A proteomic analysis of prenatal transfer of microcystin-LR induced neurotoxicity in rat offspring

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**ABSTRACT**

Recent studies showed that microcystins (MCs) can be transferred to offspring from their adults and exert notable neurotoxicity, but the exact mechanism is little known. In order to better understand cellular responses in brain tissues disrupted by prenatal transfer of MCs, this work mainly focuses on brain impairments of rat offspring. Pregnant SD rats were infused exposed to microcystin-LR (MCLR) at 10 μg/kg body weight (BW)/day or saline solution from gestational day 8 (GD8) to postnatal day 15 (PD15) of lactation. MCLR accumulation, the levels of malondialdehyde (MDA) and acetylcholine esterase (AChE) activity were detected. The results showed that MCLR enhanced toxin accumulation and MDA, but decreased GSH and the level of AChE activity in the brains of rat offspring. MCLR also caused changes to cerebrum ultrastructure, showing a sparse structure, distention of endoplasmic reticulum and swelling mitochondria. To explore the exact mechanisms, we used a proteomic analysis to identify global brain protein profiles. The proteomic results revealed that MCLR remarkably altered the abundance of 49 proteins that were involved in neurodevelopment, oxidative phosphorylation, cytoskeleton, metabolism, protein folding and degradation. Our results indicated that MCLR exerts neurotoxicity mainly by generating oxidative stress and endoplasmic reticulum stress.

**Biological significance**

The integration of proteomics and bioinformatics analyses revealed that perinatal exposure to MCLR can occur from mother to offspring and impair the brain of rat pups. MCLR has negative effects on the development of nervous system mainly by generating oxidative stress and endoplasmic reticulum stress.

**Keywords:** Neurotoxicity, MCLR, Proteomics, Offspring, Generation transfer

1. **Introduction**

Blooms of cyanobacteria occur worldwide in nutrient-rich waters and can pose a public health threat when toxin-producing species are involved. Among cyanotoxins, microcystins (MCs), produced by the genera Microcystis, Anabaena, Anabaenopsis, Aphanizomenon, Fischerella, Planktothrix, and the terrestrial Hapalosiphon are the most common all over the world [1–3]. MCs comprise a family of more than 80 structurally related toxins, among which, MCLR is the most common and toxic variant [4].
MCs are generally associated with hepatotoxicity. The typical toxicological action of MCs is to inhibit serine/threonine protein phosphatase in the liver cells [5,6], followed by hyper-phosphorylation of numerous cellular proteins, thereby resulting in the collapse of the cytoskeleton and the loss of cellular integrity. Prolonged exposure to MCs and the ensuing reactive oxygen species (ROS)-induced damage to DNA have been shown to be a powerful tumor promoter in rodents, but its impact as a carcinogen is yet to be established in humans [7-9].

Several studies have demonstrated that MCs could accumulate and induce neurotoxicity in both fish and mammals. More recently, several OATPs/Oatps (rodent Oatp) appear to be specifically required for the active uptake of MCs into brain [10-12]. In fish, Baganz et al. reported changes in the spontaneous locomotor behavior of zebrafish (Danio rerio) and sunbleak (Leucaspius delineatus) after MCLR exposure [13,14] and Cazenave et al. showed changes in swimming activity of Jenynsia multidentata fed with microcystin-RR (MCRB) [15], suggesting the probable neurotoxicity of MCs. In mammals, spatial learning and memory loss were observed in rats after intra-hippocampal infusion with MCLR [15,17]. Furthermore, a reduction in brain size was reported in progeny of Swiss Albino mice exposed to cyanobacterial bloom extract containing MCs [18], suggesting probable neurotoxicity. Therefore, it is assumed that MCs are able to enter the brain and to exert neurotoxic effects. Indeed, 116 (89%) of 131 patients of a hemodialysis unit in Caruaru, Brazil, suffered from the freeze-dried surface blooms collected from Lake Dianchi in China by the method reported by Dai et al. [25]. Briefly, the extraction of microcystis cells was sequentially applied to an octadecylsilis cartridge and semi-performance preparative liquid chromatograph system (Waters 600, United States). The content of purified MCLR was over 95%, and its identity was confirmed by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS, Thermo Electron Corporation, Waltham, MA). All reagents obtained from various commercial sources were analytical or higher grades, and the chemicals used for electrophoresis were obtained from Bio-Rad Laboratories (Hercules, California, USA).

2.2. Animals

Ten female Sprague–Dawley (SD) [Crl:CD(SD)] rats (12 weeks of age) and 10 male SD rats (12 weeks of age) were supplied by the Wuhan University Laboratory Animal research Center (Hubei, China). The rats were housed under controlled conditions of 12 h light/dark cycle, 50 ± 5% humidity and 20 ± 1 °C. The animals were allowed free access to food and water. The animals were treated humanely and with regard for alleviation of suffering. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory (permit number SCXK 2008-0004).

Female and male rats were randomly assigned to the treatment and control groups. They were allowed to acclimatize for 5 days before being paired to mate. The day on which a vaginal plug was detected in the morning and sperm were found on a vaginal smear was considered GD1. On the evening of GD8, the dams were implanted subcutaneously with Alzet osmotic pumps (model 2004; Alza Corp., Palo Alto, CA, USA) following the manufacturer’s recommendations. The pumps were designed to deliver vehicle alone (0.9% saline solution) or a MCLR solution (10 μg MCLR/kg BW/day). These pumps continued to release solution at a constant rate (0.25 μL/h) for 4 weeks. It should be noted that the actual delivered dose of MCLR decreased as the pregnancy progressed because the weight of the mother at GD8 was used to calculate the MCLR dose, and the body weight increased from this point throughout pregnancy. In rats, blastocyst implantation in the uterus occurs until the 5th gestational day, and organogenesis occurs from the 6th day until the 16th day of gestation. Fetal development occurs from 16th day of gestation until the 21st day [24]. Thus, GD8 (during the stable organogenesis stage) was chosen for the start of exposure. During the 4 weeks (28 days) of maternal MCLR exposure, the dams delivered naturally, and litters were euthanized on postnatal day 15. The brains of rat pups were rapidly extirpated, frozen in liquid nitrogen, and maintained at −80 °C until being processed for analyses.

2.3. Ultrastructure analysis

Newborn rats were then decapitated and the cerebral tissues were removed. Serial, coronal, ultrathin sections of cerebral tissues were obtained and cut into 1 mm spaced pieces, placed in 2.5% glutaraldehyde phosphate buffer (pH = 7.4) and fixed for 2–4 h, followed by 1% osmium...
tetroxide fixation, ethanol dehydration and embedding in 812 epoxy resin. Ultrathin sections were stained with Uranyl Acetate-Lead Citrate stain and observed with transmission electron microscope (TEM)-FEI Tecnai G2 20 TWIN.

2.4. Lipid peroxidation and GSH level detection

Lipid peroxidation was determined according to a kit protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on the thiobarbituric acid (TBA) method [26]. GSH

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**Fig. 1** – Confirmation of extracted MCLR by LC/MS/MS and MCLR contents in the brains of rat pups after 10 μg/kg/day perinatal MCLR exposure for 28 days.
content was measured according to Griffith [27]. Each assay was carried out by triplicate. Three pups from every dam in both groups were randomly chosen for the GSH level and lipid peroxidation assays.

2.5. Protein phosphatase activity analysis and MCLR accumulation

PP activity was analyzed according to the method of Fontal et al. [28] with some modifications. 0.2 mg brain tissue was sonicated in 5 volumes of homogenizing buffer containing 5 mM Tris–HCl (pH 7.5), 0.5 mM EGTA, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride and was homogenized with a glass homogenizer [29]. The supernatant was recovered by centrifugation (20,000 g) at 4 °C for 30 min. Briefly, 35 μL of liver homogenate was mixed with 5 μL of NiCl₂ (40 mM), 5 μL of 5 mg mL⁻¹ bovine serum albumin (Sigma, USA) and 35 μL of phosphatase assay buffer (50 mM Tris–HCl, 0.1 mM CaCl₂, pH 7.4). These samples were incubated at 37 °C for 10 min. Then, 120 μL of 100 mM 6,8-difluoro-4-methylumbelliferyl phosphate (GIBCO, Molecular Probes) was added, and the samples were incubated at 37 °C for another 30 min. PP activity was analyzed using a fluorescence microplate reader at 355 nm (excitation) and 460 nm (emission). Three samples of rat pups from every dam in both groups were randomly chosen for this analysis.

MCLR content in the brains of rat pups was analyzed according to our previous study with minor modification [30,31]. Brains of ten neonatal rats from different dams in both groups were randomly chosen and analyzed. Briefly, lyophilized samples (0.2 g dry weight) were homogenized for MCLR analysis. Quantitative analysis of MCLR in the toxin-containing fraction was performed with a Finnigan LC-MS system comprising a thermo surveyor auto sampler, a surveyor MS pump, a surveyor mass spectrometer equipped with an atmospheric pressure interface ion source (LC-ESI-MS). The limit of detection for the MCLR was 0.01 μg mL⁻¹.

2.6. AChE activity

AChE activity in brains was detected using the spectrophotometric method reported by Ellman et al. [32] Homogenate (10%) of brains was prepared in 30 mM sodium phosphate buffer, pH 8.0, containing 1% Triton X 100 to release the membrane bound enzyme and it was centrifuged at 12,500 g for 30 min at 4 °C. The mercaptan formed as a result of the hydrolysis of the ester reacts with an oxidizing agent 5,5′-dithiobis (2-nitrobenzoate) read at 412 nm in Shimadzu UV2550 spectrophotometer. Three pups from every dam in both groups were randomly chosen for the analysis.

2.7. Proteome analysis

Protein extraction: Protein extraction was performed as described in our previous study with slight modification [33]. The procedure was carried out at 0–4 °C. Briefly, the frozen brain samples homogenized in the lysis buffer containing 2 M thiourea, 7 M urea, 50 mM DTT, 4% CHAPS, 50 mM Tris base, 0.2% Bio-Lytes 4/6, 1 mM protease inhibitor cocktail, 1% RNase and 1% DNase and the supernatant was obtained at 12,000 g for 1 h at 4 °C, then the supernatant was dialysed for desalination and freeze-dried. The protein powder was adjusted with a rehydration buffer for a final volume of 300 μL. Three pools for each group were prepared. Protein concentration was determined by the Bradford assay using BSA standards.

2-DE analysis: 450 μg of each protein sample was mixed with a rehydration buffer and then loaded onto IPG strips of linear pH gradient 4–7 17 cm (BioRad, Hercules, California, USA) in a PROTEAN IEF cell (BioRad, Hercules, California, USA) using the following program: 30 min at 250 V, 30 min at 1000 V, 5 h at 10,000 V constant for a total of 60,000 Vh. After the first dimension was run, each strip was equilibrated with about 6 mL equilibration buffer containing 6 M urea, 0.375 M Tris–HCl, pH 8.8, 20% glycerol, 2% SDS, 2% DTT for 15 min at room temperature, followed by the same buffer with 2.5% iodoacetamide (instead of DTT) for 15 min. Subsequently a 10% SDS-PAGE second dimension was performed. Electrophoresis was carried out at 15 mA/gel for 15 min, followed by about 7 h run at 25 mA/gel until the bromophenol blue front reached the edge of the gels. The gels were then visualized by silver strain plus kit (BioRad, Hercules, California, USA). Triplicate 2-DE gels were performed for each group.

Image capture and analysis: The gel images were captured on an Image Scanner GS-800 (BioRad, Hercules, California, USA). PDQuest software package (version 8.0, BioRad, Hercules, California, USA) software was used to match and analyze the images. Each set of gel replicates for both samples was combined into average gels, which represented spots that were reproducibly present on each set of the triplicate gels. The spots that were changed conspicuously in the control groups or exposure groups were then selected for further MS analysis. Only about two fold increase or decrease changes between the MCLR-treated and control groups were considered as regulated spots. The selected spots were analyzed with MALDI TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, USA). Mass spectra (m/z 800–4000 Da) were acquired in a positive ion mode. GPS explorer software (v3.6, default parameters, Applied Biosystems) was used to generate the peak lists for the database search against Swiss Prot 57.15 Rattus norvegicus protein database (515203 sequences) using Mascot search engine (v2.2). The identification parameters were set as follows: species, Rattus norvegicus; enzyme, trypsin; allow for one missed cleavage site; fixed modification, carbamidomethyl (C); variable modification, oxidation (M); peptide charge, 1+; monoisotopic; mass tolerance, ±50 ppm for the precursor ions and ±0.5 dalton for fragment ions. Mascot protein score is −10 × Log(P), where P is the probability that the observed match is a random event. Proteins were considered to be identified if the protein scores were greater than 51, indicating a probability (<5%) that the observed match is a random event.

Bioinformatics: The classification and functions of the proteins identified were obtained by searching Gene Ontology (http://www.geneontology.org) and then annotated with GO terms by Blast2GO software. Metabolic pathways were identified by using KEGG PATHWAY (http://www.genome.jp/kegg/pathway.html) and molecule annotation system (MAS 3.0) (http://www.bioinfo.capitalbio.com/mas3/).
3. Results

3.1. MCLR accumulation and PP activity in the brain of neonatal rats

Toxin content and PP activity in the brain of neonatal rats are shown in Fig. 1. No MCLR was detected in the control groups while MCLR enhanced toxin accumulation in MCLR exposed groups. The toxin contents were $3.75 \pm 0.94$ ng/g dry weight (DW) in brains after exposed MCLR. The ESI LC/MS$^2$ spectra for MCLR detection are shown in Fig. 1A, B. However, PP activity is shown in Fig. 2C, G.

3.2. Changes in brain ultrastructure in control and maternal MCLR-treated rat pups

TEM observation of control neonatal rat brains showed normal cerebrum and nerve cells with cytoplasm rich in rough endoplasmic reticulum and mitochondria (Fig. 2A, C, D). All microvessels consisted of a single layer of endothelial cells, forming a lumen, surrounded by a single layer of basement membrane. The basement membrane was surrounded by astrocyte, which separates the microvessels from neutrophil (Fig. 2A). Several erythrocytes were observed in the lumen of the microvessels. Astrocytes appeared in close contact to the microvessels. Organelles in all cells were well preserved and mitochondria in all cells showed a normal pattern of cristae. There was no swelling or vacuolization of the mitochondria. The nucleus membrane was conspicuous (Fig. 2C, G).

3.3. Measurement of lipid peroxidation, GSH content and AChE activity in the brain of neonatal rats

Level of LPO in brains of rat pups is shown in Fig. 3A. MDA is a well-known oxidation product of polyunsaturated fatty acids in lipoproteins and is often used as a biomarker of oxidative stress. The concentration of MDA, as a marker of LPO, significantly increased (206%) after maternal MC exposure. Fig. 3B also shows the level of the antioxidant substance GSH. GSH content was decreased 27.68% after maternal MC exposure. AChE activity in the cerebral cortex shows a significant decrease in the MCLR treated groups compared to the control groups, which presented in Fig. 3C.

3.4. Proteome analysis

Protein samples from the brain of control and maternal MCLR exposure were subjected to 2-DE. Quantitative spot comparisons were made with image analysis software. On average, more than 1400 proteins spots were detected in each gel using silver staining by the PDQuest software. A total of 49 protein spots were found to differ significantly in abundance ($\geq 2$-fold or $\leq 0.5$-fold; $P < 0.05$) in two groups (Fig. 4 and Table 1), 17 of which seemed to correspond to an up-regulation and 32 to a down-regulation significantly. These altered protein spots were excised for identification using MALDI-TOF-TOF MS. All of the protein spots were successfully identified with C.I. % (confidence interval %) values greater than 95% and the matched proteins came from the Swiss Prot database.

Metabolic pathways were identified by using KEGG PATHWAY (Fig. S1). Map of global KEGG metabolic pathway in rat showing significantly altered proteins in metabolic pathways. Of the identified proteins, three proteins were found to be related to the neurodevelopmental proteins and three were related to the oxidative phosphorylation; nine proteins were involved in protein translation, maturation and degradation. Nine proteins were implicated in metabolism. Four proteins were characterized as cytoskeleton proteins. Other seven proteins were categorized into signal transduction, regulation of transcription, endocytosis and other functions.

To gain information about the processes disrupted by MCLR, GO annotations and pathway analysis were performed with Blast2GO software and MAS 3.0 for each validated protein. The Blast2GO annotations of biological processes revealed several proteins implicated in system development, metabolic progress, catabolic progress, cellular progress and response to stimulus (Fig. 5A). At the function level, the target proteins were involved in protein binding, hydrolase activity,
purine ribonucleoside binding, purine ribonucleotide binding and purine ribonucleoside triphosphate binding.

Metabolic pathways were analyzed by using MAS 3.0. These MCLR-responsive target proteins were divided into five classes by bioinformatics analysis based on both the gene correlations and KEGG pathway analysis, which were involved in neurodevelopment, protein folding and degradation, metabolism, oxidative phosphorylation and cytoskeleton proteins (Fig. 5B).
neurotransmission in the central and peripheral nervous systems [37]. The duration of action of ACh at the synaptic clefts is critically dependent on AChE activity [38]. It has been demonstrated that ROS could cause dysfunction of the defensive antioxidant system of cells by altering enzyme activity and act as a factor for neurodegeneration [39]. Thus, the reduction of AChE activity in this study may be due to the ROS induced tissue damage and the decrease of AChE activity may eventually impair learning and memory function. Using the proteomic approach, we analyzed the brain protein expression profiles of rat pups. Data from the present study showed that maternal exposure to MCLR could alter 49 differentially expressed proteins and these proteins were assigned into several groups such as neurodevelopmental proteins, oxidative phosphorylation, protein translation, maturation and degradation, metabolism, cytoskeleton proteins and so on. Dihydropyrimidinase-related proteins (DRPs), which are intracellular proteins, are involved in the axonal outgrowth and path finding through the transmission and modulation of extracellular signals [40,41]. These DRPs are expressed mainly in fetal and neonatal brain, suggesting that the encoded proteins have a physiological role in the development of the central nervous system [42]. Since it was reported that mice with DRP-1/CRMP1 deficiency exhibited impaired spatial learning and memory [43], it is worth mentioning that learning and memory performances may be improved in response to spaceflight. Recently it was reported that DRP-2 protein was involved in neural development in bovine [44], suggesting a crucial role of this protein in promoting correct synaptogenesis and neuronal differentiation and migration processes [45]. Li et al. reported that the increased expression of DRP2 suggests a potential involvement of DRP2 in MC-induced neurotoxicity in zebrafish larvae [46]. However, in our study all the three DRPs were down expressed significantly in MCLR exposure groups compared with control brain. It may be concluded that the impairment in the developmental process of the nervous system and MCs may affect the ability of learning and memory of juvenile and adult in the future.

Three proteins (Atp5b, Ndufs1, Ndufa10) could be attributed to a altered oxidative phosphorylation, which are related to the neurodegenerative disease such as Parkinson’s disease and Alzheimer’s disease (AD). Previous studies have demonstrated that reduced ATP levels occurred in AD [47,48] and ATP-synthase (Atp5b) was an intracellular target for MCLR [49]. NADH [ubiquinone] dehydrogenase 1 alpha subcomplex subunit 10 (Ndufa10) and NADH-ubiquinone oxidoreductase 75 kDa subunit (Ndufs1) are subunits of NADH ubiquinone

4. Discussion

There have been many studies on the toxic effects of MCLR on aquatic organisms and human beings, but little effort has been devoted to exploring the neurotoxicity of MCLR, especially in progeny. The purpose of this study to investigate the molecular mechanism involved in the maternal transfer of MCs and by which neurotoxicity is triggered in rat pups. In this study, after 28 day (perinatal period) maternal MCLR exposure, MCLR was detected in the brains of rat pups indicating that brains of neonatal rats are under the risk of MCLR attack. Our result elucidates that MCLR could penetrate the placental barrier, indicating the public should pay more attention to MCs-induced hazards to the gravida.

After 28 day maternal MCLR exposure, MCLR content in the brains was 3.75 ± 0.94 ng/g DW. This is in line with studies that the levels of MCLR found in brain from aquatic animals (30.53 ng/g DW) and mammals (41.6 ng/g DW) [33,34]. The accumulation of MCLR had been reported to cause damage to the tissue of organism. Consistent with this, the ultrastructure of the cerebrum and levels of MDA, GSH and AChE activity were detected to be changed in MCLR-treated neonatal rats in our study. We found that the ultrastructure of cerebrum was conspicuously changed by MCLR which is in agreement with the prior findings [35,36], especially the endoplasmic reticulum conspicuously changed by MCLR which is in agreement with the prior findings [35,36], especially the endoplasmic reticulum and mitochondria which may play vital roles in the ER stress and oxidative stress. MCLR was found to inhibit antioxidant GSH level and MDA was increased which indicated that oxidative stress occurs in the brains. Meanwhile, we observed a significant decrease in AChE activity in the brain of MCLR exposure groups. AChE plays a major role in the Ach-cycle, including the release of ACh which is required for cholinergic neurotransmission in the central and peripheral nervous systems [37]. The duration of action of ACh at the synaptic clefts is critically dependent on AChE activity [38]. It has been demonstrated that ROS could cause dysfunction of the defensive antioxidant system of cells by altering enzyme activity and act as a factor for neurodegeneration [39]. Thus, the reduction of AChE activity in this study may be due to the ROS induced tissue damage and the decrease of AChE activity may eventually impair learning and memory function.

Using the proteomic approach, we analyzed the brain protein expression profiles of rat pups. Data from the present study showed that maternal exposure to MCLR could alter 49 differentially expressed proteins and these proteins were assigned into several groups such as neurodevelopmental proteins, oxidative phosphorylation, protein translation, maturation and degradation, metabolism, cytoskeleton proteins and so on. Dihydropyrimidinase-related proteins (DRPs), which are intracellular proteins, are involved in the axonal outgrowth and path finding through the transmission and modulation of extracellular signals [40,41]. These DRPs are expressed mainly in fetal and neonatal brain, suggesting that the encoded proteins have a physiological role in the development of the central nervous system [42]. Since it was reported that mice with DRP-1/CRMP1 deficiency exhibited impaired spatial learning and memory [43], it is worth mentioning that learning and memory performances may be improved in response to spaceflight. Recently it was reported that DRP-2 protein was involved in neural development in bovine [44], suggesting a crucial role of this protein in promoting correct synaptogenesis and neuronal differentiation and migration processes [45]. Li et al. reported that the increased expression of DRP2 suggests a potential involvement of DRP2 in MC-induced neurotoxicity in zebrafish larvae [46]. However, in our study all the three DRPs were down expressed significantly in MCLR exposure groups compared with control brain. It may be concluded that the impairment in the developmental process of the nervous system and MCs may affect the ability of learning and memory of juvenile and adult in the future.

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Fig. 2 - Changes of ultrastructure were in microvessels, endoplasmic reticulum, mitochondrion and nucleus of cerebrum in the maternal MCLR-treated rat pups. Representative electron photomicrographs were shown. Cerebral ultrastructure in normal rats (A, C, E, G). (A) Representative area of normal ultrastructure appearance of microvascular consists of endothelial cells and erythrocyte, surrounded by a single layer of basement membrane, astrocytes and neutrophil. (C, G) showed the normal endoplasmic reticulum and the evident nuclear membrane. The mitochondria were ovoid or spherical, and their cristae and matrix were clear in (E, G). Changes in cerebral ultrastructure in maternal MCLR-treated rat pups (B, D, F, H). (B) In the MCLR-treated rat pups, basement membrane of microvascular was slightly crushed and areas of edema are in contact with the microvascular basement membrane. (D) exhibited remarkable swollen endoplasmic reticulum in the cerebral cells. Swollen and vacuolated mitochondria can be observed in (F). (G) showed the formation of numerous large vacuoles and lysosomes in the cytoplasm. Meanwhile heterochromatin was obviously reduced and the nucleus membrane was inconspicuous. E—erythrocyte, En—endothelial cell, BM—basement membrane, Ast—astrocyte, ER—endoplasmic reticulum, NM—nucleus membrane, N—nucleus, M—mitochondrion, V—vacuole, L—lysosome.
oxidoreductase (complex I of the respiratory chain). Ndufa10, Ndufs1 and ATP synthase (complex V) all belong to the mitochondrial electron transport and oxidative phosphorylation system [50]. Recent reports have suggested that mitochondrial enzyme deficiencies contribute to the progression of the pathology of neurodegenerative diseases [49,51]. Regulation of Atp5b is considered to form part of oxidative stress responses intervening in mitochondrial membrane potential and permeability transition [52,53]. Oxidative stress is a common feature of many different forms of neurodegenerative disease and mitochondria are susceptible to be damaged by ROS [54]. In summary, our study indicated that MCLR is a new promoter for neurodegenerative disease, and the neurotoxicity of MCs is likely to be triggered by mitochondrial damage with impaired electron transport and obvious impairment of ATP production.

In the current study, we found that MCLR exposure increased the expression of nine proteins known to be related to protein translation, maturation and degradation (Table 1). Most of the proteins affected by MCs are involved in protein folding (Hsp90aa1, Hsp90ab1, Hspa8, Hspa13, Crkl, Pdia3, GRP78/Hspa5) and others are involved in protein translation (Eif3) or protein degradation (Psmd13). Hsp90aa1, Hsp90ab1, Hspa8, Hspa13, Crkl and Eif3h were down regulated, whereas GRP78, Pdia3 and Psmd13 were up regulated. Their variations could be explained by oxidative stress, which subsequently results in oxidation of proteins, eventually preventing their normal activity or leading to misfolding. HSP90 proteins play an important role in the folding of newly synthesized proteins and refolding denatured proteins after stress [55,56]. HSF70 proteins protected against apoptosis by inhibiting caspase cascade activation [57]. MCs have long been related to the induction of HSPs. Previous study also found that the expression of Hsp90b1 and Hspa9 was significantly up-regulated in the zebrafish larvae accompanied with MCLR-induced oxidative stress [46]. Eif3 is identified as the unique hypusine-containing protein, and it participates in the translational process [58]. The down-regulation of HSP90 proteins and Eif3h might contribute to reducing the new protein production for alleviating stress. In addition, the up-regulation of Psmd13, GRP78/Hspa5 and Pdia3 also responded to the cell stress. Psmd13 could be induced in multiple tissues of mouse including brain by exogenous chemical treatment and has a role in degradation of abnormal protein [59]. Pdia3 can act as molecular chaperones by inhibiting the aggregation of unfolded/misfolded proteins at the ER. Some studies showed that MCs could trigger oxidative stress and ER stress [60,61]. Subsequently, cellular mechanisms to diminish ER stress can be activated through up-regulation of molecular chaperones, which could enhance ER protein folding capacity and increase activity of ER-associated degradation pathways for the removal of misfolded proteins [62]. In this study, the noticeable dilatation of endoplasmic reticulum was observed in the brain, which is in line with the proteomic analysis that ER stress resulted in several proteins related to the MC stress. Thus, it is conceivable that the induction of various HSPs could be treated as markers of MC-induced oxidative stress and ER stress; the unfolded protein response triggered by which may play major roles in neurotoxicity of MCs.

Cytoskeleton disruption has been suggested as one of the first striking cytotoxicities caused by MCLR. Recently, it has been reported that MCLR triggered reorganization of microtubule and actin cytoskeleton components, leading to a loss of their filamentous distribution [35,36]. In agreement with the previous findings, our study demonstrated that MCLR could induce varied expression of the cytoskeleton and its associated proteins in rat brain, indicating that the neurotoxicity of MCLR is involved in disruption of neuronal cytoskeletal architecture. We found that abundances of four cytoskeleton proteins, α-tubulin, cytoplasmic dynein 1 intermediate chain

Fig. 3 – The MDA and GSH level (A, B) in brains after 10 μg/kg perinatal MCLR exposure for 28 days. (C) Acetylcholine esterase activity in the brain of control and MCLR groups of neonatal rats. The values are expressed as mean ± SD (N = 15), P < 0.05 (※).
2 and actin-related protein, and one actin-interacting protein calponin-3 were remarkably altered in exposed brains. Fu et al. showed that some cells lose microtubules after MC treatment [63]. Zhao et al. reported that the MCRR affects the normal microfilament network in testis [64]. Together with previous ultrastructure results, variations of cytoskeletal proteins indicated that MCLR caused cellular damage in the brain due to cytoskeletal disruptions.

Metabolic proteins in our studies had various expressions in response to maternal MCLR treatment. In this study, nine proteins involved in metabolism were identified. Serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PP1ca), aldehyde reductase AFAR2 subunit (Akr7a2), glutamine synthetase (Glul), pyruvate kinase 2 (Pkm2), alpha-enolase (Eno1), phosphoglycerate mutase 1 (Pgam1), and malate dehydrogenase (Mdh1) were up-regulated, whereas peroxiredoxin-4 (Prdx4) and dipeptidyl aminopeptidase-like protein 6 (Dpp6) were down-regulated in MCLR-treatment groups. Particularly, Pkm2, Eno1, Pgam1 and Mdh1, which are involved in glycolysis, were totally inducible by MCLR. Hereby, MCLR induced dysfunction of carbohydrate metabolism which is in accordance with a previous study that MCLR attack results in depletion of the hepatocyte.

Fig. 4 – Representative 2-DE gels of the proteins from the brains in the rat pups of the control and MCLR-treated groups. (A) 2-DE gel image with proteins expressed in the control condition; (B) 2-DE gel image with proteins expressed in the 10 μg/kg MCLR exposure condition. The proteins of the samples were separated by 2-DE and visualized by silver staining. Protein spots that were altered by MCLR exposure are labeled with characters. The molecular weights (MW) and pI scales are indicated. Each gel is representative of three independent replicates.
<table>
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<td>62499/6.64</td>
<td>1.9E-13</td>
<td>0.000005</td>
<td>167</td>
<td>1049</td>
<td>IFNL4PR</td>
<td>30%</td>
<td>Nervous system development, hydrolase activity</td>
</tr>
<tr>
<td>B1</td>
<td>53850628</td>
<td>NADH-ubiquinone oxidoreductase 75 kDa subunit (Ndufs1)</td>
<td>80331/5.65</td>
<td>0.000001</td>
<td>98</td>
<td>1442</td>
<td>FEAPLFNAR</td>
<td>20%</td>
<td>Oxidative phosphorylation</td>
<td></td>
</tr>
<tr>
<td>A21</td>
<td>54792127</td>
<td>ATP synthase subunit beta (Atp5b)</td>
<td>56318/5.19</td>
<td>1.9E-17</td>
<td>0.000001</td>
<td>206</td>
<td>1028</td>
<td>LVLEVAQHLGESTVR</td>
<td>36%</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>B5</td>
<td>170295834</td>
<td>NADH(ubiquinone) dehydrogenase 1 alpha subcomplex subunit 10 (Ndufa10)</td>
<td>40753/7.64</td>
<td>0.000001</td>
<td>30.447</td>
<td>107</td>
<td>698</td>
<td>LVLEVAQHLGESTVR</td>
<td>46%</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>A9</td>
<td>28467006</td>
<td>Heat shock protein HSP 90-alpha (Hsp90aa1)</td>
<td>85161/4.93</td>
<td>2.4E-24</td>
<td>0.2178</td>
<td>275</td>
<td>1245</td>
<td>GVDSDSPLN1SR</td>
<td>21%</td>
<td>Unfolded protein binding</td>
</tr>
<tr>
<td>A32</td>
<td>148747365</td>
<td>Heat shock protein HSP 90-beta (Hsp90ab1)</td>
<td>83571/4.97</td>
<td>2.4E-11</td>
<td>0.1228</td>
<td>248</td>
<td>1214</td>
<td>HSFVEIQLEQFR</td>
<td>25%</td>
<td>Unfolded protein binding</td>
</tr>
<tr>
<td>A17</td>
<td>56605658</td>
<td>Crk-like protein (Crkl)</td>
<td>33958/6.26</td>
<td>7.5E-13</td>
<td>0.4861</td>
<td>160</td>
<td>605</td>
<td>VSHYISLNNR</td>
<td>24%</td>
<td>Protein binding</td>
</tr>
<tr>
<td>A4</td>
<td>148747550</td>
<td>Heat shock 70 kDa protein 13 (Hsp13)</td>
<td>51820/5.43</td>
<td>0.00056</td>
<td>0.139</td>
<td>71</td>
<td>888</td>
<td>LPEDQILPDGDH</td>
<td>20%</td>
<td>Stress response, peptide-independent ATPase activity</td>
</tr>
<tr>
<td>A31</td>
<td>21312044</td>
<td>Eukaryotic translation initiation factor 3 subunit (Eif3h)</td>
<td>25356/4.81</td>
<td>2.4E-22</td>
<td>0.3883</td>
<td>258</td>
<td>1214</td>
<td>CMIDQAHQEERPIR</td>
<td>24%</td>
<td>Initiation of protein synthesis</td>
</tr>
<tr>
<td>A30</td>
<td>25742763</td>
<td>78 kDa glucose-regulated protein precursor (GRP 78/Hspa5)</td>
<td>72473/5.07</td>
<td>0.000004</td>
<td>2.2718</td>
<td>102</td>
<td>1244</td>
<td>SDDIEVLYGGSTR</td>
<td>18%</td>
<td>Cellular response to glucose starvation, anti-apoptosis</td>
</tr>
<tr>
<td>A14</td>
<td>21312044</td>
<td>26S Proteasome non-ATPase regulatory subunit 13 (Psmd13)</td>
<td>34726/6.06</td>
<td>0.0017</td>
<td>2.54427</td>
<td>70</td>
<td>583</td>
<td>VVIDAR</td>
<td>24%</td>
<td>Degradation of ubiquitinated proteins</td>
</tr>
<tr>
<td>A19</td>
<td>8393322</td>
<td>Protein disulfide-isomerase A3 (Pdia3)</td>
<td>57044/5.88</td>
<td>4.7E-09</td>
<td>2.6404</td>
<td>122</td>
<td>931</td>
<td>ILNDFISYLRQ</td>
<td>11%</td>
<td>Protein disulfide isomerase activity</td>
</tr>
<tr>
<td>A13</td>
<td>26758274</td>
<td>Peroxiredoxin-4 precursor (Prdx4)</td>
<td>31216/6.18</td>
<td>1.2E-21</td>
<td>0.2893</td>
<td>248</td>
<td>535</td>
<td>DYGVYLEDGSHTLR</td>
<td>42%</td>
<td>Oxidoreductase activity, antioxidant protein</td>
</tr>
<tr>
<td>A31</td>
<td>12408298</td>
<td>Dipeptidyl aminopeptidase-like protein 60 (Peppl)</td>
<td>97925/5.95</td>
<td>0.019</td>
<td>0.000001</td>
<td>26</td>
<td>1675</td>
<td>SI1GFVFCR</td>
<td>1%</td>
<td>Protein synthesis</td>
</tr>
<tr>
<td>B8</td>
<td>13928710</td>
<td>Serine/threonine-protein phosphatase 1P1-alpha catalytic subunit (PP1ca)</td>
<td>38829/5.94</td>
<td>3.8E-31</td>
<td>2.26838</td>
<td>343</td>
<td>689</td>
<td>ICGDHQHYQYDRLR</td>
<td>58%</td>
<td>Protein amino acid dephosphorylation</td>
</tr>
<tr>
<td>Spot ID</td>
<td>Accession number (gi)</td>
<td>Protein identity</td>
<td>MW/pI Expectation value</td>
<td>Fold change</td>
<td>MASCOT score</td>
<td>Total ions</td>
<td>SC Function category</td>
<td>Other functions</td>
<td></td>
<td></td>
</tr>
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<td>---------</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>B9 19705537</td>
<td>Aldehyde reductase AFAR2 subunit (Akr7a2)</td>
<td>41105/8005</td>
<td>6E – 15</td>
<td>2.90886</td>
<td>181</td>
<td>672</td>
<td>QVETELFPCLR 32%</td>
<td>Oxidation reduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7 142346512</td>
<td>Glutamine synthetase (GluI)</td>
<td>42982/6.64</td>
<td>7.5E – 11</td>
<td>2.41005</td>
<td>140</td>
<td>800</td>
<td>DIVEAHYR 31%</td>
<td>Glutamine biosynthesis, positive regulation of synaptic transmission, Glycolysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3 16757994</td>
<td>Pyruvate kinase 2 (Pkm2)</td>
<td>58294/6.63</td>
<td>7.5E – 17</td>
<td>2.06025</td>
<td>200</td>
<td>3043</td>
<td>EEAHAVTHR 25%</td>
<td>Glycolysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B13 158186649</td>
<td>Alpha- enolase (Eno1)</td>
<td>47440/6.16</td>
<td>6E – 11</td>
<td>2.05815</td>
<td>141</td>
<td>858</td>
<td>AGYTDQVVI GMDVAASEFYR VILAAHGNLR 35%</td>
<td>Glucose metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B16 114326546</td>
<td>Phosphoglycerate mutase 1 (Pgam1)</td>
<td>28928/6.67</td>
<td>3E – 12</td>
<td>2.24661</td>
<td>153</td>
<td>493</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>B17 15100179</td>
<td>Malate dehydrogenase (Mdh1)</td>
<td>36631/6.16</td>
<td>0.000017</td>
<td>2.06762</td>
<td>86</td>
<td>619</td>
<td>FVEGLPINDFSR 11%</td>
<td>L-malate dehydrogenase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoskeleton A2 396941662</td>
<td>Cytoplasmic dynein 1 intermediate chain 2 isoform 1(Dync1i2)</td>
<td>71533/5.11</td>
<td>0.0065</td>
<td>0.00001 C</td>
<td>61</td>
<td>1165</td>
<td>EIAVGSDEQQVI YDGGEQIAVPR 7%</td>
<td>Cytoskeleton</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A15 269315877</td>
<td>Actin-related protein 6 (Actr6)</td>
<td>47317/5.48</td>
<td>2.4E – 17</td>
<td>0.000001 C</td>
<td>205</td>
<td>868</td>
<td>IFHIDTNALHVP 30%</td>
<td>Cytoskeleton</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A18 58865558</td>
<td>Tubulin alpha-1C chain (Tuba1c)</td>
<td>50590/4.96</td>
<td>4.7E – 17</td>
<td>0.0463</td>
<td>202</td>
<td>859</td>
<td>AVFIDQTEVDIEVR 23%</td>
<td>Cytoskeleton, protein polymerization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A20 58865557</td>
<td>Tubulin alpha-1C chain (Tuba1c)</td>
<td>50590/4.96</td>
<td>3.8E – 21</td>
<td>0.4831</td>
<td>243</td>
<td>859</td>
<td>AVFIDQTEVDIEVR 29%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A25 58865557</td>
<td>Tubulin alpha-1C chain (Tuba1c)</td>
<td>50590/4.96</td>
<td>0.00000022</td>
<td>0.3771</td>
<td>95</td>
<td>859</td>
<td>AVFIDQTEVDIEVR 23%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A26 58865557</td>
<td>Tubulin alpha-1C chain (Tuba1c)</td>
<td>50590/4.96</td>
<td>0.00000022</td>
<td>0.3771</td>
<td>95</td>
<td>859</td>
<td>AVFIDQTEVDIEVR 23%</td>
<td></td>
<td></td>
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<tr>
<td>B6 9506501</td>
<td>Calponin-3(Cnn3)</td>
<td>35638/5.47</td>
<td>0.018</td>
<td>2.1713</td>
<td>56</td>
<td>676</td>
<td>YDKQAEELDR 19%</td>
<td>Calmodulin binding</td>
<td></td>
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<tr>
<td>Other functions A22 158138568</td>
<td>Serum albumin precursor (Alb)</td>
<td>70682/6.09</td>
<td>3.8E – 18</td>
<td>0.1801</td>
<td>213</td>
<td>1250</td>
<td>DVFLGTFLYEYSR 25%</td>
<td>Maintenance of mitochondrial localization, Regulation of synaptic plasticity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A23 9507245</td>
<td>14-3-3 protein gamma (Ywhag)</td>
<td>28456/4.8</td>
<td>3E – 11</td>
<td>0.0033</td>
<td>144</td>
<td>448</td>
<td>EHMQPTHPIR 19%</td>
<td>Signal transduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A29 9507245</td>
<td>14-3-3 protein gamma (Ywhag)</td>
<td>28456/4.8</td>
<td>0.00001</td>
<td>0.00001 C</td>
<td>64</td>
<td>468</td>
<td>EHMQPTHPIR 15%</td>
<td></td>
<td></td>
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<tr>
<td>B1 62078997</td>
<td>WD repeat-containing protein 1 (Wdr1)</td>
<td>66824/6.15</td>
<td>0.00013</td>
<td>0.26202</td>
<td>78</td>
<td>1252</td>
<td>VFAFLQFVR 12%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B15 6968499</td>
<td>Acidic leucine-rich nuclear phosphophoryn 32 family member A(Anp32a)</td>
<td>28718/3.99</td>
<td>2.4E – 16</td>
<td>2.98243</td>
<td>195</td>
<td>288</td>
<td>SLDFNCVSNLAYR 19%</td>
<td>Regulation of transcription</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B10 155369650</td>
<td>Myosin light polypeptide 6 (Myl6)</td>
<td>17135/4.46</td>
<td>0.047</td>
<td>2.69037</td>
<td>52</td>
<td>308</td>
<td>VLGPNKSDMN 37%</td>
<td>Actin-dependent ATPase activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other functions B12 11693142</td>
<td>Proliferating cell nuclear antigen (Pcna)</td>
<td>29072/4.57</td>
<td>0.00000019</td>
<td>2.36419</td>
<td>106</td>
<td>513</td>
<td>SEGFTYR 24%</td>
<td>DNA polymerase processivity factor activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B4 350534044</td>
<td>Stabilin-2 precursor(Stab2)</td>
<td>161856/7.28</td>
<td>0.058</td>
<td>2.4708</td>
<td>25</td>
<td>3888</td>
<td>ALASDLPR 1%</td>
<td>Endocytosis</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* The fold changes (mean values ± SD, n = 3) are indicated as compared to the controls. Only the fold changes (≥2-fold or ≤0.5-fold) are shown with their corresponding spot on the other gel. Values > 1 indicate up-regulations, and < 1 indicate down-regulations.

b SC indicates the sequence coverage of the protein in percentage obtained by MS/MS identification.

c 0.000001, the spot disappeared in the MCLR exposed group.
Fig. 5 – Distribution of GO annotations of biological processes and molecular function by Blast2GO for identified proteins. (A) Multi-level pie chart of combined graph of GO biological process annotations with a cut-off of seven proteins per annotation (if fewer than seven proteins, then it is not displayed). (B) Multi-level pie chart of combined graph of GO molecular function annotations with a cut-off of five proteins per annotation (if fewer than five proteins, then it is not displayed). (C) The network of proteins shown to be altered by MCLR was divided into five classes based on both KEGG and GenMAPP pathways analysis: neurodevelopmental-related proteins; translation, maturation and degradation proteins; metabolism proteins; oxidative phosphorylation proteins; cytoskeleton proteins.
Fig. 6 – Representative 2-DE and Western blot analysis. (A) The magnified images of protein spots from the 2-DE gels are shown in the upper part and the gel pictures presented here were from two independent experiments \(n = 3\). (B) Western blot showed that equal protein amounts of brains from control groups and MCLR exposure groups were separated by gel electrophoresis and immunoblotted with antibodies against tubulin alpha 1C chain, dihydropyrimidinase-related 2, glutamine synthetase and calponin-3. One membrane out of the three independent replicates is presented. GAPDH was used as internal loading control. (C) The line charts below show the protein levels based Western blot results. The values represent the average fold changes \(P < 0.05\). The values of protein abundance are the average %Vol of spots in three replicated gels.
glycogen content and increasing demand for energy with an incidental cellular metabolic exhaustion [36]. In addition, glucose which supplies energy is essential for the maintenance of nervous system [65]. Glucose metabolism is critical to help cells have a better defense response against oxidative stress and mitochondrial damage. Furthermore, the expression of Akr7a2 and Glul that are related to the detoxification is greatly changed after MCLR exposure. Akr7a2 is likely to metabolize genotoxic ketones and aldehydes from endogenous metabolic pathways, such as MDA [66]. Glul participates in ammonia detoxification, maintaining amino acid balance, nucleotide biosynthesis and regulation of neurotransmitters [67,68]. Additionally, Prdx4, belonging to a ubiquitous family of antioxidant proteins, acts as antioxidants and catalyzes elimination of hydrogen peroxides and other reactive oxygen species with the help of reducing systems [69]. Therefore, it is reasonable to draw the conclusion that the down-regulation of Prdx4 combined with the decreased level of GSH level indicated that MCLR induced oxidative stress in the brain. Overall, the
MCLR-induced metabolism change in brains was supposed to be an indirect evidence of neurotoxicity due to MCLR attack. Other special proteins seem also to be disturbed by MCLR. 14-3-3 protein is a family of serine/threonine-binding proteins that have anti-apoptotic functions and exert their anti-apoptotic activity through the Bad protein, a proapoptotic member of the Bcl-2 family [70,71]. The down-regulation of 14-3-3 protein may lead to activation of the apoptotic pathway. Acidic leucine-rich nuclear phosphoprotein 32 family member A (Anp32a), an inhibitor of protein phosphatase 2A (PP2A), is a novel molecular target of sphingoid bases which could regulate cellular signaling events and inflammatory gene expression [72]. So the overexpression of this protein was a defense of the brain cell after MCLR attack. Proliferating cell nuclear antigen (PCNA) has been identical to cyclin and it is a nuclear factor involved in DNA replication and repair of proliferating cells. PCNA can associate with several procaspases to block their activation for DNA replication and repair of proliferating cells. 14-3-3 protein may lead to activation of the apoptotic pathway. Acidic leucine-rich nuclear phosphoprotein 32 family member A (Anp32a), an inhibitor of protein phosphatase 2A (PP2A), is a novel molecular target of sphingoid bases which could regulate cellular signaling events and inflammatory gene expression [72]. So the overexpression of this protein was a defense of the brain cell after MCLR attack. Proliferating cell nuclear antigen (PCNA) has been identical to cyclin and it is a nuclear factor involved in DNA replication and repair of proliferating cells. PCNA can associate with several procaspases to block their activation for presenting anti-apoptotic activities [73,74]. These reports and the present results altogether suggest that MCs may induce apoptosis and inflammatory in the brain [12,13].

5. Conclusions

In summary, all the results supported that prenatal transfer of MCLR induced neurotoxicity in rat offspring and its mechanisms refer to the disruption of cytoskeleton, oxidative stress and ER stress in the brain. In addition, protein profiles revealed that MCLR neurotoxicity may also be induced by the variances of neurodevelopment-related proteins and macromolecule metabolism with a concomitant interference with protein translation, maturation and degradation. Especially, MCLR affected the process of protein folding and degradation. However, more future works are needed to focus on neurotoxicity of the gravida and the progeny.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.11.015.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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