies have investigated the effects of atrazine, pesticides and microcystins on these parameters in animals (Hussien et al., 2013; Vasanthi et al., 2014). However, there is little information on the effects of cyanobacterial neurotoxins or PSPs on these markers of hepatic detoxification and metabolism in fish (Apte and Krishnamurthy, 2011; Azevedo et al., 2013).

This study investigated the hepatotoxic and neurotoxic effects of sublethal doses of A. flos-aquae DC-1 aphanotoxins/PSPs on liver histology and ultrastructure, ALT, AST, ROS, T-AOC, and AChE and MAO activities in zebrafish. These results will further our understanding of the role of the liver in the metabolism and detoxification of cyanobacterial neurotoxins/PSPs in fish.

2. Materials and methods

2.1. Chemicals

PSP standards including STXs (dcSTX, STX, neoSTX) and gonyautoxins (GTX1–5, dcGTX2, 3) were provided by the National Research Council of Canada, Halifax, NS, Canada. All other chemicals were the highest grade from other sources, unless stated otherwise.

2.2. Toxic preparation

The strain of A. flos-aquae DC-1 was sampled from Lake Dianchi water blooms, cultivated in sterile BG11 vehicle, harvested and stored at −20 °C for analysis. Toxins were extracted twice using 0.01 mol L⁻¹ acetic acid solution, purified by precipitation, filtered and concentrated to dryness in a rotary evaporator (R-210; Buchi, Flawil, Switzerland), and finally passed through Sep-Pak C18 cartridges (Waters, Milford, MA, USA).

Toxins were analyzed using an LC20A high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) with fluorescence monitoring (LC20A, RF-10AXL, Shimadzu). The data were processed using Shimadzu Class-CR10 software (Shimadzu).

STXs and GTXs extracted from the A. flos-aquae DC-1 strain were determined by comparing their chromatograms with those of the reference standards. The concentrations of toxin were determined by the factor response (peak area/toxin concentration), as described previously (Diener et al., 2006). The overall toxicities of the extracted samples were calculated as STX equivalents based on the amount of toxin and its relative toxicity compared with STX (Asp et al., 2004; Usup et al., 2004). Purified toxins were stored at −20 °C.

2.3. Toxic exposure dosage

A total of approximately 300 fish were used to determine toxin dosage. The exposure dosage was determined according to previous studies (Bruce, 1985; Lu and Tomchik, 2002) with some modifications. Briefly, a single fish was injected (i.p.) with a starting dose (5 µL, content 9.28 µg/kg, 6.4 µg STXeq/kg toxicity, diluted to 30 µL with 0.01 mol L⁻¹ acetic acid) of crude aphanotoxins using a 50-µL single-use sterile microsyringe. If this fish died within 24 h, the subsequent fish was injected with a lower dose (diluted with 0.01 mol L⁻¹ acetic acid), but if the injected fish survived longer than 24 h, the subsequent fish was injected with a higher dose. The procedure was continued until the injected fish survived for 24 h, at which point the next fish was injected with a higher dose. Repeating this procedure established the doses causing 0 and 100% death. This series of experiments identified 5.3 and 7.61 µg STXeq/kg body weight as the low and high doses, respectively. The lower dose produced obvious behavioral signs of toxicity without death, while the higher dosage produced severe behavioral signs in all fish, but low mortality (30%). Control fish were injected with vehicle alone (0.01 mol L⁻¹ acetic acid).

2.4. Preparation of liver samples

A total of 150 healthy male zebrafish (per endpoint) were utilized in the present study (Zebrafish Center of the State Key Laboratory, Wuhan, China). All the fish were maintained under the same fertilization, batch and culture conditions. After acclimatization for 10 days, the fish were randomly separated into control, low- and high-dose groups. The low- and high-dose fish were injected with 30 µL 0.01 mol L⁻¹ acetic acid intraperitoneally containing aphanotoxins at 5.3 or 7.61 µg STXeq/kg, respectively. Fish injected with 30 µL 0.01 mol L⁻¹ acetic acid vehicle alone comprised the control group. Five fish from each group were killed by embedding in ice granules at −8 °C at 1, 3, 6, 9, 12 and 24 h after exposure. The livers were frozen at −40 °C for analysis of physiological parameters, or fixed in 10% neutral formaldehyde fixation solution for histological analysis, or fixed in 2.5% w/v glutaraldehyde for ultrastructural analysis. Protein concentrations in liver extracts were determined according to the Coomassie Brilliant Blue G-250 method (Bradford, 1976). All the endpoints were analyzed in triplicate.

2.5. Liver histology

Livers from zebrafish were fixed in 10% neutral formaldehyde solution for more than 24 h, rinsed under running water, dehydrated in a series of graded ethanol baths, cleared in xylol, and embedded in paraffin wax. The sections (6 µm) were stained...
with hematoxylin–eosin and observed under a Nikon microscope (Nikon, DS-U3, Japan).

2.6. Examination of hepatic ultrastructural abnormalities

Livers from fish in each group at each time point were prefixed using 2.5% w/v glutaraldehyde for more than 2 h, rinsed three times in 0.1 mol L⁻¹ phosphate-buffered saline (pH 7.4), and post-fixed for 2 h in 1% w/v aqueous osmium tetroxide at −4°C. Liver tissues were then embedded in Epon 812 and sequential ultra-thin sections were cut using an ultramicrotome (Leica Ultracut R, Germany). The sections were stained with uranyl acetate and lead citrate for transmission electron microscopy (JEM-1230, JEOL, Japan).

2.7. Detection of biochemical parameters

ALT, AST, ROS, T-AOC, MAO and AChE were detected using commercial diagnostic kits (Nanjing Jiancheng Bioengineering Institute, China), according to the manufacturer’s instructions. The optical densities (OD) of the reaction products were examined in 96-well microtiter plates (Sunrise Remote/Touch Screen, F039300; Salzburg, Austria). The ALT and AST activity units were expressed as enzyme activity units per mg protein (U mg⁻¹ protein⁻¹). One unit ROS decreased 1 μmol L⁻¹ H₂O₂ mg protein⁻¹ min⁻¹ (U mg⁻¹ protein⁻¹). One unit of T-AOC was defined as the amount that increased the absorbance by 0.01 OD value in reaction solution per mg of protein per min at 37°C (U mg⁻¹ protein⁻¹). AChE activity was computed according to 1 U mg⁻¹ protein⁻¹ when 1 μmol of substrate was hydrolyzed per mg of protein per 6 min. One activity unit of MAO activity was defined as the amount that increased the absorbance by 0.01 at 37°C (U mg⁻¹ protein⁻¹). All experiments were performed in triplicate.

2.8. Statistical analyses

Data were analyzed using statistical software (SPSS 16.0, Chicago, IL, USA). Each result for ALT, AST, ROS, T-AOC, AChE and MAO was the mean of three independent experiments. Statistical significances of differences between groups were assessed using one-way analysis of variance (ANOVA) with least significant difference post hoc tests. Values of p < 0.05 indicated statistical significance, and p < 0.01, indicated greater significance.

3. Results

3.1. Analysis of toxins

HPLC analysis revealed that the aphantoxins extracted from cultured A. flos-aquae DC-1 algae included three toxic components, neoSTX, GTX1 and GTX5, in accordance with the control toxin standards. The total PSP toxin content of A. flos-aquae DC-1 was 9.52 ng mg⁻¹ dry weight. The overall toxicity of the samples was 6.51 ng STX eq/mg dry weight, according to the relative toxicity ratios of GTX1, GTX5 and neoSTX versus STX, respectively. HPLC-FLD analysis confirmed that the extracted toxins had a purity of 69.57%. GTX1 was the predominant toxin, accounting for 34.04% of total PSPs, followed by GTX5 and neoSTX with 21.28% and 12.77% of total PSP toxicity, respectively. However, C toxin standards (N-sulfo carbamoyl-11-hydroxysulphate) in PSPs were unavailable and their toxin profiles were therefore not analyzed. Further studies are needed to analyze the additional molecules present in the same samples.

3.2. Changes in ALT activity

ALT activity in zebrafish livers was significantly advanced in the aphantoxin-exposed groups compared with the normal control fish (p < 0.05). At 3–12 h after exposure, the mean ALT activities in the high- and low-dose groups were 3.15 and 1.67 times higher than in the control group, respectively. The ALT level in the liver was increased at 1 h post-exposure, and further increased at 3–12 h after exposure, reaching a maximum at 12 h, with average increases of 2.49 and 1.81 times in the high- and low-dose groups versus the control group, respectively. ALT activity in exposed animal liver gradually recovered at 24 h (Fig. 1).

3.3. Changes in AST activity

AST activity in zebrafish livers was significantly increased in the aphantoxin-exposed groups compared with the normal control fish (p < 0.05). At 3–12 h after exposure, the AST activities in the high- and low-dose groups were average 1.69 and 1.49 times higher than in the control group, respectively. AST activity in the liver was advanced at 1 h after injection, and further increased at 3–12 h after exposure, reaching a maximum at 12 h, with average increases of 1.78 and 1.55 times in the high- and low-dose groups versus the control group, respectively. AST content in injected animal liver gradually recovered at 24 h (Fig. 2).

3.4. Changes in liver histology

Liver histology was changed in the zebrafish exposed to aphantoxins. The two toxin-treated groups showed similar histological alterations during the initial stage (from 1 to 3 h), with hyperemia at 1 h followed by edema at 3 h post-exposure. However, edema increased in the high-dose group from 6 to 12 h, with vacuolization and necrosis of hepatocytes, while progressive nucleus condensation and apoptosis occurred in the low-dose group. Partial recovery occurred in both treated groups at 24 h (Figs. 3 and 4).
3.5 Changes in hepatic ultrastructure

Transmission electron microscopy revealed abnormalities in hepatic ultrastructure and hepatocytes in the aphantoxin-exposed groups. The toxin-treated groups had widened intercellular spaces, cytoplasm and organelle edema, vacuolization, disruption of the cell framework and organelles, reduced glucogen, lipid accumulation, nuclear distortion and formation of apoptotic-like bodies. Hepatocytes in the treated groups showed edema indicated by enhanced light transmission at 1 h post-exposure, followed by edema of organelles including the mitochondria, endoplasmic reticulum and nuclear membranes, characterized by intracellular vacuoles, at 3 h after exposure. Organelle and cell edema were further aggravated at 6 h post-exposure, and some vacuoles became enlarged, causing nucleus deformation as a result of squeezing by the vacuoles. However, the structural alterations in the cells and organelles differed between the high- and low-dose groups. Rupture or fusion of the vacuoles and destruction of organelles occurred in the high-dose group and increased light transmission suggested necrotic changes, while apoptotic-like bodies developed in the low-dose group at 9–12 h. Partial recovery occurred in both exposed groups from 12–24 h. These results suggested that low concentrations of aphantoxins predominantly caused apoptosis, while higher concentrations caused necrosis (Fig. 5).

3.6 Changes in ROS content

The ROS content in zebrafish livers was significantly increased in the aphantoxin-exposed groups compared with the normal control group \( (p < 0.05) \). At 1–12 h after exposure, the mean ROS contents in the high- and low-dose groups were 3.15 and 2.55 times higher than in the control group, respectively. However, there was no evident difference in ROS content at 12–24 h after exposure. The ROS content in the liver was increased at 1 h after exposure, with a more obvious increase at 3–12 h after exposure. Maximum ROS contents were reached at 12 h and 9 h, with average increases of 3.78 and 2.89 times, in the high- and low-dose groups, respectively, compared with the control group. ROS content in exposed animal livers gradually recovered at 24 h (Fig. 6).
3.7. Changes in T-AOC activity

T-AOC activity in zebrafish livers was significantly decreased in the aphantoxin-exposed groups compared with the control group \((p < 0.05)\). Mean liver T-AOC activities in the exposed zebrafish groups were significantly reduced at 3–12 h after administration, to 61.38% and 49.27% of the control values in the high- and low-dose groups, respectively. There was an evident increase at 1 h and partial recovery at 12–24 h after injection. The mean minimum T-AOC activities occurred at 12 h post-exposure, with values of 69.09% and 52.31% in the high- and low-dose groups, respectively. However, mean T-AOC activities were increased by 1.45 and 1.27 times at 1 h post-exposure in the high- and low-dose groups, respectively. T-AOC activities partially recovered between 12 and 24 h post-exposure (Fig. 7).

3.8. AChE inhibition

AChE activities were examined in the livers of aphantoxin-exposed and control zebrafish to indicate the effects of exposure on neurotransmission. AChE activities were significantly reduced in the aphantoxin-exposed groups \((p < 0.05)\), with average decrease of 37.04% and 59.72% in the high- and low-dose groups, respectively, compared with the control group. Liver AChE activities began to decrease at 1 h after exposure in the exposed groups, and continued to decrease in time- and concentration-dependent manners. The most severe inhibitions of AChE activities occurred at 12 h, with average reductions of 59.72% and 37.04% in the high- and low-dose groups, respectively, compared with controls. AChE activities in the exposed fish recovered partly after 12 h, but remained different from the control group at 24 h post-exposure (Fig. 8).

3.9. MAO inhibition

MAO activities were significantly decreased in the livers of aphantoxin-exposed zebrafish livers compared with the control zebrafish \((p < 0.05)\), with average decreases of 63.51% and 44.98% in the high- and low-dose groups, respectively. Liver AChE activities in the exposed groups began to fall at 1 h post-exposure, with further time- and concentration-dependent reductions. The most severe inhibitions of MAO activities occurred at 12 h, with average reductions of 76.26% and 57.5% in the high- and low-dose groups, respectively, compared with the control. MAO activities in exposed fish partially recovered after 12 h, but remained different from the control group at 24 h post-exposure (Fig. 9).

4. Discussion

The results of this study showed that aphantoxins/PSPs increased ALT and AST activities and affected liver histology and ultrastructure in zebrafish, reflecting functional and structural liver damage. The toxins increased the ROS content and reduced T-AOC activity in zebrafish liver, demonstrating oxidative stress. Furthermore, significant inhibition of AChE and MAO activities suggest that neurotransmitter conduction function was inhibited in both sympathetic and parasympathetic/vagus nerve/neurons. All these alterations were dose- and time-dependent. Overall, these results demonstrate that aphantoxins can induce oxidative stress by increasing ROS and inhibiting the antioxidant function of T-AOC, subsequently increasing ALT and AST secretion and damaging the liver structure by inhibiting the cholinergic and adrenergic nervous function. However, C toxin standards in PSPs were unavailable, and the toxic effects of the additional unknown molecules could therefore not be identified in the present study. Further studies are needed to investigate the effects of these toxins.
Fig. 5. Ultrastructural abnormalities in zebrafish liver following aphantoxin exposure. (A) Control. (B) Original cytoplasmic edema (△) and accumulation of lipid droplets (☆) at 1 h. (C) Edema of organelles and cytoplasmic vesiculation (↑) and accumulation of lipid droplets (☆) at 3 h. (D) Larger lipid droplets (☆☆), fused cytoplasmic vesiculation (☆☆) and nucleus deformation (△) at 6 h. (E and F) Severe vesicle fusion (△) at 9–12 h in the high-dose group. (G) Apoptotic-like body formation (△△) at 12 h in the low-dose group. (H) Widening of the intercellular space (▲) at 3 h. Bar: A, C, D, E = 2 μm; B, F, G, H = 1 μm.
hepatic damage. Similar findings have also been described in goldfish (Carassius auratus) exposed to microcystin under experimental conditions (Malbruck et al., 2003).

Increases in ALT and AST in zebrafish liver may represent a metabolic compensation mechanism to react with environmental toxicants by altering and adapting their metabolic functions (Malarvizhi et al., 2012). The entry/accumulation of toxins in the liver may increase protein and carbohydrate metabolism to account for the energy crisis or need for detoxification during stress, leading to increased ALT and AST activities (De Smet and Blust, 2001; Malarvizhi et al., 2012). The results of the current and previous studies thus suggest that ALT and AST activities in zebrafish liver may be induced by aphantoxins/PSPs in response to the increased metabolism associated with hepatic dysfunction and liver structural damage (Lotesele et al., 2013).

4.2. Liver morphology

Histological and ultrastructural alterations are important biomarkers in toxicological research. These are especially significant in the liver, because of its important detoxification function (Apte and Krishnamurthy, 2011; Abdel-Moneim et al., 2012). In the current study, aphantoxins led to histological and ultrastructural alterations including hyperemia, cell and organelle edema, widened intercellular spaces, shrinkage or deformation of the cell and nucleus, cellular vacuolization, glycogen reduction and lipid accumulation. These indicate serious histological and ultrastructural damage, with consequent effects on liver function, as seen in previous studies (Perendija et al., 2011; Abdel-Moneim, 2014). The cell and organelle edema and cellular vacuolization indicate the beginning of necrosis, while the formation of apoptotic-like bodies, and shrinkage/deformation of the cell and nucleus demonstrate the beginning of apoptosis (Perendija et al., 2011). Previous studies also suggested that edema of hepatocytes, nuclei and organelles were indicative of excessive cell metabolism, while widened intercellular spaces, hyperemia and cytoplasmic vacuolization suggested damaged liver function (Shiojiri et al., 2012). The accumulation of lipid droplets may reflect increased lipid and/or decreased protein synthesis, leading to reduced lipid-protein conjugation/formation in zebrafish liver (Giari et al., 2007). Furthermore, the decrease in glycogen inclusions in aphantoxin-exposed fish hepatocytes may be attributed to the increased mobilization of energy stores associated with toxic stress (Delfuli et al., 2006; Abdel-Moneim, 2014). Our results, together with
those of previous studies, suggest that aphantoxins may cause histological and ultrastructural alterations and cell death in zebrafish liver through both apoptotic and necrotic pathways, which may represent important aphantoxin toxicity indicators (Abdel-Moneim et al., 2012).

4.3. ROS and T-AOC activities

We also investigated the effects of aphantoxin/PSPs on ROS content and T-AOC activity in zebrafish livers. Increased ROS and decreased T-AOC reflected an imbalance between ROS generation and elimination by antioxidants (Boveris et al., 2008). Previous studies showed that ROS content was increased and T-AOC activity was reduced in livers of goldfish (C. auratus), common carp (Cyprinus carpio) and zebrafish (D. rerio) after administration of chlorpyrifos, carbon tetrachloride and other toxicants, respectively (Ma et al., 2013; Dong et al., 2013; Jia et al., 2014). In the present study, we revealed that aphantoxins led to ROS up-regulation and T-AOC down-regulation, suggestive of oxidative stress in zebrafish liver, as supported by previous studies (Yu et al., 2006; Jia et al., 2014).

ROS generation is often considered as a metabolic response aimed at decomposing toxins and thus alleviating liver damage, while the decomposition of ROS may reflect the T-AOC capacities of both non-enzymic and enzymic systems to toxin oxidation, as shown previously (Yin et al., 2007). The situation is especially evident in the liver, compared with other organs, because the liver is an important organ for detoxification (Apte and Krishnamurthy, 2011). The basic ROS induced by toxicants include free radicals of superoxide anions, HO2 and OH− radicals (Malhotra and Kaufman, 2007). The T-AOC level induced by a variety of toxicants represents the total antioxidant capacity of non-enzymic and enzymic systems (Yang et al., 2014). Hydrolysis during detoxification in zebrafish liver may thus be associated with increased production of superoxide anions and the subsequent induction of a series of cascade reactions to generate H2O2 and OH− radicals, leading to ROS accumulation (Malhotra and Kaufman, 2007). In addition, the ROS generated by detoxification and toxin decomposition consume antioxidant substances, which may also lead to the depletion of T-AOC (Yu et al., 2006). The accumulation of ROS may thus also reflect an imbalance between generation and elimination, because of decreased total antioxidant elimination capacity (Boveris et al., 2008).

4.4. AChE activity

We also investigated alterations of AChE activities in zebrafish liver exposed to aphantoxins. AChE inhibition reflected slow/reduced nerve information exchange due to neurotransmitter inactivation/decomposition to acetylcholine (Martins-Silva et al., 2011). Previous studies showed that AChE inhibition induced by insecticides, heavy metals, and other chemical toxicants mainly occurred in fish brains and affected their behavioral performance (Ricchetti et al., 2011; Pereira et al., 2012). The main symptoms were altered swimming motility, motor incoordination, and paralysis (da Silva et al., 2011; Martins-Silva et al., 2011). However, there is little information on AChE inhibition in fish liver after exposure to toxicants. In this study, we observed significant down-regulation of AChE activities after exposure to aphantoxins, suggesting reduced neurotransmitter inactivation in zebrafish liver. Several recent studies have reported AChE inhibition in the liver of fish exposed to herbicides and other stressors, which results support the present study (Gordon et al., 2010; Fan et al., 2013).

Decreased AChE activity may be associated with damage to cholinergic neurons that innervate/modulate liver, because AChE is located in the synapse between neurons/non-neurons in these pathways (Azevedo et al., 2013). Aphantoxins may reduce the synthesis of AChE in these neurons, thus inhibiting neurotransmission via voltage-gated sodium channels (Hussien et al., 2013). The present and previous results suggest that aphantoxins/PSPs may damage the structure and function of the liver by inhibiting AChE activity and thus affecting cholinergic neurotransmission in zebrafish liver (Azevedo et al., 2013).

4.5. MAO activity

We also studied the effects of aphantoxins on MAO activity in zebrafish liver. MAO inhibition represents reduced inactivation of monoaminergic neurotransmitters (Hussien et al., 2013). MAO inhibition has previously been investigated mostly in the brains of mammals, including humans, while MAO activity in fish remains poorly understood (Yagodina et al., 2006). For example, Hussien et al. (2013) found that the pesticide cypermethrin might cause MAO inhibition in rat brains, while similar MAO inhibition was also reported in the brains of mice after lead exposure (Liu et al., 2013a,b). The present findings showed that MAO activity in zebrafish liver might be significantly reduced by aphantoxin exposure, suggesting reduced inactivation of monoaminergic neurotransmission. Several recent studies have reported MAO inhibition in the livers of fish and other aquatic animals (lamprey Lampetra fluviatilis) induced by several chemicals, thus strengthening the present findings (Basova and Yagodina, 2011; Yagodina and Basova, 2013).

Reduced MAO activity may be the result of reduced synthesis in the sympathetic neurons that innervate/modulate liver function in fish, with subsequent effects on the decomposition/conduction of monoaminergic neurotransmitters (Finberg, 2014). Aphantoxins damaged the hepatocytes, indicated by edema and vacuolization, affecting the hepatic function of monoaminergic neurotransmitters or MAO synthesis, as indicated previously (Abdel-Moneim, 2014). We previously demonstrated that aphantoxins induced alterations in both the ultrastructure and physiology of brain neurons in zebrafish, causing mitochondrial edema and expansion of the endoplasmic reticulum (Zhang et al., 2011, 2013a,b,c). These results suggest that MAO synthesis may be inhibited because MAO is located in the outer mitochondrial membrane (Yagodina and Basova, 2013). Alternatively, MAO activity reduction may be related to its increased consumption in hepatocytes attempting to maintain the normal detoxification functions of the liver (Apte and Krishnamurthy, 2011). Our results, together with those of other studies, indicate that aphantoxins cause a reduction in MAO activity in zebrafish livers through inhibiting its synthesis and/or increasing its consumption in attempts to alleviate toxic damage (Apte and Krishnamurthy, 2011; Finberg, 2014).

5. Conclusion

In summary, the results of this study demonstrated that aphantoxin exposure increased ALT and AST activities and histological and ultrastructural damage, suggesting both functional and structural alterations in zebrafish liver. Toxin exposure led to increased ROS content and reduced T-AOC activity, suggestive of oxidative stress, while inhibition of AChE and MAO activities suggested inhibition of neurotransmission. All the toxic effects were dose- and time-dependent. Overall, these results demonstrate that aphantoxins/PSPs induce oxidative stress via increased ROS and inhibition of T-AOC, leading to subsequent damage to liver function and structure in terms of ALT and AST activities, and histological and ultrastructural alterations. All these changes may be induced by inhibition of the cholinergic and adrenergic systems that
innervate zebrafish liver, suggesting that aptamophos/PSPs have neurotoxic effects in zebrafish liver. ALT, AST, histology, ultrastructure, ROS, T-AOC, AChE and MAO may represent important bioindicators for investigating aptamophos and cyanobacterial blooms in nature.

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