



Effects of acute exposure to microcystins on hypothalamic-pituitary-adrenal (HPA), -gonad (HPG) and -thyroid (HPT) axes of female rats

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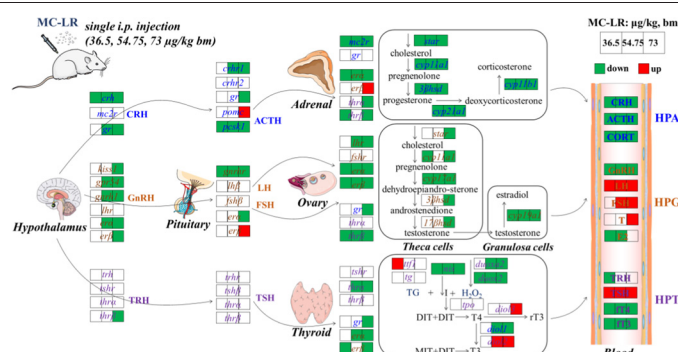
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HIGHLIGHTS

- Effects of MC-LR on the endocrine system of female rats were studied.
- MCs caused pathological damage to hypothalamus, pituitary, adrenal, ovary, thyroid.
- MC-LR changed concentrations of hormones of HPA, HPG and HPT axes in serum.
- MC-LR affected transcription of genes for hormones synthesis along the axes.
- Effects of MCs on interaction of endocrine axes need to be clarified.

GRAPHICAL ABSTRACT



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ABSTRACT

Microcystins (MCs) are common, well-known cyanobacterial toxins that can affect health of humans. Recently, it has been reported that MCs affect endocrine functions. In the present study, for the first time, histopathology, concentrations of hormones and transcription of genes along the hypothalamic-pituitary-adrenal (HPA), hypothalamic-pituitary-gonad (HPG) and hypothalamic-pituitary-thyroid (HPT) axes were examined in rats exposed to microcystin-LR (MC-LR). Female, Sprague-Dawley (SD) rats were exposed acutely to MC-LR by a single intraperitoneal (i.p.) injection at doses of 0.5, 0.75, or 1 median lethal dose (LD₅₀), i.e. 36.5, 54.75, or 73 µg MC-LR/kg body mass (bm) then euthanized 24 hours after exposure. Acute exposure to MC-LR significantly increased relative mass of adrenal in a dose-dependent manner, but relative mass of hypothalamus, pituitary, ovary and thyroid were not significantly different from respective mass in controls. However, damage to all these tissues was observed by histology. Along the HPA axis, lesser concentrations of corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and corticosterone (CORT) were observed in blood serum of

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exposed individuals, relative to controls. For the HPG axis, concentrations of gonadotropin-releasing hormone (GnRH) and estradiol (E2) were significantly less in rats treated with MC-LR, but greater concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone (T) were observed. Along the HPT axis, MC-LR caused greater concentrations of thyroid-stimulating hormone (TSH), but lesser concentrations of thyrotropin-releasing hormone (TRH), free tetra-iodothyronine (fT4) and tri-iodothyronine (fT3). Significant positive/negative correlations of concentrations of hormones were observed among the HPA, HPG and HPT axes. In addition, profiles of transcription of genes for synthesis of hormones along the endocrine axes and nuclear hormone receptors in adrenal, ovary and thyroid were significantly altered. Therefore, these results suggested that MC-LR affected HPA, HPG and HPT axes and exerted endocrine-disrupting effects. Effects of MC-LR on crosstalk among these three axes need further studies.

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1. Introduction

Recently, due to the combined/interactive effects of eutrophication and changes in climate, cyanobacterial blooms and associated cyanotoxin contamination have been increasing worldwide (Carmichael and Boyer, 2016; Svirčev et al., 2019). These blooms pose risks to ecosystems and health of humans. One of the most common bloom-forming cyanobacteria in freshwater ecosystems is *Microcystis aeruginosa* and it is notorious for production of toxic microcystins (MCs), which were originally referred to as “Fast-Death Factor” (Hughes et al., 1958; Konst et al., 1965; Carmichael et al., 1988; Harke et al., 2016). MCs are a group of cyclic heptapeptides, with the general structure of cyclo-(D-Ala1-L-X2-D-Masp3-L-Z4-Adda5-D-γ-Glu6-Mdha7), in which amino acid residues 2 (X) and 4 (Z) are variable L-amino acids. Until now, more than 279 MCs have been described (Bouaïcha et al., 2019; Chen et al., 2021). Among them, due to their toxicity, ubiquity and prevalence, MC-LR, which contains leucine (L) at position 2 and arginine (R) at position 4, respectively, is the most widely studied (Wu et al., 2006; Loftin et al., 2016; Mantzouki et al., 2018).

Numerous cases of lethal poisonings related to hazardous cyanobacterial blooms and production of MCs have been documented worldwide (Hilborn and Beasley, 2015; Backer and Miller, 2016; Wood, 2016). Exposure to MCs occurs mainly through three routes: direct contact, oral ingestion and inhalation (Meneely and Elliott, 2013; Lee et al., 2017; Massey et al., 2018; Chen et al., 2021). In 1996, exposure to MCs through hemodialysis occurred in Caruaru, Brazil (Azevedo et al., 2002). After dialysis with inadequately treated water from the Tabocas Reservoir, most patients exhibited acute neuro-toxicity and sub-acute hepato-toxicity, and the “Caruaru Syndrome” caused 52 deaths. The main modes of action and toxic mechanisms of MCs are inhibition of protein phosphatases (PPs) and induction of oxidative stress (Chen et al., 2016a; Chen and Xie, 2016). MCs can also cause various effects at the cellular level, including damage to DNA (genotoxicity), disruption of cytoskeletal architecture, dysfunction of mitochondria, endoplasmic reticulum stress (ERS) and de-regulation of the cell cycle, all of which can result in autophagy, apoptosis, programmed cell death or necrosis (Campos and Vasconcelos, 2010; Žegura et al., 2011; Chen et al., 2016a; Chen and Xie, 2016; Máthé et al., 2016; Valério et al., 2016; Žegura, 2016; AlKahtane et al., 2020).

For over two decades and more recently, increasing studies have reported endocrine-disrupting effects of *Microcystis* and MCs (Bury et al., 1996; Li et al., 2008a,b, 2020; Oziol and Bouaïcha, 2010; Rogers et al., 2011; Štěpánková et al., 2011; Sychrová et al., 2012; Jia et al., 2014, 2018; Chen et al., 2016a, 2020; Hou et al., 2016, 2017, 2018; Zhao et al., 2016a,b; Zhang et al., 2019; Al-Badrani et al., 2020; Mallia et al., 2020; Wang et al., 2020b; Pan et al., 2021; Table 1). In vertebrates, the endocrine system includes hypothalamic-pituitary-adrenal/interrenal (for fish) (HPA/HPI), hypothalamic-pituitary-gonad (HPG) and hypothalamic-pituitary-thyroid (HPT) axes (Brüggemann et al., 2018). Each axis regulates different processes via axis-specific hormones. The HPA axis mainly controls bodily responses to stress by glucocorticoids, cortisol or corticosterone (CORT), while the HPG axis coordinates

reproduction by steroid hormones, and the HPT axis regulates energy metabolism and development by thyroid hormones (THs).

Several studies have investigated effects of MC-LR on the HPG axis in female and male fishes (Table 1), with sex-dependent effects being observed (Qiao et al., 2013; Liu et al., 2016a; Cheng et al., 2018; Lin et al., 2020). However, compared with well-clarified disruption of the HPG axis and reproduction by exposure to MC-LR in male rats (Li et al., 2008b; Chen et al., 2013; Wang et al., 2016) and male mice (Chen et al., 2011; Wang et al., 2012, 2018; Xiong et al., 2014; Ding et al., 2018; Jin et al., 2021), only several studies focused on female mice (Wu et al., 2014, 2015). Also, MC-induced HPT axis and thyroid dysfunction have been mostly studied in fish (Table 1), while the evidence of effects on this axis in mammals is limited (Zhao et al., 2015b; Chen et al., 2019). Until now, there have been no studies concerning stress and HPA axis in mammals in response to exposure to MCs, and only several studies have been carried out in fish (Li et al., 2008b; Chen et al., 2018b; Wang et al., 2020a). Therefore, a comprehensive evaluation of endocrine-disrupting effects of MCs on the HPA, HPG and HPT axes in mammals especially for females was thus needed.

In this study, for the first time, a comprehensive study was performed to investigate the effects of MC-LR on the HPA, HPG, and HPT axes in female rats. Female Sprague-Dawley (SD) rats were exposed to MC-LR by single intraperitoneal (i.p.) injections, and median lethal dose (LD₅₀) was calculated by the up and down method from the mortality rate observed 24 h after the administration. Then, rats were exposed to single i.p. injections of a range of concentrations of MC-LR and samples were collected after 24 h. Histopathology of hypothalamus, pituitary, adrenal, ovary, and thyroid were analyzed. In addition, concentrations of hormones in serum and gene expressions of HPA, HPG, and HPT axes were measured. Results of the present study provide a comprehensive perspective to understand the endocrine-disrupting effects of MCs.

2. Materials and methods

2.1. Chemicals and reagents

MC-LR with purity of over 95% was bought from Taiwan Algal Science Inc. (Taiwan, China). Other chemicals and reagents used in this study were of analytical or higher grades.

2.2. Animals

Healthy, female, Specific Pathogen Free (SPF), Sprague-Dawley (SD) rats, about 6–7 weeks old, 180–200 g, were obtained from Hubei Laboratory Animal Research Center of Hubei Province, China. Rats were kept as previously described (Chen et al., 2013, 2016b) with small modifications. Rats were held at 22–25 °C and 50–65% relative humidity with a regime of 12 h light/dark in stainless steel cages that contained wood chips as bedding. Rats were given free access to de-chlorinated tap water and standard rodent pellet food (GB 14924.3-2010). All

Table 1

Summary of studies of effects of *Microcystis* and MCs on hormones along the hypothalamic-pituitary-gonad (HPG), -thyroid (HPT) and -adrenal/-interrenal (for fish) (HPA/HPI) axes in fishes and mammals.

	HPG	HPT	HPA/HPI
Fish (♂)	Qiao et al., 2013; Liu et al., 2016a; Su et al., 2016; Chen et al., 2017a; Cheng et al., 2018; Hou et al., 2018; Lin et al., 2018, 2020	Liu et al., 2016b; Cheng et al., 2017; Gao et al., 2020	Wang et al., 2020a
Fish (♀)	Qiao et al., 2013; Zhao et al., 2015a; Liu et al., 2016a, 2018; Hou et al., 2016; Cheng et al., 2018; Kawan et al., 2019; Lin et al., 2020	Liu et al., 2016b; Cheng et al., 2017; Gao et al., 2020	No studies
Fish (adult, sex not analyzed)	No studies	Li et al., 2008a	Li et al., 2008a
Fish (embryo/larvae, sex not differentiated)	No studies	Yan et al., 2012; Xie et al., 2015; Cheng et al., 2017; Zuo et al., 2020	Chen et al., 2018b
Fish (juvenile, during sex differentiation)	No studies	Liu et al., 2015a,b; Hu et al., 2020	Bury et al., 1996
Mammals (♂)	Li et al., 2008b; Chen et al., 2011, 2013; Wang et al., 2012, 2016, 2018; Xiong et al., 2014; Zhang et al., 2017; Ding et al., 2018; Jin et al., 2021	Zhao et al., 2015b	No studies
Mammals (♀)	Wu et al., 2014, 2015	Chen et al., 2019	No studies

procedures performed on rats were approved by the Institutional Animal Care and Use Committee (IACUC).

2.3. Estimation of median lethal dose (LD_{50})

After acclimation for a week, acute LD_{50} for the intraperitoneal (i.p.) route was determined by Dixon's up and down method (Dixon, 1965). All animals were fasted overnight, about 12 hours (h), before administration of a single i.p. injection of MC-LR dissolved in saline, 0.9% NaCl solution. Values of LD_{50} and 95% confidence interval (CI) were calculated from the mortality rate observed 24 h after the administration. The range of doses was chosen according to results of previous studies (Li et al., 2015; Chen et al., 2016b).

2.4. MC-LR exposure

After acclimation for a week, rats were randomly assigned to four groups, a vehicle control and three exposed groups. Rats were administered with MC-LR at doses of 50%, 75% or 100% of the LD_{50} by single i.p. injections, which corresponded to 36.5, 54.75 or 73 μ g MC-LR/kg body mass (bm), respectively. The control rats were injected with the same volume of vehicle, i.e. saline solution (0.9% NaCl).

24 h after exposure, rats were euthanized by isoflurane inhalation before final collection of blood and organs. There were five rats in each group ($n = 5$). Samples of blood were collected by retro-orbital bleeding following anesthesia by isoflurane. The blood was allowed to clot, and serum was isolated as the supernatant after 10 min of centrifugation at 3000 g. The serum was frozen at -80°C until analysis of hormones. Hypothalamus, pituitary gland, adrenal gland, ovary, and thyroid from individual animals were collected, washed, weighed, and fixed with 4% paraformaldehyde for histological observations. Indices of relative mass of organs were calculated according to each organ/body mass (Eq. (1)).

$$\text{Index} = \text{organ mass/body mass} \times 100\% \quad (1)$$

2.5. Histopathology

Histopathological analyses were conducted as previously described (Chen et al., 2013, 2017b). In Brief, individual fixed tissues in paraformaldehyde were dehydrated and embedded in paraffin blocks and cut with a microtome to yield sections of 4- μ m thickness. These sections were routinely processed by staining with hematoxylin and eosin (H&E) and mounted on glass slides. The slides were observed under a light microscope for micro-structure of tissues (400 \times).

2.6. Quantification of hormones in serum

Concentrations of corticosterone (corticotropin-releasing hormone, CRH; corticotropin-releasing factor, CRF), corticotropin (adrenocorticotropic hormone, ACTH), 17-deoxycortisol (corticosterone, CORT), gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E2), testosterone (T), thyroliberin (thyrotropin-releasing hormone, TRH; thyrotropin-releasing factor, TRF), thyrotropin (thyroid-stimulating hormone, TSH), free thyroxine (free tetra-iodothyronine, fT4) and free tri-iodothyronine (fT3) were determined by using the commercially available kits with comparison to external standards (Elabscience Biotechnology Co., Ltd., Wuhan, China). CRH, ACTH, CORT, GnRH, T, E2, TRH, fT4 and fT3 were determined using competitive ELISA and LH, FSH and TSH were determined using sandwich ELISA. The minimum detectable concentration (MDC) levels for hormones are as follows: 0.19 ng/mL for CRH, 9.38 pg/mL for ACTH, 0.24 ng/mL for CORT, 9.38 pg/mL for GnRH, 0.94 mIU/mL for LH, 1.88 ng/mL for FSH, 0.17 ng/mL for T, 1 pg/mL for E2, 4.69 pg/mL for TRH, 0.75 ng/mL for TSH and 0.94 pg/mL for fT4 and fT3. If necessary, some samples were diluted to meet the range of standard curves.

2.7. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Expressions of mRNA for genes from rat tissues were measured by use of qRT-PCR, following previous laboratory protocols (Chen et al., 2017b, 2018b). Tissues were homogenized and centrifuged to remove extra tissue particles or debris. Total RNA was isolated from approximately 30 mg of sample, by use of the E.Z.N.A. Total RNA Kit I following the manufacturer's standard protocol (Omega Bio-tek, Inc., Georgia, USA). Concentrations of total RNA were determined by measuring reading value of the absorbance at 260 nm (A260) by use of a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA) and purity of RNA was assessed by the A260/280 ratios with values between 1.8 and 2.1. Genomic DNA (gDNA) was removed with gDNA Eraser Buffer and complementary DNA (cDNA) was synthesized with oligo dT primer and random 6 mers from 1 μ g of total RNA using PrimeScriptTM RT reagent Kit with gDNA Eraser (Takara Bio Inc., Kusatsu, Japan).

qRT-PCR was performed with the gene-specific primers using LightCycler[®] 480 SYBR Green I Master (Roche Diagnostics, Indianapolis, USA) on a Bio-Rad CFX96 Real-Time System (Bio-Rad, USA). Sequences for rat-specific primers used for real time PCR are provided in Supplementary Table 1. The thermal cycle was set as follows: pre-denaturation at 95 $^{\circ}\text{C}$ for 2 min, followed by 40 cycles of denaturation at 95 $^{\circ}\text{C}$ for 10 s, annealing at 62 $^{\circ}\text{C}$ for 15 s, and elongation at

72 °C for 30 s. Melt curve analysis of amplification products was performed to confirm generation of a single PCR product and specificity of primers. Each sample was run in 3 tubes, and PCR reactions with deionized water instead of the addition of the template were used as blanks. Threshold Cycle (Ct) values for the selected genes were normalized against the housekeeping gene *gapdh* (internal control) values in the same sample. The relative fold change was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.8. Statistical analyses

SPSS statistics 19 (SPSS Inc., Chicago, Ltd., USA) was used for statistical analyses. All data are presented as mean \pm standard error (SE). The Kolmogorov-Smirnov test was used to check for normality of data distributions and Levene's test was used to determine homogeneity of variances. Data were $\text{Log}_{10}(X)$ transformed to approximate normality if necessary. If data could not meet the normality even after Log-transformation, non-parametric analysis was performed. Statistically significant differences between treatments and the corresponding control were analyzed by one-way analysis of variance (ANOVA) and Dunnett's test. Pearson correlation analysis was used to determine the relationship between concentrations of hormones after exposure to MC-LR. Significant differences between were identified by p -value < 0.05 (*), < 0.01 (**) or < 0.001 (***)

3. Results

3.1. Median lethal dose (LD_{50}) of MC-LR

Doses of intraperitoneal (i.p.) injection of MC-LR administered to female Sprague-Dawley (SD) rats by up and down method and the outcome observed at 24 h are shown in Table 2. Clinical signs included hypoactivity, lethargy, prostration, piloerection, convulsions, and slow respiration in rats exposed up to 73 $\mu\text{g}/\text{kg}$, body mass (bm), and these signs began several hours following dosing and increased in severity until death. The LD_{50} of MC-LR to rats was 73 $\mu\text{g}/\text{kg}$, body mass (bm), with 95% confidence interval (CI) of 46.98–103 $\mu\text{g}/\text{kg}$, bm. Based on these results, 36.5, 54.75, 73 $\mu\text{g}/\text{kg}$, bm, i.e. 0.5, 0.75, or 1 LD_{50} , were selected for subsequent exposures.

3.2. Indexes of relative mass of organs

There were no significant differences in relative mass of hypothalamus, pituitary, ovary or thyroid between control and rats treated with MC-LR (Fig. 1). However, exposure to MC-LR resulted in significant increased relative mass of adrenal.

3.3. Histopathological observations

3.3.1. Hypothalamus

In hypothalamuses of control females, neurons exhibited clear structures and consistent morphology (Fig. 2). Occasional shrinkage of neurons and darkened staining of cells were observed in hypothalamuses

Table 2

Procedure on up and down methods for determination of median lethal dose (LD_{50}) by intraperitoneal (i.p.) injection and outcome of female rats at 24 h after exposure.

Animal ID	Dose ($\mu\text{g}/\text{kg}$, body mass)	Outcome
1	55	Survival
2	73	Survival
3	97	Death
4	73	Death
5	55	Survival
6	73	Death

of rats exposed to 54.75 and 73 μg MC-LR/kg bm, and the boundary was unclear between nucleus and cytoplasm.

3.3.2. Pituitary

In pituitary glands of controls, cells were densely packed, with abundant sinusoidal capillaries and a small amount of connective tissue. The structure of basophil, eosinophil and chromophobe cells were clear and the morphology was uniform. In rats exposed to 54.75 and 73 μg MC-LR/kg bm, some parenchymal cells lost cytoplasm, and fragmentation and lysis of nuclei were observed in eosinophilic cells. Telangiectasis was observed in rats exposed to 73 μg MC-LR/kg bm.

3.3.3. Adrenal

In controls, adrenal glands were divided into zona glomerular, zona fasciculata and zona reticularis, and the zonal band cells were arranged in bundles. Cytoplasmic loss of adrenocortical cells was observed in rats exposed to 36.5 μg MC-LR/kg bm. Edema and hyperemia were observed in rats exposed to 54.75 $\mu\text{g}/\text{kg}$ bm. Rats exposed to 73 $\mu\text{g}/\text{kg}$ bm exhibited frank pathologic changes, including hyperemia, diffuse vacuolar degeneration, nuclear lysis and loss.

3.3.4. Ovary

The ovary in the control group showed abundant granulosa cells with uniform morphology. Vacuolation and slight hyperemia appeared in rats exposed to 36.5 μg MC-LR/kg bm, while rats exposed to 54.75 or 73 μg MC-LR/kg bm exhibited obvious hyperemia, cytoplasmic loss, abnormal nuclear change and nuclear dissolution. Necrosis of local granular cells was seen in ovaries of rats exposed to 73 μg MC-LR/kg bm.

3.3.5. Thyroid

The thyroid follicular cells in the controls had normal morphology and clear boundaries. The follicular epithelial cells were arranged as a single layer of cubes, and the cells were filled with colloid. Broken nuclei, necrosis of follicular epithelial cells and reduced intracellular colloid were observed in rats treated with 36.5, 54.75 and 73 μg MC-LR/kg bm, and there were also exfoliated epithelial cells in the follicular cavity. Pathological lesions were the most severe in rats treated with 73 μg MC-LR/kg bm.

3.4. Concentrations of hormones in serum

3.4.1. HPA axis

Compared with controls, concentrations of corticotropin-releasing hormone (CRH), adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) were significantly less in serum of rats treated with MC-LR (Fig. 3A).

3.4.2. HPG axis

Exposure to MC-LR caused significantly lesser concentrations of gonadotropin-releasing hormone (GnRH), but greater concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Fig. 3B). When treated with 73 μg MC-LR/kg, bm, concentrations of testosterone (T) were greater. Relative to the controls, significantly lesser concentrations of estradiol (E2) were observed after exposure to 36.5 or 54.75 μg MC-LR/kg bm.

3.4.3. HPT axis

Concentrations of thyrotropin-releasing hormone (TRH) were significantly less in serum of rats treated with 36.5 μg MC-LR/kg bm, but no significant changes were observed in rats exposed to 54.75 or 73 μg MC-LR/kg bm (Fig. 3C). Concentrations of thyroid-stimulating hormone (TSH) were significantly greater in MC-LR-treated rats than those of controls. Exposure to MC-LR resulted in significantly smaller concentrations of free tetra-iodothyronine (fT4) and free tri-iodothyronine (fT3).

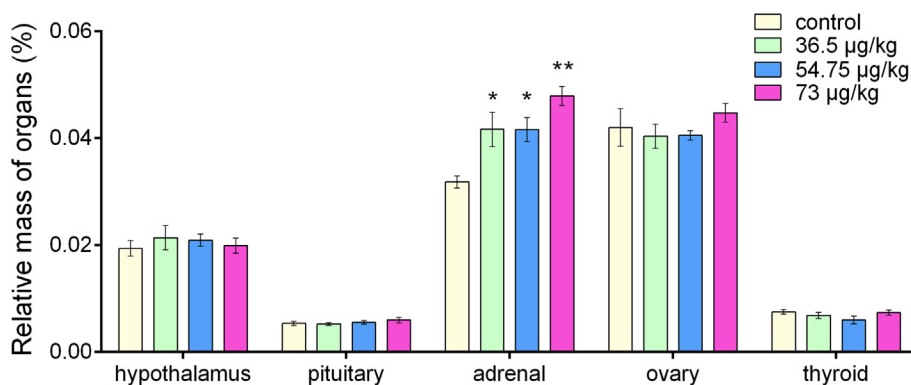


Fig. 1. Relative masses of hypothalamus, pituitary, adrenal, ovary and thyroid of female rats at 24 h after exposure to 36.5, 54.75 or 73 µg microcystin-LR/kg body mass by intraperitoneal (i. p.) injection. Values are presented as the mean ± standard error (SE), n = 5. *, ** or *** indicates p < 0.05, <0.01 or <0.001 versus control, respectively.

3.5. Correlation of concentrations of hormones in serum

3.5.1. HPA axis

There were significant positive correlations among concentrations of CRH, ACTH and CORT (Fig. 4).

3.5.2. HPG axis

There was a significant positive correlation between concentrations of LH and FSH, and both of which were negatively correlated with concentrations of GnRH. Concentrations of FSH and T were positively correlated.

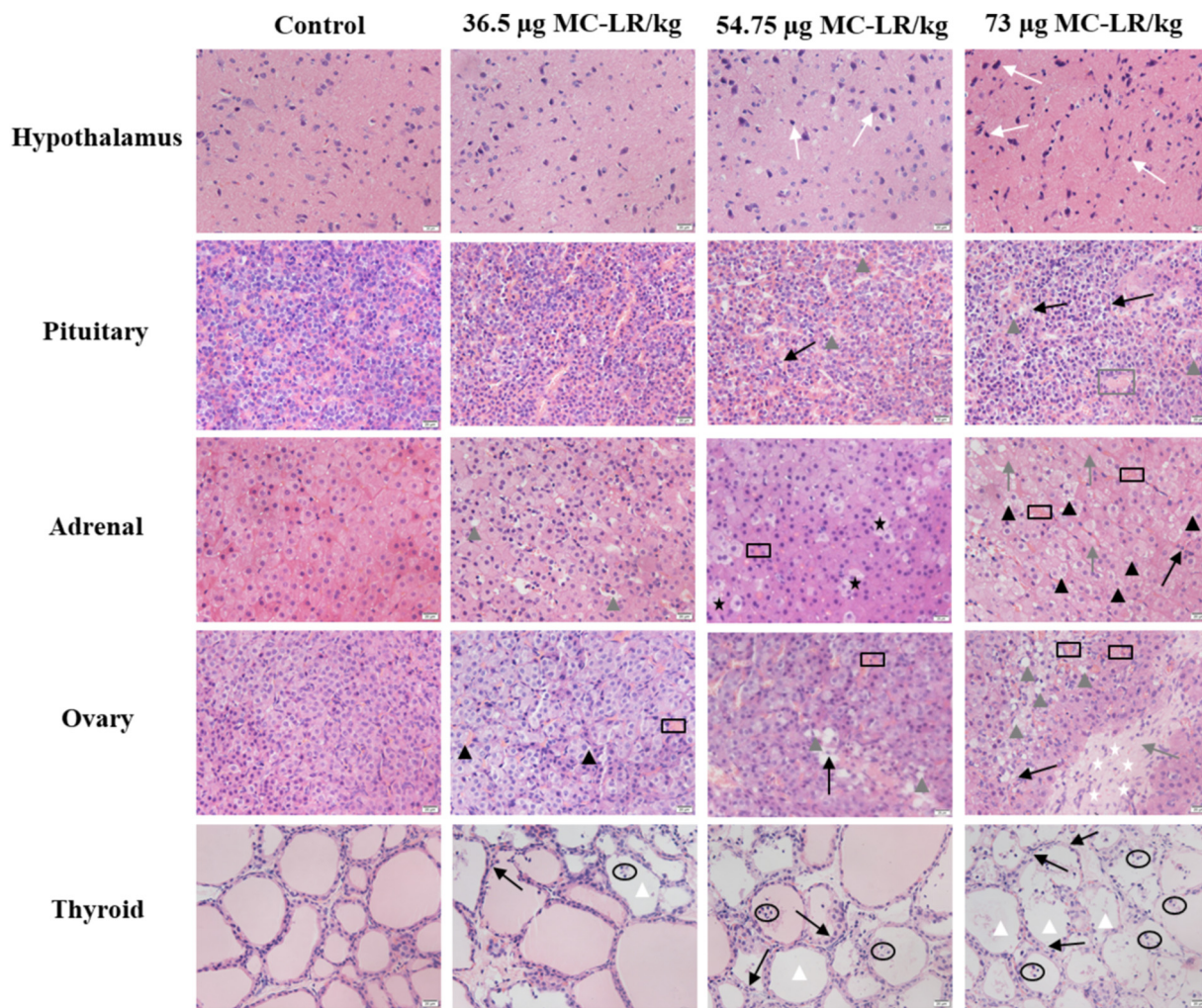


Fig. 2. Microstructural changes of hypothalamus, pituitary, adrenal, ovary and thyroid of female rats at 24 h after exposure to 36.5, 54.75 or 73 µg microcystin-LR/kg body mass by intraperitoneal (i.p.) injection. 400×, bar = 20 µm. Symbols: white arrow: unclear boundary between nucleus and cytoplasm; grey arrow: nuclear loss; black arrow: fragmentation or lysis of nuclei; white arrowhead: reduced intracellular colloid; grey arrowhead: cytoplasmic loss; black arrowhead: vacuole; grey box: telangiectasis; black box: hyperemia; white star: necrosis; black star: edema; circle: exfoliated cells.

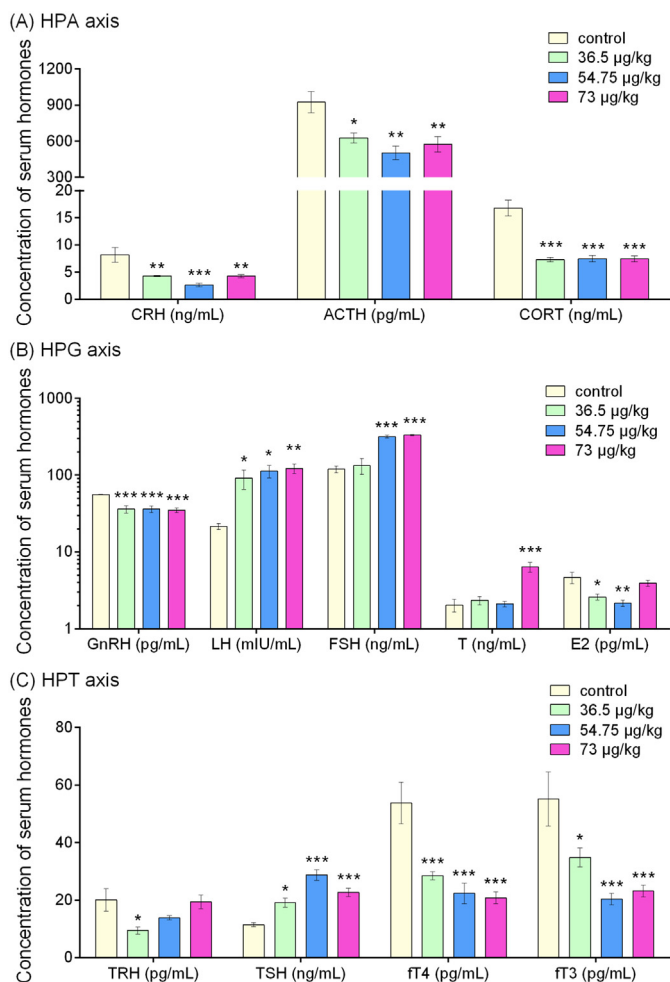


Fig. 3. Concentration of hormones of the hypothalamic-pituitary-adrenal (HPA), -gonad (HPG) and -thyroid (HPT) axes in serum of female rats at 24 h after exposure to 36.5, 54.75 or 73 µg MC-LR/kg body mass by intraperitoneal (i.p.) injection. Values are presented as the mean \pm standard error (SE), $n = 5$. *, ** or *** indicates $p < 0.05$, < 0.01 or < 0.001 versus control, respectively. CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; CORT, corticosterone; GnRH, gonadotropin-releasing hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; T, testosterone; E2, 17 β -estradiol; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone; ft4, free tetra-iodothyronine; ft3, free tri-iodothyronine.

3.5.3. HPT axis

Concentrations of ft4 and ft3 were positively correlated, and both of which were negatively correlated with concentrations of TSH.

3.5.4. HPA and HPG axes

Concentrations of CRH, ACTH and CORT were positively correlated with concentrations of GnRH, but were negatively correlated with concentrations of LH. Concentrations of ACTH and CORT were negatively correlated with concentrations of FSH, but were positively correlated with concentrations of E2.

3.5.5. HPA and HPT axes

Concentrations of CRH, ACTH and CORT were negatively correlated with concentrations of TSH, but were positively correlated with concentrations of ft4. Concentrations of ACTH and CORT were positively correlated with concentrations of ft3.

3.5.6. HPG and HPT axes

Concentrations of GnRH were negatively correlated with concentrations of TSH, but were positively correlated with concentrations of ft4.

Concentrations of LH and FSH were positively correlated with concentrations of TSH, but were negatively correlated with concentrations of ft4 and ft3. Concentrations of E2 were positively correlated with concentrations of TRH, ft4 and ft3, but were negatively correlated with concentrations of TSH.

3.6. Expressions of genes among HPA, HPG and HPT axes

3.6.1. HPA axis

In the hypothalamus, expressions of mRNA for crh and gr were down-regulated compared with those of the control (Fig. 5A), while there was no significant alteration of expression of mc2r. In the pituitary, MC-LR caused significant down-regulation of transcriptions of crhr1 and pcsk1. Expression of gr was significantly down-regulated by exposure to 73 µg MC-LR/kg bm, while expression of pomc was significantly up-regulated. No significant effects on mRNA expression of crhr2 were observed. In the adrenal gland, except for no significant differences in amounts of mRNA for gr, levels of mRNA for mc2r, star cyp11a1, 3 β hsd, cyp21a1, cyp11b1 were significantly less.

3.6.2. HPG axis

In the hypothalamus, except for no significant alteration in expression of lhr, mRNA expressions of kiss1, gpr54, gnhr1, er α and er β were down-regulated compared with those of the control (Fig. 5B). In the pituitary, MC-LR caused significant down-regulation of transcriptions of gnhr1. Expression of er α was significantly down-regulated by exposure to 73 µg MC-LR/kg bm, while expression of er β was significantly up-regulated. There were no significant alterations in transcription of lh β or fsh β . In the ovary, except for no significant alteration in expression of fshr, expressions of lhr, er α , er β , star, cyp11a1, 3 β hsd, cyp17a1, 17 β hsd and cyp19a1 were down-regulated.

3.6.3. HPT axis

In the hypothalamus, compared with the control, transcripts of thr β were less in rats exposed to 73 µg MC-LR/kg bm (Fig. 5C), while there were no significant changes in expressions of trh, tshr or thr α . In the pituitary, no significant effects on mRNA expressions of trhr, tsh β , thr α or thr β were observed. In the thyroid, there were no significant alterations in abundances of transcripts of tshr, thr β , tg or tpo. Transcriptions of thr α , duox2, duoxa2, nis and diol1 were significantly less. When exposed to 36.5 µg MC-LR/kg bm, expression of ttf1 was significantly up-regulated. Treatment with 54.75 or 73 µg MC-LR/kg bm caused significant up-regulation of diol2 and diol3.

3.6.4. Nuclear receptors across axes

In the adrenal gland, relative to controls, exposure to MC-LR resulted in significant down-regulation of er α , thr α and thr β , but up-regulation of er β (Fig. 5D). When treated with 73 µg MC-LR/kg bm, expression of gr was significantly down-regulated in both ovary and thyroid. In the ovary, transcripts of thr β were less in rats exposed to MC-LR, while there were no significant changes in expression of thr α . Abundances of transcripts of both er α and er β were lesser in the thyroid.

4. Discussion

In the current study, for the first time, histopathology, hormones and gene expressions of the hypothalamic-pituitary-adrenal (HPA), hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-gonad (HPG) axes were examined in rats treated with microcystin-LR (MC-LR). The median lethal dose (LD₅₀) of MC-LR to female SD rats via intraperitoneal (i.p.) injection observed at 24 hours (h) in this study was 73 µg/kg, body mass (bm), which is similar to values of LD₅₀ to rats reported in previous studies (Table 3). Acute exposure to MC-LR significantly increased relative mass of adrenal in a dose-dependent manner, but relative mass of hypothalamus, pituitary, ovary or thyroid showed no significant changes. However, damage to

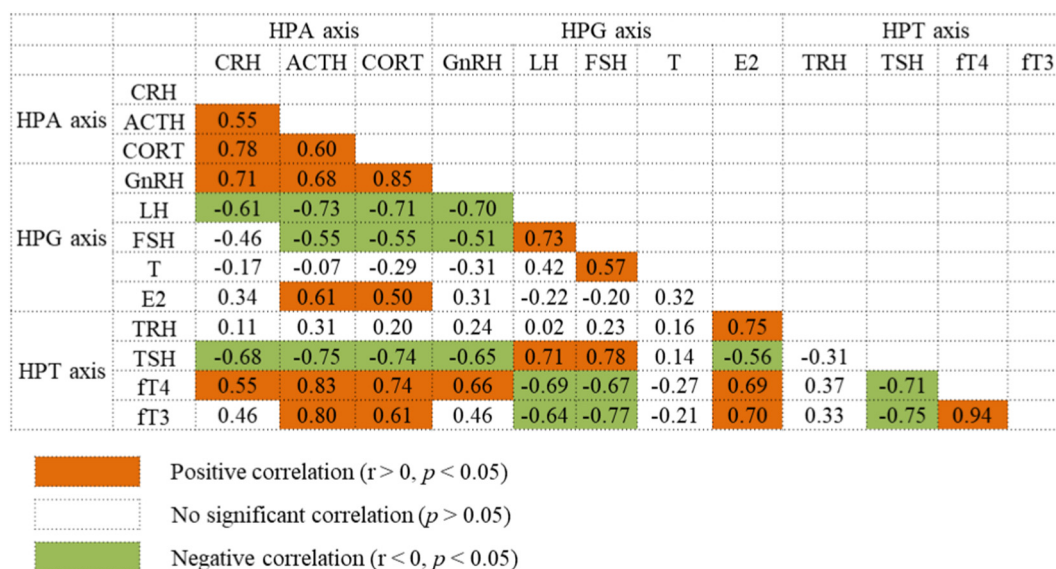


Fig. 4. Correlation coefficients (r) between concentration of hormones of the hypothalamic-pituitary-adrenal (HPA), -gonad (HPG), and -thyroid (HPT) axes in serum of female rats at 24 h after exposure to MC-LR by intraperitoneal (i.p.) injection. $n = 20$.

all these tissues was observed by histology. In addition, hormonal profiles in serum and expression of genes among HPA, HPT and HPG axes were significantly altered (Fig. 6). Therefore, the overall results suggested that MC-LR affected endocrine systems and exerted endocrine-disrupting effects.

In this study, changes of MC-LR on concentrations of hormones were not in dose-dependent manners. 36.5 μg MC-LR/kg, bm, i.e. 0.5 LD₅₀ to female rats, the smallest dose used in this study, resulted in strong effects on concentrations of corticotropin-releasing hormone (CRH), corticotropin-releasing hormone (ACTH) and corticosterone (CORT) along HPA axis as those caused by the lethal dose of 73 μg /kg, bm. The dose makes the poison (Chen et al., 2018a). Therefore, it was hypothesized that the threshold for endocrine-disrupting effects of MC-LR might be lesser than 36.5 μg /kg, bm. However, out of expectation but interestingly, non-monotonic dose-responses (NMDRs) of hormones and genes were observed. Concentrations of estradiol (E2) of HPG axis and thyrotropin-releasing hormone (TRH) of HPT axis were lesser in rats exposed to 36.5 or 54.75 μg MC-LR/kg bm, but there were no changes in 73 μg MC-LR/kg bm group. Transcription of *tcf1* was greater only in 36.5 μg MC-LR/kg bm group. Significant down-regulation of *cyp11a1* in ovary, *thr β* in adrenal gland and *er β* in thyroid were observed in rats exposed to 36.5 or 73 μg MC-LR/kg bm, but not in rats treated with 54.75 μg MC-LR/kg bm. Unlike the typical dose-responses in traditional concepts in toxicology, NMDRs are non-linear relationship between dose and effect and contain at least one point in the tested dose range where the slope of the response curve changes sign/directions, which result in U-shaped or an inverted U-shaped profile (Vandenberg et al., 2012). NMDRs are remarkably common in studies of endocrine-disrupting chemicals (EDCs), which can have effects at lesser doses which are not predicted by effects at greater doses. Therefore, further studies are required to explore endocrine disorder induced by smaller concentrations of MC-LR.

Animals survive by maintenance of homeostasis, a complex dynamic but harmonious equilibrium, which is constantly disturbed by intrinsic and/or extrinsic factors, including physical, chemical and biological stimuli, i.e. "stressors" (Magiakou et al., 1997). In the presence of stressors, maintaining homeostasis needs activation and coordination of responses involving the nervous, endocrine and immune systems, collectively called the stress response (Smith and Vale, 2006). The HPA axis is responsible for stimulation of adrenal corticosteroids cortisol or corticosterone (CORT) in response to stress (Keller-Wood, 2015). As the primary neurohormone of HPA axis, hypothalamic synthesized

corticotropin-releasing hormone (CRH) triggers bio-synthesis and release of corticotropin (adrenocorticotrophic hormone, ACTH) by the pituitary (Fig. 6). Subsequently, ACTH acts on cortex of the adrenal gland to produce corticosteroids. Negative feedback regulation by corticosteroids limits hypothalamic synthesis of CRH and pituitary synthesis of ACTH. In the current study, lesser concentrations of CRH, ACTH and CORT were observed in serum of rats exposed to MC-LR, compared with controls. Significant positive correlations were also observed among concentrations of CRH, ACTH and CORT. Lethal doses of MC-LR exposure seemed to induce a global inhibition of the HPA axis activity, which plays a key role in many important physiological functions such as endocrine, immune, metabolism, energy, neuro-regulation and behavior (Pereiro et al., 2014). However, treatment with lysed cells of *Microcystis aeruginosa*, extracts of *Microcystis* or pure MC-LR caused greater concentrations of cortisol in juvenile brown trout (Bury et al., 1996), crucian carp (Li et al., 2008a), larvae zebrafish (Chen et al., 2018b) and adult male zebrafish (Wang et al., 2020a). No significant changes of concentrations of cortisol were observed in zebrafish or Nile tilapia exposed to *Microcystis* spp. (Ziková et al., 2010; Kist et al., 2011). For these differences in responses, a possible explanation is species-specific variations between fish and mammals and magnitude of exposures. Also, samples were collected only at 24 h after exposure in this study, time-course experiments especially for early time points might be needed. Moreover, in this study, most genes along the HPA axis, including *crh* and *gr* in hypothalamus, *crhr1*, *gr* and *pcsk1* in pituitary, and *mc2r*, *star*, *cyp11a1*, *3 β hsd*, *cyp21a1* and *cyp11b1* in adrenal gland, were significantly down-regulated in rats exposed to MC-LR. These results may be responsible for the global inhibition of the HPA axis activity and lesser concentrations of CRH, ACTH and CORT observed in serum of rats exposed to MC-LR. Actually, this is the first study investigating the effects of MCs on HPA axis in mammals.

The HPG axis includes an inter-communicating set of neural and endocrine tissues and regulates reproductive functions and fertility by production of a variety of hormones, including gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH) and sex steroids (Bliss et al., 2010). The hypothalamic pulsatile release of GnRH stimulates the bio-synthesis and secretion of gonadotropins LH and FSH by binding to GnRH receptor (GnRHR) of gonadotropic cells in the pituitary. Then, LH and FSH acts at the gonads to trigger a series of set points of synthesis and secretion of sex steroids, testosterone (T) and estradiol (E2), which exert negative feedback effects on hypothalamic secretion of GnRH (Bliss et al., 2010). In the

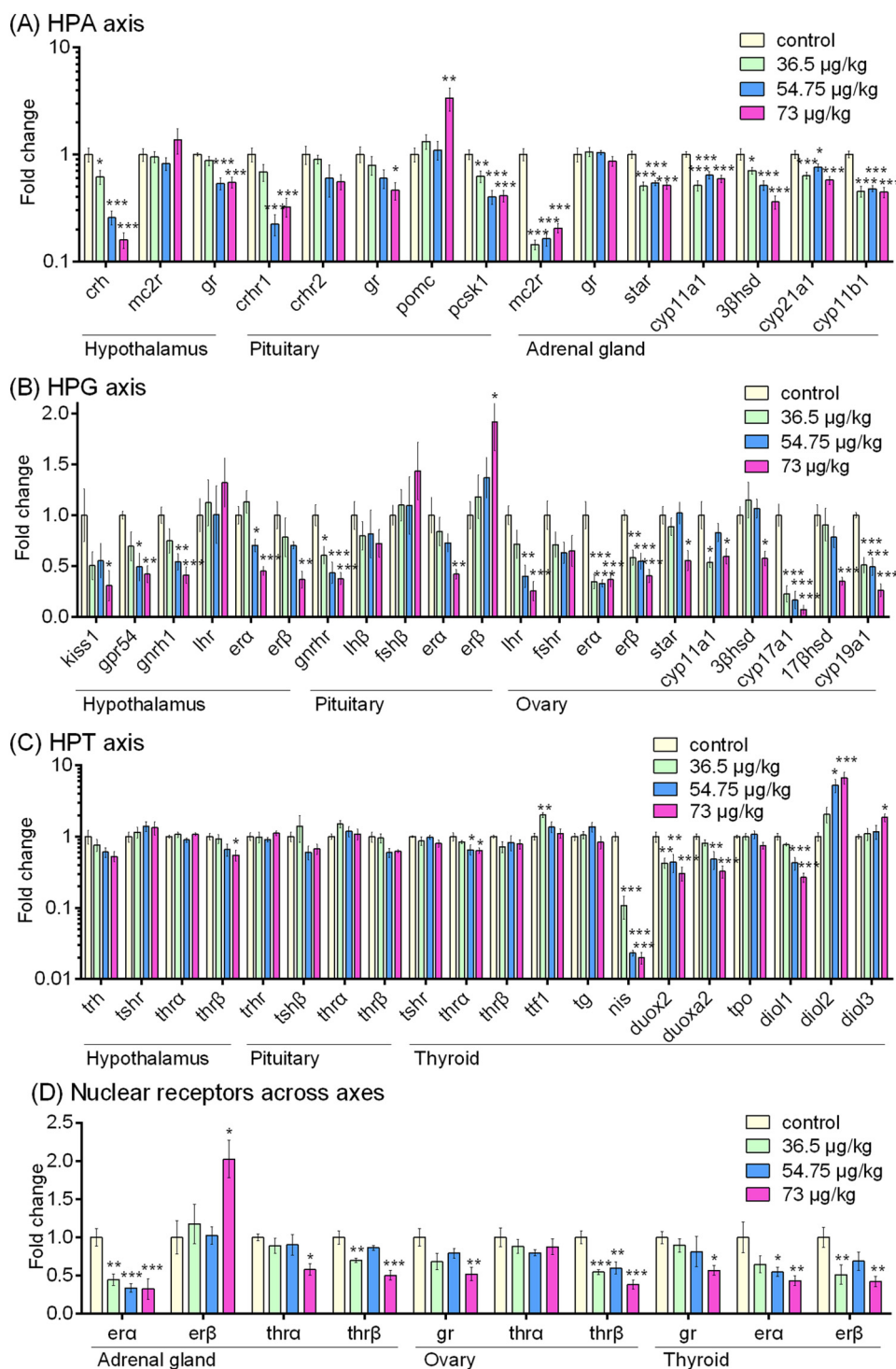


Fig. 5. Transcription of genes for synthesis of hormones along the hypothalamic-pituitary-adrenal (HPA), -gonad (HPG) and -thyroid (HPT) axes and nuclear hormone receptors across axes in adrenal, ovary and thyroid of female rats at 24 h after exposure to 36.5, 54.75 or 73 µg MC-LR/kg body mass by intraperitoneal (i.p.) injection. Values are presented as the mean ± standard error (SE), n = 5. *, ** or *** indicates p < 0.05, <0.01 or <0.001 versus control, respectively. Full names of genes are shown in Supplementary Table 1.

Table 3
Median lethal dose (LD₅₀) of MC-LR to rats by intraperitoneal (i.p.) injection.

Animals	Gender	Time	LD ₅₀ (µg/kg, body mass)	References
Sprague-Dawley rats	♀	24 h	73	The present study
Sprague-Dawley rats	♀, ♂	24 h	80–120	Hooser et al., 1989
Fischer 344 rats	♂	25 h	72–122	Miura et al., 1991
Wistar rats	♂	48 h	106	Moreno et al., 2005
Sprague-Dawley rats	♂	24 h	82.7	Li et al., 2015; Chen et al., 2016b

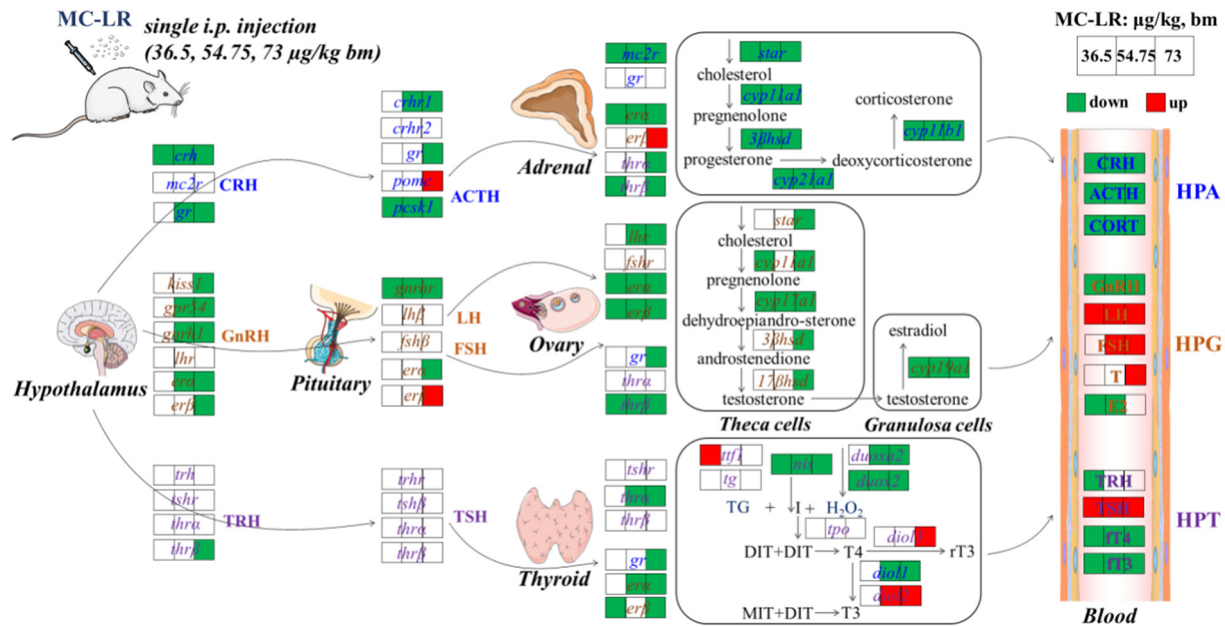


Fig. 6. A schematic summary of effects of acute exposure to microcystin-LR (LR) on the hypothalamic-pituitary-adrenal (HPA), -gonad (HPG), and -thyroid (HPT) axes in female rats. Female, Sprague-Dawley (SD) rats were exposed acutely to MC-LR by single intraperitoneal (i.p.) injections at doses of 0.5, 0.75 or 1 median lethal dose (LD_{50}), i.e. 36.5, 54.75 or 73 µg MC-LR/kg body mass (bm) and were sacrificed at 24 h after exposure.

present study, compared with controls, concentrations of GnRH and E2 were significantly lesser in rats treated with MC-LR, whereas greater concentrations of FSH, LH and T were observed. Concentrations of FSH and T were positively correlated, while both concentrations of LH and FSH were negatively correlated with concentrations of GnRH. Changes of transcriptions of genes along the HPG axis were also observed. Acute exposure to MC-LR might cause the observed effects by negative feedback, i.e. greater concentrations of FSH, LH and T could inhibit biosynthesis and/or release of GnRH by the hypothalamus. Similarly, greater concentrations of serum LH and T, lesser hypothalamic *gnrh1* transcription and greater pituitary *lh β* transcription were observed in male BALB/c mice treated with 30 µg MC-LR/kg, bm by a single i.p. injection, while concentrations of serum FSH and pituitary transcription of *fsh β* showed no significant changes (Wang et al., 2012). However, neither concentrations of GnRH, FSH, LH or T, nor transcriptions of genes along the HPG axis were altered in male C57BL/6 mice or male SD rats which received a single i.p. injection of 30 µg MC-LR/kg, bm (Xiong et al., 2014; Wang et al., 2016). These inconsistent results might be due to differences in species, strains, and/or genders. Also, the doses used in the current study, i.e. 36.5, 54.75 and 73 µg MC-LR/kg, bm, were greater than 30 µg MC-LR/kg, bm used in male C57BL/6 mice or male SD rats (Xiong et al., 2014; Wang et al., 2016). In fact, dose-dependent changes of serum concentrations of hormones were observed in male BALB/c mice exposed to MC-LR (Wang et al., 2012). Greater concentrations of serum LH and T were only observed in mice treated with a single i.p. injection of 30 µg MC-LR/kg, bm, while no significant changes were observed in mice treated with 3.75, 7.5 or 15 µg MC-LR/kg, bm (Wang et al., 2012). Results of previous studies also showed repeated sub-acute, sub-chronic or chronic exposure to MC-LR caused disruption of production and release of sex hormones and dys-regulation of HPG axis in male rats (Li et al., 2008b; Chen et al., 2013; Wang et al., 2016), male mice (Chen et al., 2011; Wang et al., 2012, 2018; Xiong et al., 2014; Ding et al., 2018; Jin et al., 2021), and female mice (Wu et al., 2014, 2015). Changes in concentrations of E2, testosterone and FSH, and altered HPG axis by treatment with MC-LR were also observed in male giant freshwater prawn (Zhang et al., 2019), female zebrafish (Qiao et al., 2013; Zhao et al., 2015a; Liu et al., 2016a; Hou et al., 2016; Cheng et al., 2018; Kawan et al., 2019), male zebrafish (Qiao et al., 2013; Liu et al., 2016a; Su et al., 2016; Hou et al., 2018; Lin

et al., 2018, 2020), male Nile tilapia (Chen et al., 2017a), and male frogs (Jia et al., 2014, 2018). Thus, MCs can target the HPG endocrine axis in a variety of species, from fish and amphibians, to mammals.

It's known that the HPG axis and GnRH, LH, FSH, T and E2 play key roles in reproductive function and fertility (Bliss et al., 2010). Reproductive toxicity of MCs, especially to reproductive peripheral organs including testis and ovary, has been widely reported (Lone et al., 2015; Chen et al., 2016a; Zhang et al., 2021). MCs were found to enter ovary of SD rats *in vivo* (Wu et al., 2014) and primary cultured mouse granulosa cells *in vitro* (Wu et al., 2015). Exposure to MC-LR also caused pathological changes and oxidative stress in granulosa cells and ovary (Wu et al., 2014, 2015). In female mammals, the theca cells secrete androgens, i.e. T, and then they are converted to estrogens, i.e. E2, by the adjacent granulosa cells (Bliss et al., 2010). In this study, damage to ovary following exposure to MC-LR was also observed by use of histology. It's not difficult to understand that toxicity to ovary would lead to disruption of synthesis and secretion of sex steroids. Recently, congener- and dose- dependent ovotoxicities of MCs (-LR, -LA, -LF and -LY) to vitrified and fresh follicles *in vitro* were observed, including decreased follicle survival rates, inhibited follicle growth and lesser E2 secretion (Wang et al., 2020b). In males, however, MCs were not able to enter and exert toxicity to Leydig cells where T is produced (Wang et al., 2012, 2013; Xiong et al., 2014). MC-LR was detected in the hypothalamus of rats and remarkable apoptotic cells were observed (Wang et al., 2016; Ding et al., 2018). Similarly, exposure to MC-LR caused histopathological damage of hypothalamus in this study. Specifically, MCs were found to enter GnRH neurons in hypothalamus of rats *in vivo* (Wang et al., 2016) and immortalized hypothalamic neuronal cells (GT1-7 neurons) of mice *in vitro* through organic anion transporting polypeptide 1a5 (Oatp1a5) (Wang et al., 2016; Ding et al., 2017, 2018; Jin et al., 2019). Exposure of GT1-7 cells to MC-LR resulted in decreased cell viability, increased release of lactate dehydrogenase (LDH), intracellular concentrations of Ca^{2+} and cAMP and activities of adenosine cyclase (AC), protein kinase a/c (PKA/C) and mitogen-activated protein kinase (MAPK) signaling pathways, which finally led to lesser synthesis of GnRH (Wang et al., 2016, 2018; Ding et al., 2017, 2018; Jin et al., 2019). Therefore, MCs can cause sexual hormone disturbance by targeting hypothalamic GnRH neurons. As expected, in this study, transcriptions of *kiss1*, *gpr54* and *gnrh1* were all down-regulated in

hypothalamus of rats exposed to MC-LR. Thus, in females, the effects of MCs on synthesis of sex hormones occur at two levels, i.e. by affecting both GnRH neurons in the hypothalamus and granulosa cells in the ovary, which are different from males, only by targeting hypothalamic GnRH neurons.

Similar to HPA and HPG axes, the HPT axis determines all the molecular and biochemical processes of production of thyroid hormones (THs), including tetra-iodothyronine (thyroxine, T4) and tri-iodothyronine (T3) (Ortiga-Carvalho et al., 2016). The thyrotropin-releasing hormone (TRH) released by the hypothalamus triggers production of pituitary thyrotropin (thyroid-stimulating hormone, TSH), which stimulates the thyroid to synthesize and secrete THs. T4 and T3 regulate the production and release of TRH and TSH by negative feedback. In this study, MC-LR resulted in greater concentrations of TSH but smaller concentrations of TRH, free tetra-iodothyronine (fT4) and free tri-iodothyronine (fT3). Concentrations of fT4 and fT3 were positively correlated, and both of which were negatively correlated with concentrations of TSH. It is suggested that acute exposure to MC-LR might disrupt bio-synthesis or secretion of the THs and inhibition of the HPT axis activity. These results are consistent with the finding in crucian carp treated with lethal doses of extracts of *Microcystis* containing 150 and 600 µg MCs/kg, bm in a previous study (Li et al., 2008a). Acute, sub-acute, sub-chronic or chronic exposure to MC-LR or MC-RR caused lesser concentrations of T4 or T3 in adult female mice (Chen et al., 2019), eggs and larvae of zebrafish (Yan et al., 2012; Xie et al., 2015; Cheng et al., 2017; Zuo et al., 2020), juvenile Chinese rare minnow (Liu et al., 2015a), juvenile zebrafish (Hu et al., 2020), adult female zebrafish (Cheng et al., 2017; Gao et al., 2020), and adult male zebrafish (Liu et al., 2016b; Gao et al., 2020). However, greater concentrations of T4 or T3 were observed in juvenile zebrafish (Liu et al., 2015b) or male mice (Zhao et al., 2015b) following sub-chronic exposure to MC-LR. These differences might be explained by variations among species, genders, developmental stages as well as different doses of MCs, different durations of exposure, and/or different temporal patterns. Sex-dependent effects of MC-LR on concentrations of T4 and T3 were also reported in zebrafish (Liu et al., 2016b; Cheng et al., 2017; Gao et al., 2020). In this study, abundances of transcripts of *duox2*, *duoxa2*, *nis* and *diol1*, which are responsible for synthesis of THs, were significantly less in thyroid of rats exposed to MC-LR. These results can explain the observed lesser concentrations of fT4 and fT3 in serum. However, one unexpected observation was that dysregulation of mRNA of genes were not observed in the pituitary, while treatment with MC-LR caused greater concentrations of TSH which is secreted by pituitary. More studies are needed to clarify this contradictory phenomenon. Anyhow, results of all of these studies revealed that MCs disrupt thyroid endocrine function and HPT axis in both fish and mammals.

In the current study, treatment with MC-LR resulted in activation of HPG axis and greater concentrations of LH, FSH and T, but inhibition of HPA and HPT axes and smaller concentrations of CRH, ACTH, CORT and thyroid hormones (THs), fT4 and fT3. Significant positive or negative correlations of concentrations of hormones in serum were observed among HPA, HPG and HPT axes. Crosstalk/crossover/interaction among these endocrine axes has well been documented previously (Kamilaris et al., 1987; Magiakou et al., 1997; Viau, 2002; Liu et al., 2011). The HPA, HPG or HPT axis does not act independently of each other and the effects can exceed the border of one axis (Brüggemann et al., 2018). Effects among these endocrine axes could be explained by 4 mechanisms. (1) There are physiological overlap among axes, such as the hypothalamus and pituitary, which are common organs of hormone bio-synthesis of all HPA, HPG and HPT axes. (2) Some hormones (eg. CRH), can regulate multiple signal transduction pathways along different endocrine axes (Magiakou et al., 1997). (3) A tissue or cell can respond to different hormonal signals. (4) Hormone-bound nuclear receptors (NRs) can regulate the expression of other types of NRs, i.e. NR cross-regulation (Bagamasbad and Denver, 2011). In this study, exposure to MC-LR resulted in down-regulation of *erα*, *thrα* and *thrβ* but

up-regulation of *erβ* in adrenal gland, down-regulation of *gr* and *thrβ* in ovary, and down-regulation of *gr*, *erα* and *erβ* in thyroid. Thus, it is likely that MC-induced changes on one endocrine axis can lead to changes of other axes. However, primary and secondary effects of MC-LR on the endocrine system cannot be discriminated in this study. In other words, which was the initial event and led to subsequent effects, activation of HPG axis or inhibition of HPA or HPT axis? Which hormone changed first? To answer these questions, time-course experiments especially for early time points might be needed. Also, the mechanisms of effects of MC-LR on the initial affected endocrine axis and hormonal functions, i.e., synthesis, secretion, transport, binding, action and elimination, warrant further investigations.

5. Conclusions

This is the first comprehensive study which reveals that microcystin-LR (MC-LR) affects histopathology, hormones and gene expressions of the hypothalamic-pituitary-adrenal (HPA), hypothalamic-pituitary-gonad (HPG) and hypothalamic-pituitary-thyroid (HPT) axes in rats. Acute exposure to MC-LR resulted in histopathological damage to hypothalamus, pituitary, adrenal, ovary and thyroid in female rats. Changes of MC-LR on concentrations of hormones in serum and expression of genes among HPA, HPT and HPG axes were observed, which suggested endocrine-disrupting effects of MC-LR. Effects of microcystins (MCs) on normal physiological functions of hormones and endocrine physiology may cause dys-regulation and abnormal functioning of the endocrine system, endocrine disease(s), and thereby adversely affect health of human and wildlife.

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CRedit authorship contribution statement

Liang Chen: Conceptualization, Data curation, Project administration, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition. **Ting Shi:** Data curation, Methodology, Investigation, Formal analysis, Visualization, Writing – review & editing. **Yu-Ting Wang:** Investigation, Formal analysis, Visualization, Writing – review & editing. **Jun He:** Methodology, Resources, Investigation. **Xu Zhao:** Investigation. **Ye-Ke Wang:** Methodology, Investigation. **John P. Giesy:** Supervision, Conceptualization, Writing – review & editing. **Feng Chen:** Investigation. **Yang Chen:** Investigation. **Xun Tuo:** Investigation. **Jun Chen:** Supervision, Resources, Data curation, Project administration, Writing – review & editing, Funding acquisition. **Ping Xie:** Supervision, Resources, Data curation, Project administration, Writing – review & editing, Funding acquisition.

Declaration of competing interest

None.

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