The role of apoptosis in MCLR-induced developmental toxicity in zebrafish embryos

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ABSTRACT

We previously demonstrated that cyanobacteria-derived microcystin–leucine–arginine (MCLR) is able to induce developing toxicity, such as malformation, growth delay and also decreased heart rates in zebrafish embryos. However, the molecular mechanisms by which MCLR induces its toxicity during the development of zebrafish remain largely unknown. Here, we evaluate the role of apoptosis in MCLR-induced developmental toxicity. Zebrafish embryos were exposed to various concentrations of MCLR (0, 0.2, 0.5, 2, and 5.0 mg L−1) for 96 h, at which time reactive oxygen species (ROS) was significantly induced in the 2 and 5.0 mg L−1 MCLR exposure groups. Acidine orange (AO) staining and terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labelling (TUNEL) assay showed that MCLR exposure resulted in cell apoptosis. To test the apoptotic pathway, the expression pattern of several apoptotic-related genes was examined for the level of enzyme activity, gene and protein expression, respectively. The overall results demonstrate that MCLR-induced ROS which consequently triggered apoptosis in the heart of developing zebrafish embryos. Our results also indicate that the p53–Bax–Bcl-2 pathway and the caspase-dependent apoptotic pathway play major roles in MCLR-induced apoptosis in the developing embryos.

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1. Introduction

In the last decade, public health concerns about toxic cyanobacteria have increased in many countries, owing to the frequent occurrence of cyanotoxins in both drinking and recreational waters. The blooms of these cyanobacteria and their cyanotoxins are harmful to aquatic biota, particularly to fish (Carbis et al., 1997; Landsberg, 2002). The hazard to fish populations has only recently been taken into consideration with the increasing occurrence of blooms and a better knowledge of the cyanotoxins (Ernst et al., 2006; Palikova et al., 2007; Stone and Bress, 2007). Among cyanotoxins, microcystins (MCs) are the most commonly reported all over the world: typically they have molecular weights ranging between 900 and 1000 Da. So far, more than 80 different structural analogues of MCs have been identified (Fastner et al., 2002), with microcystin-LR (MCLR) being the most toxic (Mirura et al., 1989).

In the aquatic environment, the concentration of MCs in surface water is general low except during periods of cyanobacterial blooms. However, MCs are readily bioavailable and can bioaccumulate in macrophytes, zooplankton, fish, and mammals including humans through the food web (Sahin et al., 1996; Ferrão-Filho et al., 2002; Pfugmacher et al., 1998; Chen et al., 2009). In humans, MCs have been identified for the first time in the serum (average 0.228 ng MCLRreq/mL) of a chronically exposed human population (fishermen at Lake Chaohu, China), together with an indication of hepatocellular damage (Chen et al., 2009). There have also been an increasing number of studies to evaluate MC contamination of fishes from natural waters with cyanobacterial blooms (Vasconcelos, 1995; Mohamed et al., 2003; Xie et al., 2005; Chen...
et al., 2006; Deblois et al., 2008). Elevated concentrations of MCs (1.54–97.4 μg g⁻¹ DW) associated with dense and toxic blooms of *Microcystis aeruginosa* were found in the blood, kidney, liver and intestine of phytoplanktivorous silver carp (*Hypophthalmichthys molitrix*) stocked in Taihu Lake (China) (Chen et al., 2006). Lower concentrations (100–821 ng g⁻¹ fresh weight) have been recorded in samples of liver, kidney, gut and muscle of farmed Nile tilapia (*Oreochromis niloticus*) in Egypt (Mohamed et al., 2003). More recently, Chen et al. (2009) examined MC contents in the muscle of 16 species of aquatic animals from Taihu Lake. For all 16 species the average MC-RR, -YR and -LR concentrations were 42.1, 3.3, and 33.1 ng g⁻¹ DW, respectively, reflecting increasing occurrence and biological contamination of MCs in China. Recent laboratory and field studies have demonstrated the accumulation of MCs in the gonads of rats, water birds, ducks, shrimp and fishes (Wang et al., 2008; Chen and Xie, 2005; Lei et al., 2008); these findings suggest that maternal transfer of MCs to offspring may be possible.

MCs have been characterized as potent inhibitors of protein phosphatases 1 and 2A in hepatocytes of animals (Eriksson et al., 1990; Falconer and Yeung, 1992). The inhibition leads to an imbalance of protein phosphorylation, which consequently causes disruption of the cell cytoskeleton and which may ultimately induce liver disease or even liver necrosis (Falconer and Yeung, 1992; Ito et al., 1997, 2000). There is evidence that these adverse effects are closely related to oxidative stress processes and free radicals (Ding et al., 1998; Gehringer et al., 2003). Damage has also been documented (Sekijima et al., 1999; Gehringer et al., 2003) as well as apoptosis, which is of great importance (Earnshaw, 1995).

In recent years, numerous studies have examined the toxic effect of MCs on the embryonic development and larval growth of aquatic organisms (Oberemm and Fastner, 1997; Oberemm et al., 1999; Wiegand et al., 1999; Liu et al., 2002; Wang et al., 2005; Wright et al., 2006; Malbrourck and Kestemont, 2006). Oberemm et al. (1999) reported that the early life stage of fishes or amphibians may be more vulnerable to cyanobacterial toxins than the juvenile or adult life stages due to lower mobility to avoid contamination early on in the life cycle of these two groups. Toxic effects of MCs on embryonic development are stage-specific (Liu et al., 2002; Jacquet et al., 2004) and MCs can result in retarded development, low hatching rate and high rates of malformation (Oberemm and Fastner, 1997; Wiegand et al., 1999; Liu et al., 2002; Jacquet et al., 2004; Wang et al., 2005; Zhang et al., 2008). However, the molecular mechanisms by which MCLR induce their toxicity during the development of zebrafish remain largely unknown. By themselves, the toxicological end points such as increased rates of morphological disorders or decreased rates of hatching success or survival do not provide sufficient information to reveal the mechanism of toxicant-induced effects. However, recent work is starting to elucidate a mechanistic pathway of action for MCLR. In our previous study, a proteomic analysis was performed on developing zebrafish embryos exposed to MCLR for 96 h post-fertilization (Li et al., 2011). The results demonstrated that MCLR was able to induce toxicity in the forms of development abnormalities, growth delay and also decreased heart rates in zebrafish embryos. The results also showed that the expression of several apoptosis-related proteins was significantly changed, suggesting the important role of apoptosis in MCLR-induced developing toxicity.

The present study was designed to investigate the role of apoptosis in MCLR-induced developmental toxicity. Firstly, we examined the reactive oxygen species (ROS) level and the target organ of MCLR-induced apoptosis. Secondly, we examined the gene and protein expression patterns as well as enzyme activity related to the apoptosis pathway (e.g., pro-apoptotic [p53, Bax, Bid, caspase-9, caspase-8 and caspase-3] and anti-apoptotic [Bcl-2]) to elucidate the potential mechanism of apoptosis induced by MCLR.

### 2. Materials and methods

#### 2.1. Chemicals

The cyanobacterial toxin MCLR was obtained from Express (Taiwan), with a purity of ≥95%, confirmed by high performance liquid chromatography (HPLC, LC-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan) following the method of Chen et al. (2009). Tricaine (MS-222 – Sigma-Aldrich), which was dissolved in deionized water, was used to anesthetize the larvae. All of the other chemicals utilized in this study were of analytical grade and the chemicals used for electrophoresis were obtained from Amersham Biosciences (Piscataway, NJ, USA).

#### 2.2. Maintenance of zebrafish and embryo toxicity test

Zebrafish maintenance and embryo collection were performed according to our previous study (Li et al., 2011). Briefly, wild-type (AB strain) zebrafish were cultured in a closed flow-through system with charcoal-filtered tap water at 28 ± 0.5 °C in a 14:10 h light:dark cycle. The fish were fed twice daily with Artemia nauplii. Spawning adults in groups of about 20 males and 10 females in tanks were prepared for collecting fertilized eggs. At 0.5–1.0 h post-fertilization (hpf) normally developed embryos (with out malformation) were selected and randomly distributed among beakers (30 embryos per beaker) containing different concentrations of MCLR (0, 0.2, 0.5, 2 and 5 mg L⁻¹) and a 50 mL solution comprising of Ca(NO₃)₂ (0.2 mM), MgSO₄ (0.13 mM), NaCl (19.3 mM), KCl (0.23 mM), and HEPES (1.67 mM) (Westerfield, 1995). The exposure time was selected at 96 hpf, because most embryonic organs are well developed by this time. The environmentally relevant or high concentrations of MCLR were selected in the present study based on earlier range-finding studies (Li et al., 2011). The control group received no MCLR. Each concentration had three replicates and each replicate consisted of a glass beaker containing 50 mL of the respective treatment solutions and 30 viable embryos. Larvae that developed normally were selected for the following experiments.

#### 2.3. Acidine orange (AO) staining

Embryo cell apoptosis was identified using AO staining which is a nucleic acid-selective metachromatic stain technique useful for studying apoptosis patterns (Chan and Cheng, 2003; Deng et al., 2009). After 96 h of exposure to the concentrations of MCLR (0, 0.2, 0.5, 2.0 and 5.0 μg L⁻¹), 10 larvae from each beaker (n = 3) were washed twice in 30% Danieau’s solution (58 mM of NaCl, 0.7 mM of KCl, 0.4 mM of MgSO₄, 0.6 mM of Ca(NO₃)₂, and 5 mM of HEPES, pH 7.4), then transferred to 5 μg mL⁻¹ of AO dissolved in 30% Danieau’s solution for 20 min at room temperature. The larvae were then washed with 30% Danieau’s solution three times for 5 min each. Before examination, the embryos were anesthetized with 0.03% MS-222 for 3 min. Apoptotic cells were identified with a fluorescence microscope (Nikon, Japan). Apoptotic cells appeared as obvious bright spots.

#### 2.4. Terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labelling (TUNEL)

For TUNEL analysis, an *in situ* cell death detection kit, POD (Roche Diagnostics GmbH, Mannheim, Germany), was used following the previous studies with slightly modification (Shi et al., 2008; Li et al., 2012). Briefly, 96 hpf embryos were fixed in 4% paraformaldehyde at 4 °C overnight, and then rinsed twice in PBS. Endogenous peroxidases were blocked by incubation in 3% hydrogen peroxide in methanol for 15 min at room temperature. After washing with PBS, the TUNEL reaction mixture was added and the fry were incubated
at 37 °C for 60 min. The embryos were then rinsed with PBS and incubated in converter POD solution for 30 min at 37 °C, and washed in PBS. Finally, specimens were reacted in diaminobenzidine for 30–90 min and were photographed with a dissecting microscope (Nikon, Japan). Apoptotic regions were identified with DAB stain (brown).

2.5. ROS

The dose-dependent generation of ROS in the larvae exposed to MCLR was measured using dichlorofluorescein-diacetate (DCFH-DA). Briefly, 10 larvae were washed with cold-PBS (pH 7.4) twice and then homogenized in cold buffer (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of MgCl₂, and 0.5 mM of phenylmethyl sulfonylfluoride (PMSF), pH 7.4). The homogenate was centrifuged at 15,000 × g at 4 °C for 20 min, and the supernatant was transferred to new tubes for further experimentation. Twenty microliters of the homogenate were added to each well of a 96-well plate and incubated at room temperature for 5 min, after which 100 μL of PBS (pH 7.4) and 8.3 mL of DCFH-DA stock solution (dissolved in DMSO, 10 mg mL⁻¹) were added to each well. The plate was incubated at 37 °C for 30 min. The fluorescence intensity was measured using a microplate reader (Molecular Device, M2, Union City, CA, USA) with excitation and emission at 485 and 530 nm, respectively. The ROS concentration was expressed in arbitrary units (DCF mg⁻¹ total proteins).

2.6. Gene expression

After exposure to MCLR, embryos were collected and preserved in TRIzol reagent (Invitrogen, NJ, USA) at −20 °C. Each concentration was measured in triplicate with a composite of 30 embryos. Isolation, purification and quantification of total RNA, first-strand cDNA synthesis, and quantitative real-time PCR (QPCR) were performed according to Li et al. (2012). Briefly, total RNA was isolated by use of TRIzol reagent, and digested by RNase-free DNase I (Promega, WI, USA) following the manufacturer’s instructions. Concentrations of total RNA were estimated at 260 nm and the quality was verified by measuring the 260/280 nm ratio. One percent agarose-formaldehyde gel electrophoresis with ethidium bromide staining was used to further verify the quality of total RNA. First-strand cDNA synthesis was performed using commercial kits (Promega, WI, USA) following the manufacturer’s instructions. QPCR was analyzed on a Chrom 4Tm detector (BioRad, USA) in sterile, 96-well PCR plates (Applied Biosystems, Foster City, CA, USA). Because glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels were not significantly different between control and treated groups following the treatment with MCLR (Li et al., 2011), GAPDH was chosen as an internal control to normalize the data. Primer sequences were designed using Primer v3 software (http://frodo.wi.mit.edu/) (Table 1). The thermal cycle was set at 95 °C for 10 min, followed by 35–45 cycles of 95 °C for 30 s, 60 °C for 20 s and 72 °C for 1 min. The fold-change of the genes tested was obtained by the 2⁻ΔΔCT method. Transcript expression was measured in triplicate.

2.7. Western blot analysis

MCLR-exposed zebrafish larvae were washed twice with PBS (pH7.4) and then homogenized in ice-cold protein extraction buffer to extract proteins (Wuhan Boster Biological Technology, China). Each set of 150 larvae was pooled for protein preparation, such that n = 1 refers to protein from these 150 embryos. The homogenates were centrifuged for 10 min at 12,000 × g and supernatants were collected. Protein concentrations were determined by the Bradford method (Bradford, 1976); western blot analysis was performed with minor modifications following Li et al. (2011). About 20 μg of protein from each sample was denatured, electro-phoresed, and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked and blots were incubated in specific antibody against p53, Bax, Bcl-2, and GAPDH (Abcam, UK), and then secondary antibodies following the manufacturer’s instructions. The NBT/BCIP system was used to evaluate the protein signal. The results of the western blots were quantified with Gene Snap software (Syngene, America).

2.8. Caspase activity

Caspase activities were determined by a colorimetric assay based on the ability of caspase-3, -8, -9 to change acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA), acetyl-Ile-Glu-Thr-Asp p-nitroanilide (Ac-IETD-pNA) and acetyl-Leu-Glu-His-Asp p-nitroanilide (Ac-LEHD-pNA) into a yellow formazan product (p-nitroaniline (pNA)), respectively. Briefly, 20 larvae from each beaker (n = 3) were washed with iced PBS (pH 7.4) and homogenized on ice for 20 min, after which they were centrifuged at 2000 × g at 4 °C for 5 min and the supernatant collected. Supernatant enzyme activity was determined using a caspase assay kit (Keygene Biotech Co., Ltd., Nanjing, China) according to the manufacturer’s instructions. Caspase activity was expressed as the percentage enzyme activity compared to the control group. All measurements were carried out in triplicate.

2.9. Statistical analysis

The data were checked for homogeneity of variance using Levene’s test and then evaluated by a one-way analysis of variance (ANOVA) test followed by a Tukey’s test using SPSS 13.0 (SPSS, Chicago, IL, USA). The value p < 0.05 was used as the criterion for statistical significance. All values were expressed as the mean ± standard error (SE).

3. Results

3.1. AO staining and TUNEL assay

At 96 hpf, no obvious apoptotic cells were observed in the control group, but considerable numbers of apoptotic cells appeared, mainly around the heart area, in the 2.0 and 5.0 mg L⁻¹ MCLR-treated groups (Fig. 1A). In addition, a small number of apoptotic cells were observed in the eye lens in the 5.0 mg L⁻¹ MCLR-treated
3.2. ROS

In MCLR-treated zebrafish embryos, ROS concentrations were significantly increased in the 2 and 5.0 mg L\(^{-1}\) exposure groups, while ROS levels remained unchanged in other exposure groups compared with the controls (Fig. 2).

3.3. Gene expression pattern of p53, Bax, Bcl-2 and Bid

The transcription level of the p53 gene was significantly upregulated, by 1.73-, and 4.00-fold in the 2.0 and 5.0 mg L\(^{-1}\) MCLR-treated embryos relative to the control group (Fig. 3). The transcriptional changes of three members of the Bcl-2 family (Bax, Bid and Bcl-2) that plays a major role in the regulation of apoptosis were examined. Bax expression was significantly induced 2.38-, 2.57-, 5.74-fold in a dose-dependent manner in 0.5, 2.0 and 5.0 mg L\(^{-1}\) MCLR-treated groups compared with the control. Bid was significantly up-regulated in the 2.0 and 5.0 mg L\(^{-1}\) MCLR-treated groups. The mRNA expression of Bcl-2 was inhibited in a concentration-dependent manner, and was significantly downregulated in the 5.0 mg L\(^{-1}\) MCLR-treated group (Fig. 3).

3.4. Western blot analysis

The effect of MCLR on protein expressions of p53, Bax, and Bcl-2 in zebrafish was shown in Fig. 4A. Protein expression of p53 was significantly induced by 2.26-, 2.46-, 3.03-, and 4.43-fold in a dose-dependent manner in the 0.2, 0.5, 2.0 and 5.0 mg L\(^{-1}\) MCLR-treated groups compared with the control (Fig. 4B). The protein
level of Bax was dramatically induced by 2.47- and 3.42-fold in the 2.0 and 5.0 mg L\(^{-1}\) MCLR exposure groups (Fig. 4B). In addition, Bcl-2 expression was significantly inhibited in a dose-dependent manner in the 0.5, 2.0 and 5.0 mg L\(^{-1}\) MCLR-treated groups compared with the control (Fig. 4B). The Bax/Bcl-2 ratio increased in a dose-dependent manner and reached a maximal value in the 5.0 mg L\(^{-1}\) MCLR-treated group for both mRNA and protein expressions (Fig. 4C).

### 3.5. Caspase activity and gene expression analysis

The transcriptional changes of three enzymes involved in caspase pathway were examined (Fig. 5A). The expression of the caspase-3 gene was significantly induced in the 0.5, 2.0 and 5.0 mg L\(^{-1}\) MCLR-treated groups (Fig. 5A). The caspase-8 gene was greatly up-regulated in the 2.0 and 5.0 mg L\(^{-1}\) MCLR-treated groups, while the caspase-9 gene was significantly increased in the 0.2, 0.5, 2.0 and 5.0 mg L\(^{-1}\) MCLR-treated groups compared with the control (Fig. 5A). Under MCLR exposure, a slight but significant increase in caspase-3, -8, -9 activities was observed at 5.0 mg L\(^{-1}\) exposure with an increase of 21.9%, 34.4%, 40.0%, respectively, compared to the control (Fig. 5B).

### 4. Discussion

Apoptosis, also called programmed cell death, is an important regulator of growth and development (Ulukaya et al., 2011). In the present study, a high percentage of apoptotic cells were observed in the heart of zebrafish larvae as revealed by AO staining and TUNEL assay. It had been demonstrated that very low levels of myocyte apoptosis are sufficient to cause a lethal, dilated cardiomyopathy (Wencker et al., 2003). Several studies have reported that MCs could accumulate in the heart of rat and fish, subsequently triggering oxidative stress (Wang et al., 2008; Qiu et al., 2009). Heart could be the target organ of MCs. Exposure of zebrafish embryo to hexabromocyclododecane (HBCD) also induces apoptosis in heart areas, accompanied with body growth retardation (Deng et al., 2009). Thus, we may speculate that the developing heart is an important potential target for MCLR toxicity in zebrafish and MCLR-induced apoptosis may result in circulation failure by depressing heart rates, eventually disturbing the early development of zebrafish.

MCLR-induced apoptosis has long been correlated with generation of ROS (Chen et al., 2005). In the present study, the content of ROS has been shown to be greatly increased among embryos of the 2.0 and 5.0 mg kg\(^{-1}\) MCLR treated groups. Consistent with the present results, our previous study showed that several heat shock proteins (HSP) were dramatically up-regulated in the 96 hpf zebrafish embryo exposed to 0.5 mg kg\(^{-1}\) MCLR (Li et al., 2011). Three points are significant in this regard: first, the induction of HSP expression has been suggested to be an early marker of oxidative stress (Polla et al., 1996; Peng et al., 2000); second, ROS-induced oxidative stress is thought to contribute to abnormal development during embryogenesis (Yamashita, 2003), and third, excessive ROS and oxidative damage were detected in HBCD and TBPPA-exposed zebrafish embryos and eventually resulted in developmental toxicity (Deng et al., 2009; Hu et al., 2009). Thus, MCLR-induced development toxicity in embryos/larvae in this study can be partially explained by ROS-induced apoptosis.

The p53 is a multi-faced nuclear phosphoprotein induced in response to cellular stress, functioning as a transcriptional transactivator in apoptosis, DNA repair, and tumour suppression pathways. The induction of p53 has been associated with MCs-induced apoptosis in adult zebrafish, mammals and Caenorhabditis elegans (Chen et al., 2005; Li et al., 2011; Wang et al., 2012; Ji et al., 2013). In this study, both the transcriptional level and protein expression of p53 were significantly induced, suggesting that p53 may play a pivotal role in MCLR-induced apoptosis in the early life stages of zebrafish. Typically, p53 induces apoptosis by up-regulating the transcription of pro-apoptotic genes and by down-regulating anti-apoptotic genes, including those of bcl-2 family (Perfettini et al., 2004). Both the up-regulation of pro-apoptotic genes (Bax and Bid) and the down-regulation of the anti-apoptotic gene (Bcl-2) of the
bcl-2 family were detected in this study. Elsewhere, it has been demonstrated that p53 can lead to apoptosis indirectly by down-regulating expression of Bcl-2 (Miyashita et al., 1994). Bax can promote apoptosis by homodimerizing or heterodimerizing with Bcl-2. Up-regulation of Bax and the trend of down-regulation of Bcl-2 were observed in rat testis, hepatocyte BRL-3A cells, and in vivo in hepatocytes of Balb/c mice when MCLR-induced apoptosis occurred (Chen et al., 2005; Žegura et al., 2008; Li et al., 2011). The ratio of Bax/Bcl-2 is thus crucial in determining the progress of cell apoptosis, and our study showed a dose-dependent increase of the ratio both in the gene and protein expression levels, suggesting that MCLR might trigger apoptosis via a p53–Bax–Bcl-2 pathway in zebrafish larvae. In addition, previous studies have shown that Bid–Bax–Bcl-2 is the main pathway for MCLR-induced apoptosis (Chen et al., 2005). Although the detailed mechanisms by which MCLR-induced apoptosis operate are unclear, the results from the present study indicate that the induction of p53 and the changes of the Bcl-2 family may contribute to the MCLR-triggered apoptosis in the early-life stage of fish.

The caspase pathway is another important pathway involved in MCLR-triggered apoptosis (Xiong et al., 2009). Caspase activity has also been thought to be a useful marker for assaying stress-induced apoptosis in the early-life stages of fishes, during which caspase-3 is confirmed as a key executor to be activated downstream in apoptosis pathways (Liu et al., 2007; Deng et al., 2009). Generally, two pathways of caspase activation were involved in apoptosis. The first one is initiated by the stress-mediated release of cytochrome c from the mitochondria, which promotes downstream effector caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex then leading to the characteristic phenotype of apoptosis (Fulda and Debatin, 2006; Gao et al., 2013). In the second pathway, stimulation of Fas, tumour necrosis factor receptor (TNFR) or TNF-related apoptosis-inducing ligand receptors (TRAILR) results in activation of the initiator caspase-8 (Fulda, 2009). In the present study, the gene transcription and activity of caspase-3, caspase-8 and caspase-9 were greatly increased. Our results are consistent with earlier reports that the induction of caspases were detected in MCLR-induced apoptosis in rat testis, mouse liver and murine neuron (Chen et al., 2005; Xiong et al., 2009; Feurstein et al., 2011). Taken together, we may speculate that these results indicate that caspase-dependent apoptotic pathway may contribute greatly to the MCLR-induced apoptosis in the early-life stages of zebrafish.
In summary, the present study examined the role of apoptosis in MCLR-induced developmental toxicity in zebrafish embryos, building on our previous study that showed that MCLR can significantly affect embryo development. Our results indicate that the probable mechanism of this developmental toxicity is the generation of ROS and the consequent triggering of apoptosis in the heart of developing embryos. In addition, the p53–Bax–Bcl-2 pathway and the caspase-dependent apoptotic pathway play major roles in MCLR-induced apoptosis in the developing embryos. However, the exact mechanisms still need to be further investigated.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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