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RESEARCH ARTICLE

Dietary exposure to methyl mercury chloride induces alterations in hematology, biochemical parameters, and mRNA expression of antioxidant enzymes and metallothionein in Nile tilapia

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Abstract

Methyl mercury chloride "MMC" (CH_3ClHg) is an ubiquitous environmental toxicant that causes a variety of adverse effects. In the present study, we investigated the effects of sub-chronic toxicity of MMC on Nile tilapia (*Oreochromis niloticus*) through the evaluation of growth performance and hematological, biochemical, and oxidative stress biomarkers. From 150 healthy fish, five equally sized treatment groups were created: a control (CT) group fed with a basal diet and four MMC treatment groups exposed to 0.5, 1, 1.5, and 2 mg of MMC per kg of basal diet for 60 days. MMC exposure significantly reduced the growth performance and survival of *O. niloticus* and decreased red blood cell count and hemoglobin concentration. Treated fish exhibited normocytic normochromic anemia in addition to leucopenia, lymphopenia, granulocytopenia, and monocytopenia. Moreover, MMC exposure significantly affected liver function, including a reduction in the total protein levels while increasing cholesterol and triglyceride levels. It also markedly increased the production of stress biomarkers such as glucose and cortisol levels. Furthermore, MMC significantly elevated the levels of hepatic enzymes, induced tissue damage, and caused inflammation, as indicated by the upregulation of mRNA expression of hepatic metallothionein. Finally, MMC exposure induced oxidative stress by altering the antioxidant status of the liver and downregulating the mRNA expression of superoxide dismutase, glutathione peroxidase, and glutathione S-reductase. In conclusion, MMC toxicity induced hematological and biochemical alterations, leading to an enhanced state of oxidative stress in *O. niloticus*.

Keywords Methyl mercury chloride · *Oreochromis niloticus* · Metallothionein · GPX · GSR · Gene expression

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Introduction

Heavy metals produce several physiological, metabolic, behavioral, and ecological disorders (Soengas et al. 1996; Abu Zeid et al. 2021) in fish. Mercury (Hg), considered to be the most toxic heavy metal to the environment and humans (Altunay 2018), is used on a large scale in medicine, agriculture, industry, the military, and dentistry. Through such applications, mercury is released into the environment, entering the food chain through rapid diffusion and binding to proteins tightly in the form of methyl mercury. Methyl mercury (MeHg) is an organic mercury compound, and owing to its lipophilicity, it is more toxic than the corresponding inorganic mercury species (Fernández et al. 2016). MeHg is thought to be the most organic source of mercury and accounts for almost 95–99% of the total fraction of mercury found in the tissues of fish (Drevnick and Sandheinrich 2003).

In any chemical form at sufficient concentrations, mercury inactivates enzymes, denatures proteins, and severely

disrupts the physiological processes of any tissue with which it comes into contact (Sastry and Rao 1981; Shakoori et al. 1994). For example, low concentrations of MeHg can modify the B- and T-cell functions (growth, proliferation, and cytokine production) and affect the cellular processes, resulting in apoptosis (Shenker et al. 1993; Makani et al. 2002). In aquatic cultures, methyl, elemental, and inorganic mercury are all important forms of mercury (Beckvar et al. 1996). The main sources of freshwater mercury pollution are run-off from agricultural fields, dumping of industrial effluent, and discharge of untreated waste. Aquatic ecosystems are the most common end containers for urban and industrial waste products (Hoffman 1995). One form of mercury, methyl mercury chloride (MMC) (CH3ClHg), is acutely toxic to aquatic organisms. Inorganic mercury in the aquatic environment can be biomethylated by aquatic sulfate-reducing bacteria that occur in the sediment, forming MeHg (Compeau and Bartha 1985). MeHg is more toxic due to rapid entry and circulation via the intestinal tract and can be taken up by the brain tissue as its L-cysteine conjugate via the active transport system for amino acids (Kerper et al. 1992). In previous studies, MMC significantly reduced embryonic survival in the teleost fish *Fundulus heteroclitus* (Sharp and Neff 1982) and was a potent chemical stressor in juvenile rainbow trout, wherein it modified carbohydrate metabolism and stimulated the pituitary−thyroid axis (Bleau et al. 1996). To assess the sublethal and chronic toxicity of contaminants such as MMC, hematological parameters are often measured during fish physiological diagnoses (Kim et al. 2008).

Tilapia (*Oreochromis niloticus*) is a native Egyptian fish species. It is now cultivated worldwide as it is valuable and easy to farm and breed in various aquatic conditions (El-Sayed 2006). Nile tilapia is also a popular and highly consumed source of protein and fast-growing fish species. Presently, little is known about the effects of MMC in tilapia. Therefore, in the present study, the toxic impact of dietary MMC on *O. niloticus* was investigated through evaluation of the hematological and biochemical changes, oxidative stress, and mRNA expression resulting from the sub-chronic exposure of MMC.

Materials and methods

Chemicals

Analytical grade methyl mercury (II) chloride (CH3HgCl (linear formula), CH3ClHg (chemical formula), 442534–5G-A, Lot# 031M1357V, Cl-13%; Pcode: 1001085892) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Experimental diet preparation

In compliance with the nutritional requirements of *Oreochromis niloticus*, formulated food pellets were formed with the basal ingredients containing 27.6% crude protein and 9.12% crude lipid (Jobling 2012) (Table 1). The appropriate concentrations of MMC (0, 0.5, 1.5, and 2 mg/kg diet) were dissolved in 6 mL of 100% ethanol and then thoroughly mixed with the diet ingredients. The ethanol was evaporated during the formulation of the diet. The diet was dried at room temperature and then stored in the dark at 4°C until use. Fish were fed a basal diet at 3% of their body weight daily.

Experimental design and laboratory conditions

A total of 150 healthy *O. niloticus* fingerlings (22.8 ±0.5 g body weight) were purchased from a fish hatchery at Abbassa, Sharkia Governorate. The fish were transported to the laboratory (Faculty of Veterinary Medicine, Fish Diseases and Management Department, Zagazig University) in polyethylene bags filled with one-third dechlorinated water and two-thirds of air in the morning to protect them from the heat and sunshine. The water in the transport polyethylene bags was gradually replaced with the water from a source in the lab for

Table 1 Compositions of the formulated basal diet pellets (g/kg)

Ingredients	Items (g/kg)
Yellow corn	210
Soybean meal 48% CP	200
Fish meal	150
Corn gluten 60% CP	130
Rice bran	110
Wheat middlings	150
Premix-Mina 10[a]	10
Premix-Vitb 10[b]	10
Corn oil	30
Total	1000
Chemical composition	
Crude protein%	320.5
Crude lipid %	42.50
Crude fiber %	42.45
Crude ash %	73.01
Nitrogen-free extract[c]	518.54

[a] Composition of mineral premix kg^{-1} : manganese, 53 g; zinc, 40 g; iron, 20 g; copper, 2.7 g; iodine, 0.34 g; selenium, 70 mg; cobalt, 70 mg, and calcium carbonate as carrier up to 1 kg

[b] Composition of vitamin premix kg^{-1} : vitamin A, 8,000,000 IU; vitamin D3, 2,000,000 IU; vitamin E, 7g; vitamin K3, 1.5 g; vitamin B1, 700 mg; vitamin B2, 3.5g; vitamin B6, 1g; vitamin B12, 7 mg; biotin, 50 mg; folic acid, 700 mg; nicotinic, 2g; pantothenic acid, 7g

[c] Nitrogen-free extract = 100 − (crude protein + crude lipids + ash + crude fiber)

acclimatization to lab temperature and water quality conditions. They were acclimatized in glass aquaria (80 × 40 × 30 cm) filled with tap water (60 L, dechlorinated) for a photoperiod of 12h per day for about 1 week. Fish were allocated into five groups (30 fish/group), each group containing three replicates of 10 fish each. Group 1 (CT) was kept as control and fed a basal diet. Fish in groups 2, 3, 4, and 5 (0.5 MMC, 1 MMC, 1.5 MMC, and 2 MMC) were exposed to CH3ClHg in the basal diet mixed with 0.5, 1, 1.5, and 2 mg of MMC per kg of food, respectively.

In the experimental diets, the toxicant concentrations were selected after considering (a) Hg concentrations that had demonstrated toxicant accumulation in previous experiments (10–100 mg mercuric chloride and 0.5–10 mg methyl mercury kg^{-1}) (Berntssen et al. 2004) and (b) the current EU maximum limit for Hg in the fish feed (0.1 mg kg^{-1}) and the dietary level of methyl mercury in the basic diet was 0 mg kg^{-1} (control) (Berntssen et al. 2004). Water was replaced every 48 h, and water quality was monitored and maintained at acceptable levels according to APHA (1998) throughout the experimental period. These conditions were as follows: temperature 26 ±2 °C, pH 6.5 ±0.3, dissolved oxygen 6.3 ±0.5 mg/L, ammonia 0.01 ±0.001 mg/L, nitrite 0.03 ±0.015 mg/L, total hardness 143 ±1 mg/L, total dissolved solids 235 ±2 mg/L, conductivity 370 ±2.7 μS/cm, Ca^{+2} 36 ±0.1 mg/L, Mg^{+2} 20 ±0.5 mg/L, Na^{+} 10 ±0.5 mg/L, K^{+} 2.5 ±0.002 mg/L, HCO_3^{-} 98.5 ±2.5 mg/L, SO_4^{2-} 53 ±2 mg/L, and Cl^{-} 19 ±1 mg/L. Clinical signs and mortality rates were recorded during the exposure period.

The experiment was conducted according to the ethical guidelines for Animal Use in Research Committee (EAURC) of the Zagazig University (FISH MD-296) and the National Institute of Health (Jobling 2012).

Analysis of growth performance

At the beginning of the experiment, the body weights of the fish were determined. They were weighed every 2 weeks to determine the fish feed intake. The final body weight was determined at the end of the study (60 days). Weight gain (WG, g), daily weight gain (DWG, g day^{-1}), specific growth rate (SGR), and food conversion ratio (FCR) were determined using the following formulae:

$$\mathrm{WG\ (g)} = \text{final weight} - \text{initial weight}$$

$$\mathrm{DWG} = (\text{final weight} - \text{initial weight})/60\ (\text{number of days in the feeding period})$$

$$\mathrm{WG\ (\%)} = [(\text{final weight} - \text{initial weight})/\text{initial weight}] \times 100$$

$$\mathrm{SGR} = 100 \times [(\text{In final weight} - \text{In initial weight})/\text{no.of experimental days}]$$

$$\mathrm{FCR} = \text{total feed intake (g)}/\mathrm{WG\ (g)}$$

$$\mathrm{SR} = 100 \times (\text{final number of tested fish}/\text{initial number of tested fish})$$

Sample collection

At the end of the experiment (60 days), nine fish/group (3 fish/replicate) were collected and anesthetized using 50 mg L^{-1} benzocaine solution (Al-Nasr Pharmaceutical Chemicals Co, Egypt) (Ferreira et al. 1979). To assess the hematological parameters, the blood samples were collected by puncturing the fish caudal vessels with a syringe rinsed with EDTA previously. Additional samples were obtained without EDTA and left to coagulate (30 min/4°C) before centrifugation (15 min/3000 rpm) for serum separation. Fresh serum was used for biochemical analysis. Also, tissue samples from the liver were collected from the different experimental groups, of which 30 mg samples were washed in cold saline and snap-frozen in liquid nitrogen. The samples were then stored at −80 °C until subsequent RT-PCR analysis. Certain portions of the liver were set for 72 h in buffered formalin (10%) and transferred to 70% ethanol for histopathological analysis.

Evaluation of hematological parameters

Red blood cell (RBC) count, packed cell volume (PCV), hemoglobin (Hb) content, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and white blood cell (WBC) counts were immediately estimated at the Animal Health Research Institute using an Automated Hematology Analyzer (Sysmex XT-2000iV, Japan). The differential leucocyte count was evaluated on blood films stained with Giemsa stain.

Evaluation of serum biochemical parameters

Serum glucose levels were determined using the colorimetric method of Trinder (1969). Serum total protein, albumin, triglycerides, and cholesterol were estimated colorimetrically using the Diamond Diagnostics assay kit (Holliston, MA). The serum globulin level and the activities of the enzymes aminotransferase (ALT and AST) and alkaline phosphatase

(ALP) were estimated using assay kits (Spectrum Diagnostics, Egypt) according to the methods of Reitman and Frankel (1957) and Tietz et al. (1983). Serum cortisol levels were measured using the Coat-a-Count Cortisol Kit (Diagnostic Products Corporation, Los Angeles, CA, USA).

Real-time quantitative PCR (RT-qPCR)

Nine fish per group (three fish per replicate) were collected and euthanized with an overdose of benzocaine solution (250 mg L^{-1}) (Al-Nasr Pharmaceutical Chemicals Co, Egypt). Total RNA was extracted from 30 mg of hepatic tissue using Trizol (Invitrogen, Thermo Fisher Scientific, Inc.), and then the cDNA was synthesized using the RH (-) cDNA HiSenScript TM Synthesis Kit (iNtRON Biotechnology Co., South Korea). For analysis of the gene expression, a real-time PCR-based detection was performed in the CFX96 real-time RT-PCR system (Bio-Rad, USA) using the TOPreal™ qPCR 2X PreMIX SYBR Green (Enzynomics, Korea), following the manufacturer's instructions. The PCR cycling conditions included initial denaturation (95 °C for 15 min), followed by 40 denaturation cycles at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The oligonucleotide-specific primers (Standen et al. 2016; Afifi et al. 2016; Ghazy et al. 2017; Caxico Vieira et al. 2018) were synthesized by Sangon Biotech (Beijing, China) and are presented in Table 2. The expression levels of the target genes were normalized to that of GAPDH, and the relative fold changes in gene expression were determined based on the comparative approach $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001).

Histopathological examination

Three fish from each group per replicate were used for histopathological examinations. Samples from the liver tissue were set in 10% formalin for 24 h, dehydrated in a graded series of ethanol, cleared in xylene, and then embedded in paraffin. Sections (5 μm thick) were cut, mounted onto slides, stained with hematoxylin and eosin, and finally examined using light microscopy (Layton and Bancroft 2013).

Statistical analysis

All the data were expressed as means ± standard errors. The data were screened and normality was tested using the Shapiro-Wilk test. The homogeneity of variance was evaluated using Levene's test. The parameters were compared between groups using a one-way ANOVA followed by the Bonferroni post hoc test. The statistical analysis was conducted using the program GraphPad Prism version 8 (GraphPad Software Inc., San Diego, 189 CA, USA). p-values of < 0.05 were considered to be statistically significant.

Results

Growth performance and survival rate

Nile tilapia (*O. niloticus*) exposed to MMC at 0.5, 1, 1.5, and 2 mg/kg diet for 60 days showed significant decreases in FBW, DWG, SGR, and SR, whereas FCR increased significantly in the groups exposed to 1, 1.5, and 2 mg/kg MMC, compared to the control (Table 3). A dose-response relationship was observed between the mortality of fish and MMC levels in the diet, with the highest mortality being recorded in the group exposed to 2 mg/kg MMC (55% mortality).

Hematological parameters

All the tested concentrations of MMC caused a significant decrease in the RBC count and Hb level after 60 days. The PCV was also significantly decreased ($p < 0.05$) in the 0.5 MMC and 1 MMC groups (but not the 1.5 MMC and 2 MMC groups) compared to the control fish. The MCV and MCHC of the fish exposed to all levels of MMC did not change significantly compared to the control. However, normocytic normochromic anemia developed in the fish exposed to different concentrations of MMC for 60 days (Table 4).

Table 2 Primer sequences of target genes

Fish;β-actin;F	TGACCTCACAGACTACCTCATG	(Standen et al. 2016)
Fish;β-actin;R	TGATGTCACGCACGATTTCC	
Fish;SOD;F	GGTGCCCTGGAGCCCTA	(Afifi et al. 2016)
Fish;SOD;R	ATGCGAAGTCTTCCACTGTC	
Fish;GPx;F	CGCCGAAGGTCTCGTTATT	(Caxico Vieira et al. 2018)
Fish;GPx;R	TCCCTGGACGGACACTT	
Fish;GSR;F	TACGCCAAATCTCCCACAAA	(Caxico Vieira et al. 2018)
Fish;GSR;R	CCGACAGGTAAACACCGTAAT	
Fish;MT;R	TTTTGTTTTCAAGGTGGAACC	(Ghazy et al. 2017)
Fish;MT;R	AGAGGTTGGTGAACTTTGTGG	

Table 3 The effect of methyl mercury chloride (MMC) at concentrations of 0.5, 1, 1.5, and 2 mg/kg diet for 60 successive days on the growth performance parameters in *O. niloticus*

Parameters	Groups				
	CT	0.5 MMC	1 MMC	1.5 MMC	2 MMC
IBW (g)	19.53±0.16	19.28±0.27	19.35±0.32	19.99±0.33	19.20±0.27
FBW (g)	38.26±0.84^{a}	30.47±0.30^{b}	27.99±0.37^{c}	25.32±0.55^{d}	23.27±0.32^{e}
DWG (g day^{-1})	0.31±0.01^{a}	0.18±0.01^{b}	0.14±0.01^{c}	0.08±0.01^{d}	0.06±0.01^{d}
WG (%)	95.94±4.3^{a}	58.08±1.5^{b}	44.68±1.9^{c}	26.67±2.7^{d}	21.23±1.6^{d}
SGR	0.11±0.00^{a}	0.008±0.00^{b}	0.006±0.00^{c}	0.004±0.00^{d}	0.0032±0.00^{d}
FCR	3.20±0.12^{c}	4.43±0.11^{b}	5.48±0.24^{b}	7.82±0.87^{a}	7.80±0.75^{a}
SR	95±0.57^{a}	80±0.57^{b}	70±0.57^{b}	70±0.57^{c}	45±0.57^{d}

Values are represented as the mean ± SE (three samples/replicate; nine samples/group). The means within the same row carrying different superscripts indicate statistical significance at $p < 0.05$. *IBW* initial body weight (g), *FBW* final body weight (g), *DWG* daily weight gain (g day^{-1}), *WG* weight gain (%), *FCR* food conversion ratio, *SR* survival rate

Leucocyte counts

Fish exposed to all MMC concentrations showed a significant ($p < 0.05$) decrease in the WBC counts compared to the control fish. Evaluation of differential leucocyte counts against those of the control group indicated that the lymphocyte count was reduced in the 1.5 MMC group, granulocyte counts (neutrophils, eosinophils, and basophils) were reduced in the 1.5 MMC and 2 MMC groups, and monocyte count was reduced in all the groups exposed to MMC (Table 4).

Biochemical parameters

Fish exposed to all concentrations of MMC showed a significant decrease in the total protein levels, whereas albumin levels were significantly lower in the groups exposed to 1, 1.5, and 2 mg/kg MMC. Globulin levels decreased in the 0.5 and 2 MMC groups compared to those in the control fish. Triglyceride and cholesterol levels in fish exposed to all doses of MMC for 60 days were significantly higher ($p < 0.05$) compared to the control group (Table 5).

Stress and hepatic enzyme biomarkers

Glucose and cortisol concentrations in fish exposed to all concentrations of MMC increased significantly ($p < 0.05$) after 60 days, compared to the control. Furthermore, all MMC doses increased the levels of hepatic enzymes (ALT, AST, and ALP) compared to those in the control fish (Table 5).

mRNA expression of MT, SOD, GP_X, and GSR

The level of mRNA expression of MT in the liver tissue of MMC-exposed fish was significantly upregulated compared to that of control fish. In contrast, mRNA expression of *SOD* was significantly downregulated in MMC-exposed fish. *GPX*

Table 4 The effect of methyl mercury chloride (MMC) at concentrations of 0.5, 1, 1.5, and 2 mg/kg diet for 60 successive days on hematological parameters in *O. niloticus*

Parameters	Groups				
	CT	0.5 MMC	1 MMC	1.5 MMC	2 MMC
RBCs (10^6/mm^3)	1.50± 0.04^{a}	1.10± 0.10^{b}	0.93± 0.02^{b}	1.05± 0.06^{b}	1.09± 0.05^{b}
Hb (g/dl)	6.40± 0.17^{a}	5.78± 0.32^{b}	4.32± 0.12^{d}	5.00± 0.12^{c}	5.20± 0.12^{c}
PCV%	13.00± 1.00^{a}	10.40± 0.24^{bc}	9.20± 0.37^{c}	11.40± 0.50^{ab}	12.00± 0.63^{ab}
MCV/Fl	86.50± 6.59^{a}	97.45± 7.94^{a}	98.21± 2.53^{a}	110.33± 11.14^{a}	109.73± 5.82^{a}
MCHC%	50.22± 3.92^{a}	49.05± 3.41^{a}	47.16± 1.67^{a}	44.25±2.43^{a}	43.80± 2.51^{a}
WBCs (10^3/mm^3)	7.20± 489.89^{a}	5.80 ±514.78^{b}	5.40±244.94^{bc}	5.10± 400^{bc}	4.40±100^{c}
Lymphocyte (10^3/mm^3)	3.456±296.54^{a}	2.616±377.69^{ab}	2.636±273.67^{ab}	2.410± 260.95^{b}	2.624 ±95.37^{ab}
Granulocyte (10^3/mm^3)	3.186±232.19^{a}	2.905±159.76^{ab}	2.660± 56.21^{ab}	2.578 ±233.84^{b}	1.554±139.26^{c}
Monocyte (10^3/mm^3)	0.480±45.16^{a}	0.279±48.89^{b}	0.104±9.79^{c}	0.380±12.64^{ab}	0.295 ±43.49^{b}

Values are represented as mean ± SE (three samples/replicate; nine samples/group). The means within the same row carrying different superscripts indicate statistical significance at $p < 0.05$. *RBCs* red blood cells, *Hb* hemoglobin concentration, *PCV* packed cell volume, *MCV* mean corpuscular volume, *MCHC* mean corpuscular hemoglobin concentration, *WBCs* white blood cells

Table 5 The effect of methyl mercury chloride (MMC) at concentrations of 0.5, 1, 1.5, and 2 mg/kg diet for 60 successive days on some biochemical parameters in *O. niloticus*

Parameters	Groups				
	CT	0.5 MMC	1 MMC	1.5 MMC	2 MMC
Total protein (g/dl)	5.95±0.06^{a}	5.52±0.07^{b}	5.33±0.12^{bc}	5.1±0.16^{c}	4.46±0.03^{d}
Albumin (g/dl)	4.07±0.28^{a}	4.46±0.19^{a}	3.30±0.08^{b}	3.52±0.06^{b}	3.46±0.11^{b}
Globulin (g/dl)	1.87±0.24^{a}	1.06±0.14^{bc}	2.03±0.15^{a}	1.58±0.17^{ab}	1.00±0.15^{c}
Triglycerides (mg/dl)	77.00±2.1^{d}	109.9±3.7^{bc}	141.2±2.5^{c}	206.2±13.5^{b}	270.2±21.5^{a}
Cholesterol (mg/dl)	104.2±7.6^{d}	207.2±2.6^{c}	221.6±1.66^{c}	302.4±22.2^{b}	358.2±3.65^{a}
Glucose (mg/dl)	62.48±0.4^{d}	66.36±0.69^{c}	67.88±0.22^{c}	72.4±0.7^{b}	75.58±0.79^{a}
Cortisol (ng/ml)	0.334±0.02^{e}	0.518±0.02^{d}	0.726±0.03^{c}	0.834±0.01^{b}	0.996±0.01^{a}
ALT (IU)	10.54±0.15^{e}	11.30±0.07^{d}	11.84±0.12^{c}	12.22±0.06^{b}	12.75±0.16^{a}
AST (IU)	8.58±0.04^{d}	8.86±0.05^{c}	9.43±0.03^{b}	9.69±0.06^{b}	10.29±0.17^{a}
ALP (IU)	17.36±0.15^{d}	17.80±0.03^{c}	18.03±0.01^{bc}	18.15±0.02^{ab}	18.44±0.19^{a}

Values are represented as mean ± SE (three samples/replicate; nine samples/group). The means within the same row carrying different superscripts indicate statistical significance at $p < 0.05$. *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *ALP* alkaline phosphatase

mRNA expression was downregulated only in the 1.5 MMC and 2 MMC groups, while *GSR* expression was downregulated in the 1, 1.5, and 2 MMC groups compared to the control (Fig. 1).

Histopathological findings

Liver specimens in the control group showed normal hepatic architectures, with contact erythrocytes in the central vein, as well as normal hepatopancreas, hepatic cells, and sinusoids (Fig. 2a). Exposure to 0.5 MMC produced focal fibrosis (collagen fibers admixed with erythrocytes), engorged blood vessels, and multifocal and individual melanomacrophages among degenerated hepatocytes (Fig. 2b). Exposure to 1 MMC produced congested blood vessels with hyalinized walls and peri hepatopancreas extraverted erythrocytes with focal necrotic areas admixed with inflammatory cells and erythrocytes (Fig. 2c). Fish exposed to 1.5 MMC showed focal necrotic hepatocytes admixed with inflammatory cells, mainly lymphocytes and a few erythrocytes (Fig. 2d). Fish exposed to 2 MMC exhibited necrotic hepatopancreas, necrotic debris admixed with inflammatory cells, small pancreatic acini, and interstitial lymphocytic aggregations (Fig. 2e).

Discussion

Mercury is extremely toxic with no known safe amount of exposure. MMC is a potent toxicant to aquatic organisms (Bleau et al. 1996). Toxicity, health issues related to toxic substances, and the different approaches for ameliorating such effects have recently been extensively researched not only in fish but also in different animal models (Yan et al. 2019; Abdel-Daim et al. 2019; Alam et al. 2019; Arisha et al. 2019; Elewa et al. 2019; Galal et al. 2019; Hussein et al. 2019; Saber et al. 2020; Abdel-Daim et al. 2020a; Abdel-Daim et al. 2020b; Abu Zeid et al. 2021). Water toxicity is a major environmental risk factor affecting the aquaculture industry and results in a decreased immune response, thus, allowing the spread of infectious diseases (Ahmadifar et al. 2019). Ideally, neither children nor adults should have any mercury in their bodies, as it has no physiological role. Both prenatal and postnatal exposure to mercury or methyl mercury frequently occurs in various ways (Mergler et al. 2007; Yang et al. 2020). In the present study, FBW, DWG, and SGR were significantly reduced, while FCR was increased in *O. niloticus* exposed to MMC at all tested doses for 60 days. This indicated that MMC might negatively affect nutrient absorption, increase energy consumption related to the detoxification of toxic metabolites, and/or alter the expression of growth hormones (Cao et al. 2012), probably also because fish use most of their body energy to repair damaged cells resulting from mercury toxicity, which may lower the somatic and reproductive growth (Houck and Cech 2004). Similar results were reported by Sharp and Neff (1982), who showed reduced survival in estuarine teleosts exposed to MMC. Moreover, Yu et al. (2019) observed a marked reduction in the growth performance and survival of yellow croaker larvae exposed to MMC. Moreover, methyl mercury is known to generally affect growth performance in various fish species (Mozhdeganloo et al. 2015).

The hematology of fish is particularly sensitive to water toxicants, and hematological parameters can be assessed as indicators of xenobiotic toxicity (Sancho et al. 2000). Changes to hematological and biochemical parameters are useful in studying the toxic effects of metals on fish (Hoyle et al. 2007). We demonstrated that the sub-chronic exposure of *O. niloticus* to MMC significantly reduced the RBC count

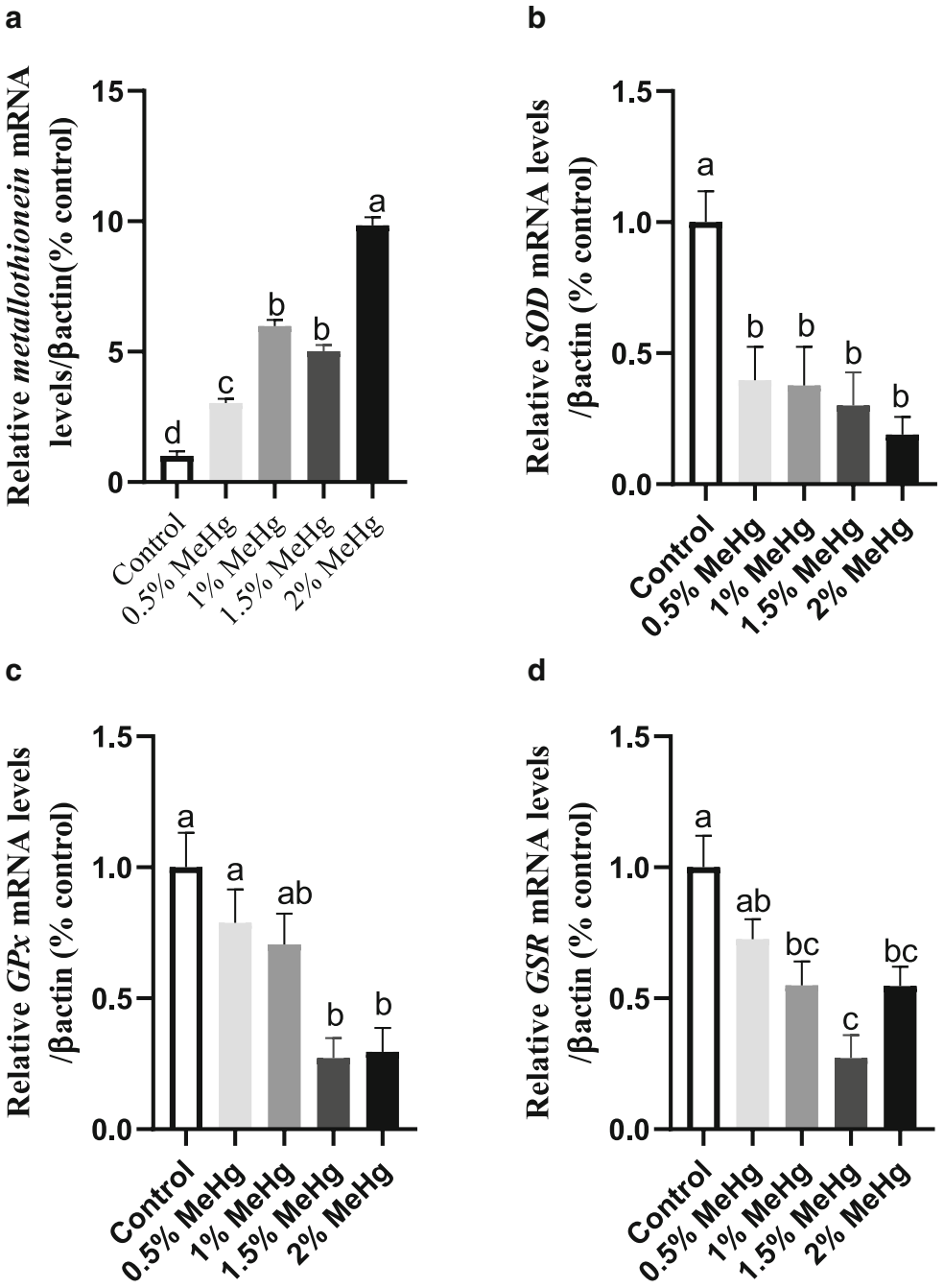

Fig. 1 Effects of the sub-chronic dietary exposure of methyl mercury chloride (MMC) for 60 days on the mRNA expression patterns of hepatic metallothionein (MT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione S-reductase (GSR) in *Oreochromis niloticus*. Data are expressed as mean ±SE (three samples/replicate; nine samples/group)

and Hb level, but MCV and MCHC remained normal. Thus, intoxication with MMC resulted in normocytic normochromic anemia in *O. niloticus* after 60 days of exposure, which may be attributed to prolonged exposure damaging the mitochondria and related functions (Zahir et al. 2005), thus disrupting and damaging the erythropoietic cells that suppress cell production from the hemopoietic tissues of fish. Similar results were reported in several previous studies (Shah and Altindağ 2005; Hedayati and Ghaffari 2013; Setiyowati et al. 2019), in which marked reductions in the erythrocytic count and hemoglobin concentration were observed in fish exposed to mercuric chloride, and these results were attributed to decreased production and circulation of RBCs.

Leucocytes have a critical role in fish immunity. They are involved at a cellular level in innate immunity and function in the production of humoral substances such as lectins, cytokines, and complement components (Vetvicka et al. 2013; Ni et al. 2014). In *O. niloticus* exposed to the various MMC concentrations for 60 days, total leucocyte count, lymphocytes, granulocytes, and monocytes were significantly reduced, which may be attributed to the reduced cell production in hemopoietic organs or the exhaustion of total leucocytes in

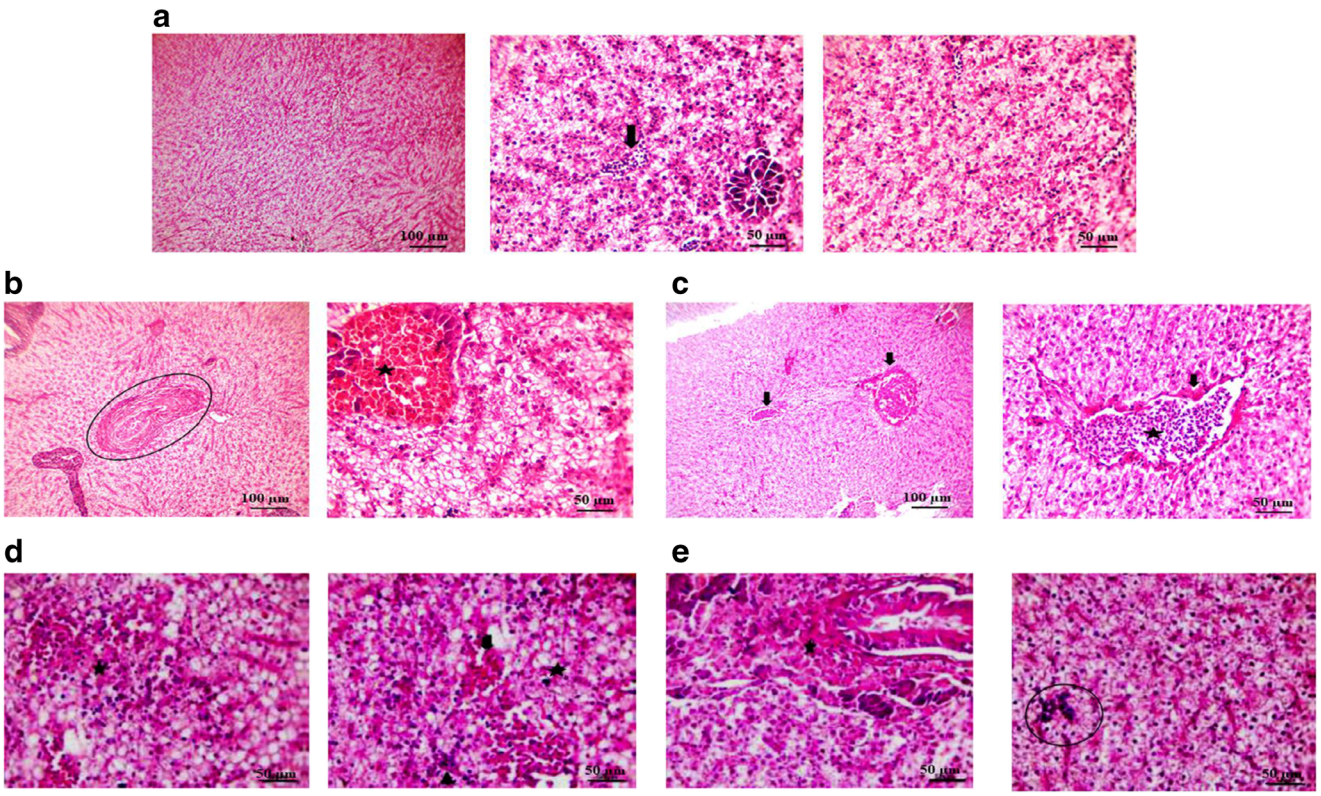

Fig. 2 Photomicrograph of hepatic tissue following exposure to 0%, 0.5%, 1%, 1.5%, and 2% MMC (A-E). **a** Liver tissue of the 0% MMC (control group); (left panel) normal hepatic architectures, (middle panel) contact erythrocytes in the central vein (arrow) beside normal hepatopancreas (star), and (right panel) normal hepatic cells and sinusoids. **b** Liver tissue of the 0.5% MMC; (left panel) focal fibrosis (circle) beside congested blood vessels (arrow), (right panel) melano-macrophages in the hepatopancreatic area (star). **c** Liver tissue of the 1% MMC; (left panel) engorged blood vessels (arrows), (right panel) congested blood vessels (star) with a hyalinized wall (arrow). **d** Liver tissue of the 1.5% MMC; (left panel) necrotic hepatocytes (star), (right panel) necrotic hepatocytes (star) admixed with lymphocytes land erythrocytes (arrow). **e** Liver tissue of the 2% MMC; (left panel) high power to show necrotic debris (star) admixed with inflammatory cells with the presence of pancreatic acini and (right panel) interstitial lymphocytic aggregation (circle)

the immune defense against toxicity. Chronic exposure to MMC can reduce leucocyte functions and cellular viability of T and B lymphocytes; it reduced cellular mitogenic responses and inhibited mixed lymphocyte responses, as well as altered the cellular processes, resulting in apoptosis and cell death (Shenker et al. 1993; Makani et al. 2002; Guzzi et al. 2012). A decrease in leucocyte levels generally reflects reduced fish immunity after exposure to toxic chemicals (Adedeji et al. 2009; Galal et al. 2018).

We observed that exposure to MMC at various concentrations reduced serum total protein, albumin, and globulin levels, which may be attributed to reduced food intake and reduction of protein synthesis by hepatocytes that have degenerated due to the accumulation of MMC in the cells. This was confirmed by the histopathological examination of the liver tissue, which showed necrosis and degeneration of the hepatocytes with necrotic debris admixed with inflammatory cells and lymphocyte aggregation. Similar findings were reported by Jasim et al. (2016). Moreover, MMC toxicity in *O. niloticus* significantly increased the serum triglyceride and cholesterol levels; this may be due to injury to the liver, which is mainly responsible for fat metabolism (Hasheesh et al. 2011), and/or increased levels of cortisol (Borges et al. 2007), which were observed in this study, probably due to MMC stress. Our results agree with those of Sayed and Moneeb (2015), who reported elevated cholesterol levels in *O. niloticus* exposed to methyltestosterone.

Toxicity in the aquatic environment affects fish at the cellular and molecular level, which in turn induces significant alterations in fish biochemical markers (Kavitha et al. 2010). Among several stress biomarkers, serum glucose levels were elevated in *O. niloticus* exposed to MMC in our study. This may have been due to (1) increased gluconeogenesis, which would be required to provide additional energy to upregulate the metabolism as a result of MMC stress (Hedayati and Ghaffari 2013), (2) decreased hepatic glycogenesis and modification of carbohydrate metabolism (Bleau et al. 1996), or (3) increased cortisol levels, which would increase the mobilization of glucose via tissue and liver glycogenolysis (Dobšíková et al. 2009). Similar results were observed in carp stressed by mercuric chloride (Setiyowati et al. 2019). Moreover, serum cortisol levels, another biomarker used as a

primary indicator of the degree of stress in fish (Barton 2002), were elevated in fish exposed to MMC at various concentrations. This may be attributed to the stimulation of the hypothalamic−pituitary−internal axis by MMC, which is a potent chemical stressor (Bleau et al. 1996). Similar results were reported for silver carp intoxicated with mercury chloride (Hedayati and Ghaffari 2013). Using ALT, AST, and ALP as indicators, we also investigated liver function. These indicators are related to nonfunctional enzymes in plasma that are presented within the hepatic cells, kidneys, muscles, and gills of fish (Hadi et al. 2009). We observed increased serum AST, ALT, and ALP levels in *O. niloticus* exposed to MMC, which may be due to the degeneration and necrosis of hepatocytes resulting from the toxicity of MMC in the liver, which in turn likely disrupted cell membrane permeability and released the enzymes from cells into circulation (He et al. 2015; Ma et al. 2015). Our results are in line with those of Qiang et al. (2017).

Metallothionein is a metal-binding protein that is used as a marker to evaluate fish biological events following exposure to MMC. It is an indicator of heavy metal toxicity, as it plays a critical role in detoxifying metals in fish (Choi et al. 2007), is a low-molecular-weight protein, and binds to several elements (Thirumoorthy et al. 2007; Singha Roy et al. 2011). In this study, MT mRNA expression was upregulated in MMC-exposed *O. niloticus*; thus, it may be a biomarker of sub-chronic toxicity as well as rapidly activating hepatic regulation of heavy metal detoxification (De Boeck et al. 2003; Cheung et al. 2005), also, as MeHg exposure induced the elevation of plasma cytokines (i.e., IL6) that induced hepatic MT (Yasutake and Nakamura 2011). Similar MT upregulation was reported in *O. niloticus* exposed to silver nanoparticles and farmed tilapia exposed to cadmium (Thummabancha et al. 2016; Qiang et al. 2017).

Reactive oxygen species (ROS) are produced in fish exposed to toxic metals. Both GSH-Px and GSR play important roles in detoxifying and scavenging the peroxides that result from oxidation, which occurs in cells subjected to free radicals. Moreover, increased levels of SOD assist in the destruction of free radicals generated in the cells (Swiergosz-Kowalewska et al. 2006; He et al. 2015). In the present study, MMC toxicity in *O. niloticus* significantly downregulated the activities of hepatic SOD, GPX, and GSR after 60 days of exposure, suggesting that sub-chronic exposure to MMC causes oxidative stress, alters the oxidation−reduction process, and results in increased damage to cellular components (Basha and Rani 2003). Furthermore, liver antioxidant enzymes are possibly exhausted during the elimination and reduction of ROS induced by MMC stress. MMC has a positively charged reaction center, due to which it is highly reactive to selenol and thiol groups; thus, it can disrupt the functions of antioxidants and proteins, resulting in increased stress (Farina et al. 2011). Therefore, MeHg can interfere with several regulators of the antioxidant response, such as glutathione (GSH) and the antioxidant selenoenzymes thioredoxin reductase (TrxR) and glutathione peroxidase (Gpx) (Antunes Dos Santos et al. 2018). The increase in ROS production and disruption of the antioxidant defense system are the primary mechanisms related to methyl mercury toxicity (Farina et al. 2011). Methyl mercury negatively affects the action and production of glutathione peroxidase (Glaser et al. 2010). Also, it can interact with GSH, leading to the formation of an excretable GS–MeHg complex (Ballatori and Clarkson 1982). This interaction decreases the levels of GSH.

Conclusion

Based on our results, oral exposure to MMC induces various toxic effects in *O. niloticus* in a concentration-dependent manner. Various effects on growth performance, RBCs, WBCs, liver function, stress biomarkers, and hepatic metallothionein and mRNA expression of antioxidant enzyme genes have been reported in this study, all of which indicate negative impacts on *O. niloticus*. These alterations can be considered as bioindicators of toxicity following dietary exposure to MMC in Nile tilapia.

Author contribution RR and EA conceived and designed the research. RR, EA, and RA conducted the experiments. AA did the gene expression and histopathological investigations. EA, BK, and RA analyzed the data and wrote the manuscript. All authors read and approved the manuscript, and all data were generated in-house, and that no paper mill was used.

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval The experiment was conducted and approved according to the Animal Care and Welfare Committee of the Zagazig University, Egypt.

Consent to participate Not applicable.

Consent to publish Not applicable.

Conflict of interest The authors declare no competing interests.

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