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Evaluation of Physiological Stress of Grass Carp Chronically Exposed to Enrofloxacin Based on IBR Index

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Abstract Enrofloxacin (ENR) is a commonly used drug in aquaculture, and it is frequently detected in the aquatic environment. Data on ENR toxicity toward aquatic species are limited. This study was aimed at using different biomarkers to evaluate the possible toxic effects of grass carp (*Ctenopharyngodon idella*) exposed to 0 (control), 1, 100, and 10,000 μg/L enrofloxacin for 21 days as a sub-chronic exposure trial, oxidative stress biomarkers (including superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and malondialdehyde (MDA)), neurotoxicity indicators (including acetylcholinesterase (ACHE) activity, nitric oxide (NO)), and digestive enzyme activities (including lipase (LPS), amylase (AMS) enzymes). In addition, an integrated biomarker response (IBR) index was utilized to evaluate the integrated toxic effects of ENR on grass carp. Our results demonstrated that ENR exposure significantly increased activities of CAT, LPS, and AMS. ENR exposure also significantly upregulated the expression levels of *sod1*, *ACHE*, *LPL*, *ATGL*, and *AMY* genes. Furthermore, histopathological changes were observed in the hepatopancreatic tissues of grass carp exposed to ENR. It was observed that higher IBR scores were noticed in the tissues of fish exposed to ENR, suggesting an induced biological response. The comprehensive biomarker index showed that CAT and ACHE activities have a higher response to ENR, and 100 μg/L has a greater impact on grass carp. These results indicate that ENR has a toxic effect on grass carp and impairs their physiological functions. This is the first study to explore the effects of ENR on grass carp, and it provides basic information for assessment of ENR effects in aquaculture.

Keywords Grass carp · Enrofloxacin · Physiological stress · Integrated biomarker response

1 Introduction

Population growth, over-reliance on fossil fuels, and climate change could threaten global food security in the future (Fry et al., 2016; Yogev et al., 2020). Aquatic food has made a significant contribution to alleviating this pressure. In order to safeguard aquaculture production, antibiotics are inevitably used to prevent and treat diseases that occur in aquaculture (Chen et al., 2020; Zhou et al., 2021). Antibiotics have been the focus of many ecotoxicological studies

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due to their persistence in the environment and high ability to produce physiological effects (Perussolo et al., 2019; Rodrigues et al., 2019). For example, antibiotics can inhibit fish survival, development, and hatching rates by primarily disrupting the intracellular redox balance and inducing oxidative stress (Yang et al., 2020).

ENR, a fluoroquinolone, exerts its antibacterial effect by inhibiting DNA gyrase (a type II topoisomerase) (Sehonova et al., 2019). It is widely used in the prevention and treatment of biological diseases in aquaculture (Li et al., 2017, 2018; Zhang et al., 2019). ENR is released into the water environment because it is not fully metabolized by the body (Ren et al., 2021). Considering that ENR can be rapidly adsorbed by soil particles and 90% dissipation time of ENR in soil and marine sediments is greater than 150 days, it will slowly desorb from soil particles (Dalla Bona et al., 2015; Wei et al., 2012). The continued release of ENR may pose a potential risk to aquatic life. At present, ENR has been found in various concentrations in aquatic environments around the world (Huang et al., 2020a; Andrieu et al., 2015; He et al., 2019; Tang et al., 2015; Teglia et al., 2019; Han et al., 2020). For example, the maximum concentration of ENR detected was 5.68 μg/L in 24 water samples from two rivers in North China (Cheng et al., 2019). The concentration of ENR in seawater around Xiamen Island was 24 ng/L (Chen et al., 2021). Previous studies have detected ENR in fish muscles. For example, the residual levels of ENR in the muscles of six fish species from the Karakaya Dam Reservoir in Turkey ranged from 0.0034 to 0.0073 mg/kg (Varol & Sunbul, 2019).

Adverse reactions of ENR released into the water environment to nontargeted organisms have been reported. For example, in juvenile giant freshwater prawn *Macrobrachium rosenbergii*, ENR inhibited the growth of shrimp, caused damage to the gill and hepatopancreas tissue, and also showed to induce oxidative stress (Zhang et al., 2019). A previous studies by Du et al. (2022). showed that ENR affects glycolysis/gluconeogenesis and the pentose phosphate pathway, which indirectly affects nutrient absorption and meat quality, in the gut of American shad. Qiu et al. showed that ENR can produce immune suppression on fish and confirmed for the first time that the immune suppression by ENR is closely mediated through alterations of the intestinal microbiome in fish (Qiu et al., 2022). However, there are few studies on toxic effects of ENR on aquatic organisms, and there is a lack of comprehensive analysis of indicators. Integrated biomarker response (IBR) is a comprehensive analysis method, which compares the specific adverse effects of harmful substances by simple calculation, screens sensitive biomarkers, and further accurately and effectively evaluates the ecological risk of the environment (Liao et al., 2021; Samanta et al., 2018). Samanta et al. integrated oxidative stress and histopathological changes in fish gills, liver, and kidneys through IBR to study the adverse effects of domestic, industrial, and hot spring on fish inhabiting polluted streams (Samanta et al., 2018). Superoxide dismutase, catalase, reduced glutathione, and malondialdehyde are sensitive markers to assess the antioxidant response and lipid peroxidation caused by chemicals in the external environment (Huang et al., 2020b). For example, a study has found that greater antioxidant was with the higher IBR index of limpets in polluted environment (Silva et al., 2018). Acetylcholinesterase and nitric oxide are sensitive biomarkers of neurotoxicity when organisms are challenged by toxicants and adverse environmental conditions (Shi et al., 2018; Iheanacho & Odo, 2020; Mukherjee et al., 2019). The activity of analyze lipase and amylase can be used as biomarkers to assess the health status of an organism (Li & Li, 2020). To date, few studies have employed IBR to quantify the effects of ENR on aquatic organisms. In this study, grass carp were exposed to different concentrations of ENR (1, 100, 10,000 μg/L) for 21 days to explore the comprehensive impact of ENR on grass carp. This will enrich our understanding of the ecological risks of ENR in aquaculture.

2 Material and Methods

2.1 Chemicals

Enrofloxacin (ENR) was purchased from Hefei Bomei Biotechnology Co. Ltd (Anhui, China) and the purity was above 98%.

2.2 Animal Experiment

Grass carps (weight 91.975 ± 13.17 g, length: 21.01 ± 0.87 cm) were obtained from Rushan fish

breeding base (Shandong, China). After 14 days of adaptation, they were randomly distributed into four treatments (15/group in three plastic tanks (20 L), $n=5$ per tank). The concentrations used included 1, 100, and 10,000 μg/L. Exposure doses of ENR were selected based on ambient concentrations and those used in production (Zhang et al., 2019; Zheng et al., 2020; Li et al., 2020b; Liang et al., 2014; Phillips et al., 2016). The water temperature was controlled at 23 °C. The photoperiod was kept at 14:10-h light/dark cycle. The fish were fed with commercial fish food (Xinda, Tianjin, China) twice a day. The water (half of the volume) of each experimental tank was replaced every 48 h to maintain the appropriate concentration and water quality. All solution samples were analyzed by HPLC equipped with a fluorescence detector, which was based on the method (Sun et al., 2014). According to the analyzed results, the measured concentration of ENR (0.92 ± 0.05, 95.14 ± 11.42, and 9012.97 ± 159.32 μg /L, corresponding to the 1, 100, and 10,000 μg /L) was within 20% of the nominal concentration, which meets the OECD guidelines (the OECD guideline for testing of chemicals No. 204, "Fish, Prolonged Toxicity Test").

All procedures and animal handling comply with the guidelines approved by the Local Animal Ethics Committee. No fish mortality occurred during the toxicity tests.

After 21 days, grass carps were euthanized with MS-222 (0.03%, Sigma-Aldrich Corp.). The weight and length of the fish were measured. Calculate the condition factor (CF, whole fish weight (g)/whole fish length $(cm)^3 \times 100$). The brain, hepatopancreas, and intestines were frozen in liquid nitrogen and stored at −80 °C.

2.3 Biochemical Biomarkers

Oxidative stress indexes (SOD, CAT, GSH, and MDA) in the hepatopancreas, neurotoxicity indexes (ACHE and NO) in the brain, and digestive enzymes (LPS and AMS) in the intestine were analyzed using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to manufacturer's protocol. References of all biomarker kits are in the supporting information (Table S1).

2.4 Histopathological Biomarker

Fragments of hepatopancreas were immobilized in 4% paraformaldehyde. They were then dehydrated in alcohol (70%, 80%, 90%, and 100%), transparentized in xylene, and incorporated into Paraplast. They were cut into 5 μm slices and stained with hematoxylin–eosin (HE). For the nuclear area, each sample captured 3 images under a × 1000 microscope. In each image, the area of 12 nuclei was measured and analyzed using ImageJ.

2.5 Quantitative Real-Rime Polymerase Chain Reaction (qPCR)

The RNA was extracted according to the manufacturer protocol from the brain, hepatopancreas, and intestines ($n=3$) using Trizol (Accurate Biotechnology Co., Ltd., Hunan, China). Then, use Evo M-MLV RT Kit and gDNA Clean for qPCR II Kit (Accurate Biotechnology Co., Ltd., Hunan, China) for reverse transcription. Use the Roche 96 Light Cycler RT-PCR system (Roche Applied Science, Indianapolis, IN, USA) to perform qRT-PCR on the target gene. The reaction system included 5 μL SYBR® Green Premix Pro Taq HS, 0.2 μL PCR forward primer (10 μmol/L), 0.2 μL PCR reverse primer (10 μmol/L), 2 μL cDNA template, and 2.6-μL DEPC-treated water. The amplification procedure used a two-step method: pre-denaturation at 95 °C for 60 s, followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s. *β-Actin* was cited in Wang et al.'s previous work (Wang et al., 2015) (F: GGCTGTGCTGTCCCTGTA, R: GGGCATAAC CCTCGTAGAT. GenBank access: M25013). *Sod1* was cited in Wang et al.'s previous work (Wang et al., 2019). NCBI primer blast program (https://www.ncbi.nlm.nih.gov/) is used to determine the biochemical biomarker-related pathway primer sequences of other genes. The primers used for qRT-PCR analysis are listed in Table 1. Functional gene primers are synthesized by Tsingke Biotechnology Co., Ltd. Use the $2^{-\Delta\Delta Ct}$ formula to calculate the gene fold change (Livak and Schmittgen, 2001). And the primer amplification efficiency of the selected genes in the study has been shown in Table S2.

Table 1 Selected genes, primer sequences, and GenBank access

Gene name	Primer sequence 5′-3′	Product size (bp)	Tm (℃)	GenBank access
Sod1	F: CGCACTTCAACCCTTACA	218	61.5	GU901214.1
	R: ACTTTCCTCATTGCCTCC			
cat	F: TTGAACCGAAACCCCGTGAA	139	60.1	MG821473.1
	R: GCCGATGTGTGTCTGGGTAA			
LPL	F: AACGAGAGCCAACAGCCAA	186	59.2	FJ436077.1
	R: GAGCACCAAGACTGAAGCC			
ATGL	F: TTCCGTGGTGTGCGTTATGT	134	60.1	HQ845211.2
	R: TGGAAGCTGGTGGAACTGTC			
AMY	F: TTCCGTGGTGTGCGTTATGT	134	60.1	FJ641975.1
	R: TGGAAGCTGGTGGAACTGTC			

2.6 Integrated Multi-level Biomarker Response

This study used SOD, CAT, GSH, MDA, ACHE, NO, LPS, and AMS activity to calculate the biomarker reaction version 2 comprehensive index (IBRv2) (Sanchez et al., 2013; Beliaeff & Burgeot, 2002) results. IBRv2 was calculated following the methods described by Beliaeff and Burgeot (2002) with modifications from Sanchez et al. (2013). Details of IBRv2 can be found in the support information (text S1).

2.7 Statistical Analysis

Mean ± standard error of the mean (SEM) was used to calculate all data. Three biological replicates were set for each treatment. Statistical analyses use SPSS statistics 19 (SPSS Inc., Chicago, Ltd., USA). The data were determined for normality and homogeneity of variances using the Shapiro–Wilk test and Levene's test, respectively. If the data does not satisfy the normal distribution and variance homogeneity, Log-transformation is performed on the data. If the data is not satisfied after Log-transformation, nonparametric analysis is used. One-way analysis of variance (ANOVA) and Dunnett's test could be used to analyze statistically significant differences between treatments and the corresponding control. The level of significance was set at $P < 0.05$ (*) and $P < 0.01$ (**).

3 Results

3.1 Growth Performance

Exposure to ENR had no effect on CF index of grass carp. CF is shown in the supporting information (Fig. S1).

3.2 Molecular and Biochemical Responses

3.2.1 Oxidative Stress Responses

Compared with the control group, the activities of SOD (Fig. 1a) and GSH (Fig. 1c) and the content of MDA (Fig. 1d) showed no significant differences after 21 days of exposure to ENR. Compared with the control group, the activity of CAT (Fig. 1b) was significantly increased in the 1 μg/L ($p < 0.05$), 100 μg/L ($p < 0.01$), and 10,000 μg/L ($p < 0.01$) ENR exposure group.

Through exposure to ENR, the transcription level of *sod1* (Fig. 1e) in hepatopancreas of grass carp in all exposed groups was upregulated compared with the control. When the concentration was 1 μg/L, it was significantly upregulated ($p < 0.01$). The transcription level of *cat* (Fig. 1f) in all exposed groups was upregulated compared with the control, but not significantly.

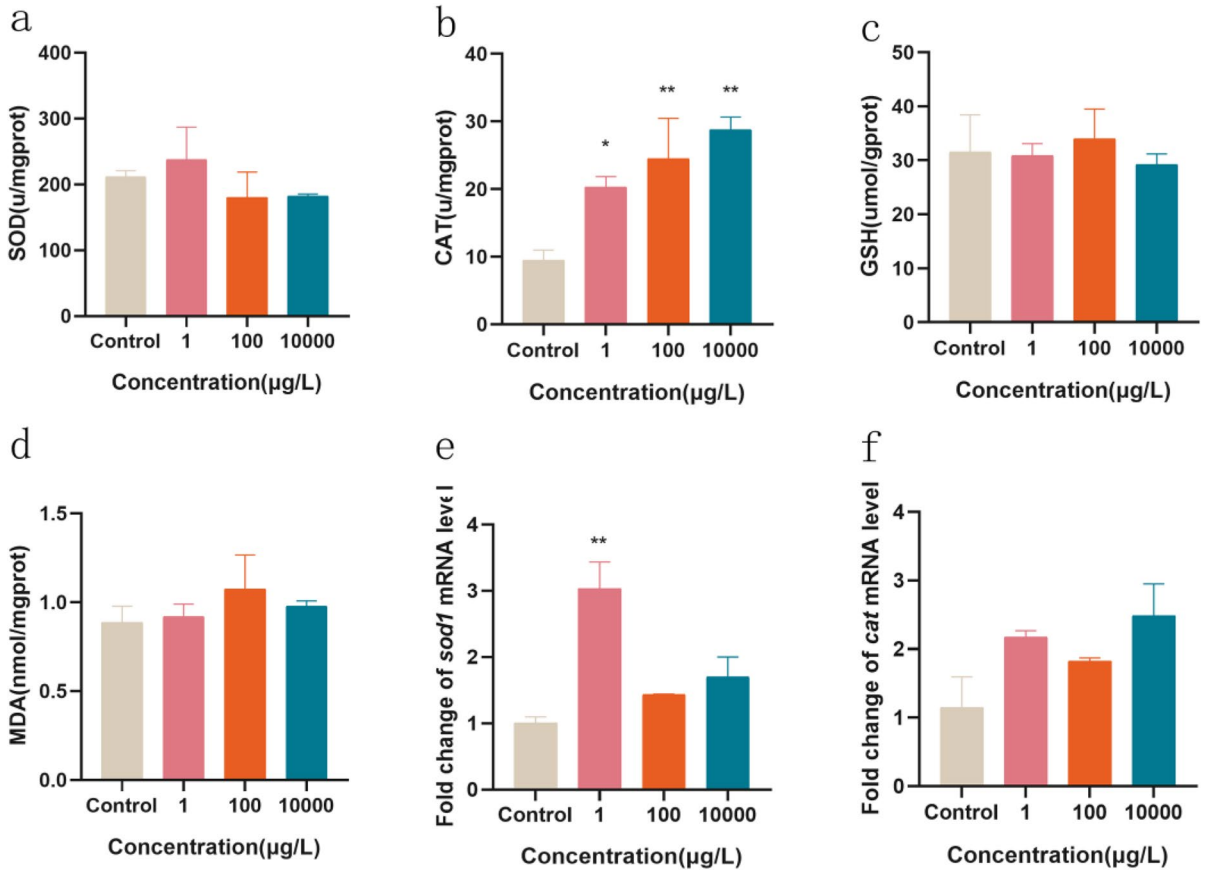

Fig. 1 Enzyme activities of SOD (**a**), CAT (**b**), GSH (**c**), content of MDA (**d**), and the transcription levels of *sod1* (**e**) and *cat* (**f**) in hepatopancreas of grass carp after 21 days of exposure to different concentrations of ENR (0, 1, 100, and 10,000 μg/L). Data are mean ± SEM ($n = 3$). *$p < 0.05$; **$p < 0.01$ relative to the control

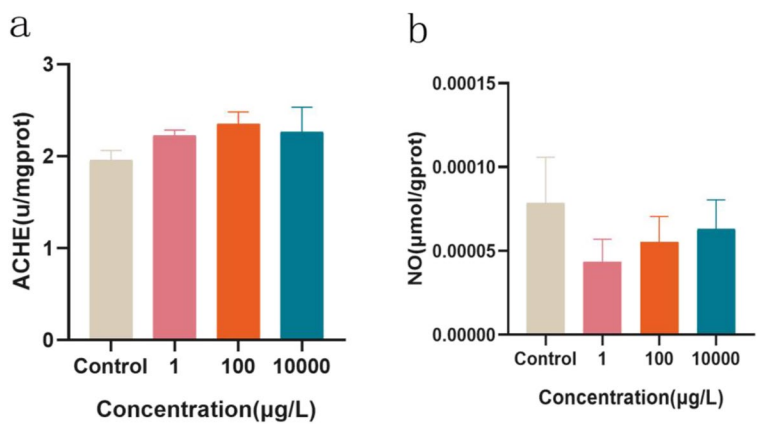

Fig. 2 Enzyme activities of ACHE (**a**) and content of NO (**b**) and the transcription levels of *ACHE* (**c**) in the brain of grass carps after 21 days of exposure to different concentrations of ENR (0, 1, 100, and 10,000 μg/L). Data are mean ± SEM ($n = 3$). *$p < 0.05$; **$p < 0.01$ relative to the control

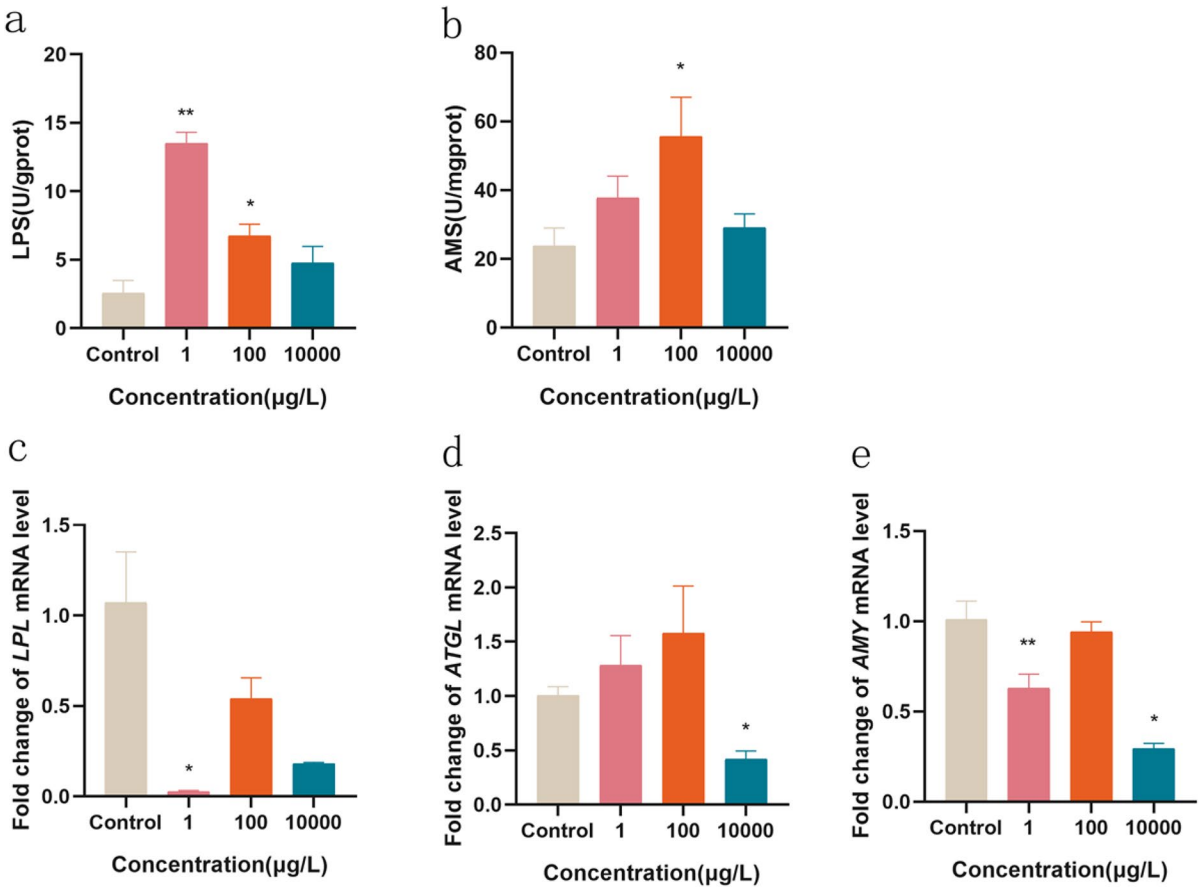

Fig. 3 Enzyme activities of LPS (**a**), AMS (**b**), and the transcription levels of *LPL* (**c**), *ATGL* (**d**), and *AMY* (**e**) in the intestines of grass carps after 21 days of exposure to different concentrations of ENR (0, 1, 100, and 10,000 μg/L). Data are mean ± SEM ($n = 3$). *$p < 0.05$; **$p < 0.01$ relative to the control

3.2.2 Neurotoxicity Responses

After 21 days of exposure to ENR, the activity of ACHE (Fig. 2a) and the content of NO (Fig. 2b) showed no significant difference compared with the control group. But the activity of ACHE increased with the increase of exposure concentration, and the content of NO was lower than the control group.

3.2.3 Digestive Enzyme Responses

Compared with the control group, LPS (Fig. 3a) activity increased significantly at exposure concentrations of 1 μg/L ($p < 0.01$) and 100 μg/L ($p < 0.05$). AMS (Fig. 3b) activity in all exposed groups was higher than that in the control group. And it was significantly increased when exposure concentration was 100 μg/L ($p < 0.05$).

The expression of *LPL* (Fig. 3c) was inhibited in the intestinal tract of grass carp exposed to ENR, and the mRNA transcription level of *LPL* was significantly downregulated ($p < 0.05$) when the concentration was 1 μg/L. Compared with the control group, the mRNA transcription level of *ATGL* (Fig. 3d) significantly decreases ($p < 0.05$) when the concentration is 10,000 μg/L. The mRNA transcription level of *AMY* (Fig. 3e) was significantly downregulated at 1 μg/L ($p < 0.05$) and 10000 μg/L ($p < 0.05$).

3.3 Hepatopancreas Histopathology

The results of histopathological examination of the hepatopancreas of grass carp are shown in Fig. 4. The

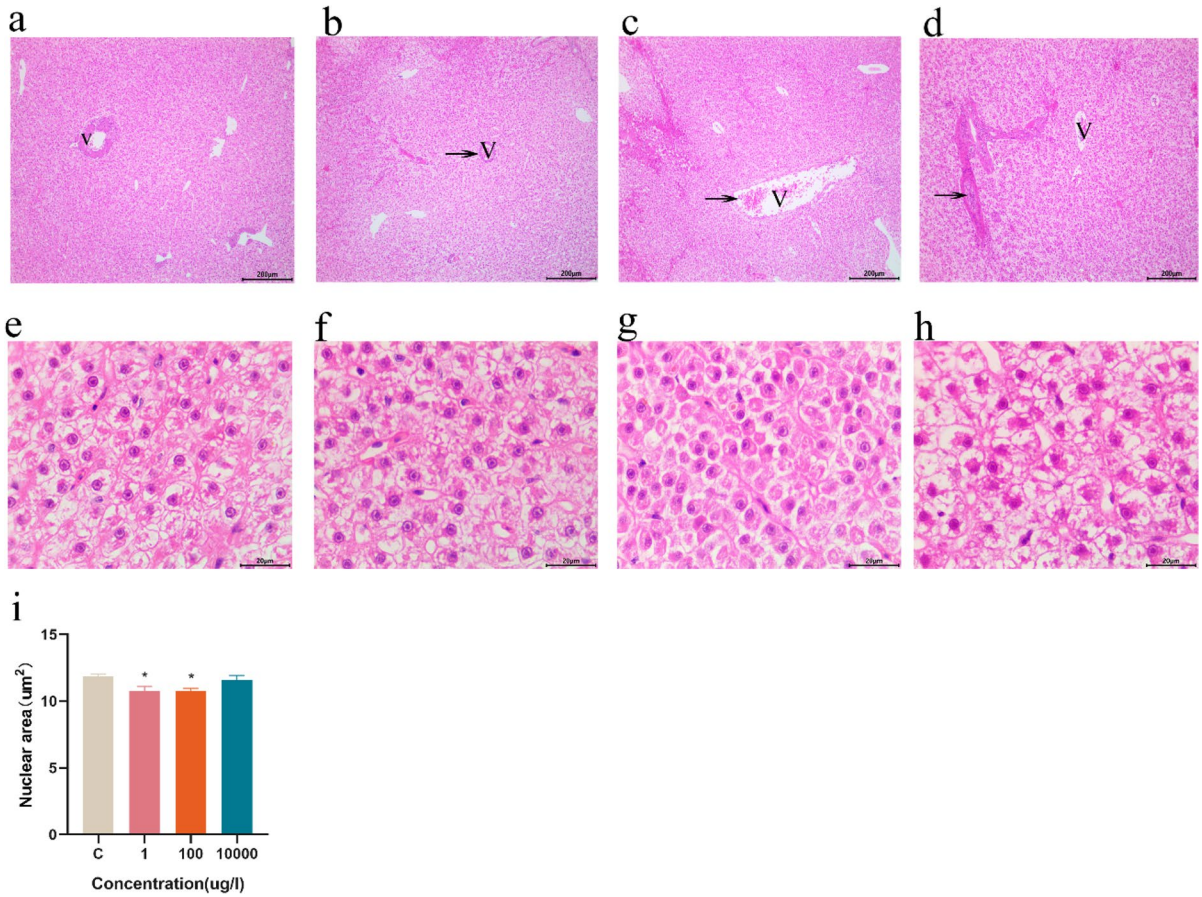

Fig. 4 Representative photomicrographs of hepatopancreas tissue sections of grass carp after 21 days of exposure to different concentrations of ENR (0, 1, 100, and 10,000 μg/L). **a** control × 100; **b** 1 μg/L × 100; **c** 100 μg/L × 100; **d** 10,000 μg/L × 100; **e** control × 1000; **f** 1 μg/L × 1000; **g** 100 μg/L × 1000; **h** 10,000 μg/L × 1000; **i** hepatopancreas cell nuclear area. Data are mean ± SEM ($n = 12$). V, blood vessel; arrow, hyperemia

structure of normal hepatopancreas tissue is neatly arranged, the cytoplasm is intact, and the nucleus is clear (Fig. 4a, e). Compared with the control group, the exposure group had vascular congestion, especially in the portal vein (Fig. 4b–d, f–h). Exposure to ENR can also cause cell nuclei to become smaller (Fig. 4i). Compared to the control group, the nuclei in the 1 μg/L ($p < 0.05$) and 100 μg/L ($p < 0.05$) groups were smaller.

3.4 Integrated Biomarker Response

In this study, eight biomarkers were selected to explore whether grass carp exposed to ENR would be toxic. The star plot of the IBR index of grass carp exposed to different concentrations of ENR is shown in Fig. 5a, and the obtained IBR value is shown in Fig. 5b. In all exposed groups, the content of NO was inhibited, while the activities of CAT, ACHE, LPS, AMS, and the contents of MDA were induced. Among all the biomarkers measured, the changes in CAT and ACHE were obvious. IBR values show that the stress caused by 100 μg/L is the highest.

4 Discussion

Oxidative stress is closely related to fish growth and health during fish culture. Fish under oxidative stress are often weak in immunity and disease resistance, so their health status and growth performance are negatively affected. Studies have shown that organisms

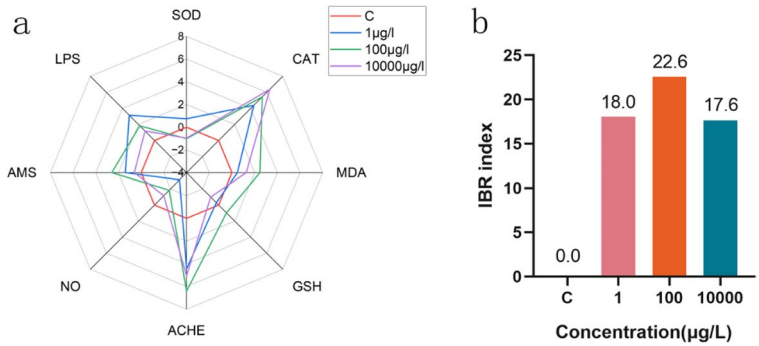

Fig. 5 **a** Related star maps and comprehensive biomarker response index (IBRv2) values of grass carp after 21 days of exposure to different concentrations of ENR (0, 1, 100, and 10,000 μg/L). Abbr.: SOD superoxide dismutase activity, CAT catalase activity, MDA malondialdehyde, GSH glutathione, ACHE acetylcholinesterase activity, NO nitric oxide, LPS lipase, AMS amylase. The area above 0 reflects induction of the biomarker and below 0 indicates reduction of the biomarker. **b** Histogram of IBRv2

exposed to pollutants increase the production of reactive oxygen species (ROS) due to increased metabolic and biotransformation activities, which further increase the antioxidant enzyme activity and/or transcription level in the body (Regoli & Giuliani, 2014). SOD and CAT play a huge role in removing active oxygen and the body's protective defense response (Zhang et al., 2017). SOD can effectively remove the superoxide anion free radicals generated during the oxidation process in organisms and convert them into H_2O_2 and O_2. GSH can be used as a cofactor to combine with exogenous compounds to degrade toxic substances out of the body (Ajima et al., 2021; Sehonova et al., 2019; Su et al., 2019). MDA is a toxic product of lipid peroxidation, which can indirectly reflect the degree of oxidative damage in the body (Zhang et al., 2017). When the antioxidant system is unable to eliminate free radicals produced by xenobiotics, the content of MDA will increase (Lin et al., 2016). For example, Sehonova et al. exposed zebrafish to 5, 10, and 500 μg/L enrofloxacin for 14 days, and the results showed that enrofloxacin can cause oxidative stress in exposed fish and lipid peroxidation was observed at the highest concentration (Sehonova et al., 2019). The enhanced expression of antioxidant genes and enzyme activity observed in this study indicated increased ROS production. However, MDA content did not change, which may be due to the antioxidant response to avoid the oxidation of macromolecules due to oxidative stress (Grott et al., 2021). In this study, SOD activity did not change significantly, while CAT activity increased significantly compared with the control, possibly because CAT involved in the removal of H_2O_2 was more active compared with SOD activity (Magara et al., 2018). It is not surprising that the gene transcription level of SOD increases without changing at the functional level. Because there are multiple regulatory steps involved between transcription and translation in eukaryotic cells, there is a delay (Defo et al., 2015).

ACHE activity has been widely recognized as a sensitive biomarker of neurotoxicity of organisms (Shi et al., 2018; Iheanacho & Odo, 2020). It not only inactivates acetylcholine, ensuring the normal function of the neuromuscular system, but also participates in neurodevelopment (Yang et al., 2018). Alterations in ACHE activity may lead to changes in cholinergic neurotransmission (Yuan et al., 2018). The increased levels of ACHE hydrolyzed the acetylcholine produced in the body, which eventually led to the reduction of neurotoxicity (Li et al., 2020a). In this study, the activity of ACHE increased. The results of this study may be due to overcompensation (Badiou et al., 2008; Pan et al., 2012). NO is a neurotransmitter that is enzymatically synthesized by nitric oxide synthase (NOS) in various cell types (Carreno Gutierrez et al., 2020; Serafini et al., 2020). NO plays an important role in cell signal transmission and neurotransmission (Jay et al., 2014). In this study, the level of NO decreased compared to the control group, which may be due to the generation of ROS that reduced the level of NO in the brain. NO reacts quickly with oxygen free radicals (O_2•-) to produce peroxynitrite anion (ONOO-), which can further form

peroxynitrous acid (ONOOH), thereby reducing the level of NO (Liu et al., 2013).

In general, most pollutants entering an organism enter the intestines, where they alter the activity of intestinal enzymes that play an important role in digestion (Adeyemi et al., 2020; Xie et al., 2019). Therefore, the activity of digestive enzymes can be used as a biological indicator of fish growth and health (Xie et al., 2019). Changes in the activity of biological digestive enzymes may change the processing of dietary inputs, which in turn may affect energy metabolism (Kong et al., 2019). Wang et al. showed that under severe ammonia stress, fish consume more energy by increasing digestive enzymes in the intestine to maintain the balance of ammonia accumulation and metabolism in the body (Wang et al., 2021). Previous studies have fed zebrafish with oxytetracycline (OTC) at a therapeutic concentration (80 mg/kg body weight) for 6 weeks. The results showed increased amylase and lipase activities, indicating that fish need more energy to resist the pressure caused by antibiotics (Limbu et al., 2020; Zhou et al., 2018). In this study, the activity of amylase and lipase increased significantly, which may be due to the increase of energy supply by fish to enhance the body's adaptability to ENR. Previous studies have shown that there was a low correlation between gene expression and enzyme activity of AMS and trypsin. For example, the study by Xie et al. showed that the activities of lipase and amylase under Cd^{2+} exposure were significantly inhibited, while significantly downregulating the activity of the respective encoded enzymes (Xie et al., 2019). In Houde et al.'s previous study, AMS gene expression was upregulated and AMS enzyme activity was inhibited under hexachlorocyclopentadiene exposure (Houde et al., 2013). Our research results show that the activities of lipase and amylase are significantly increased, while the mRNA expression level of related enzymes is significantly downregulated. These results suggest that gene expression and enzyme activity may show different responses under different conditions (Schwarzenberger & Fink, 2018). The difference between mRNA expression level and related biochemical reactions needs further study (Kim & Jung, 2016; Defo et al., 2015).

Histopathology can reflect the health status of organisms exposed to a variety of environmental pollutants (Correia et al., 2020; Lam et al., 2013). It can be employed as biomarkers of contaminant exposure and consequences (Iftikhar et al., 2022). Because of its sensitivity to aquatic pollution, the hepatopancreas is often used as a histopathological index to assess the health of fish (Yancheva et al., 2016). Our histopathological results indicate that grass carp hepatopancreas may be affected by ENR. The hepatopancreas of fish exposed to ENR will show congestion and a decrease in the area of the nucleus. Congestion may be caused by increased blood pressure after exposure to poison (Bernet et al., 1999). Compared with the control group, the area of hepatocyte nucleus is smaller. Similar to the results of this study, Zhang et al. exposed *Macrobrachium rosenbergii* to 5 mg/L ENR for 14 days and found that its hepatopancreas nucleus had coagulated and became smaller (Liu et al., 2015; Zhang et al., 2019). A small nucleus is a sign of apoptosis. Previous studies have shown that ENR can induce hepatocyte apoptosis (Liu et al., 2015; Zhang et al., 2019).

The IBR index is considered to be a general description of the "health" of the animal in the environment (Adeyemi et al., 2020). It is used to prove the environmental stress caused by animals exposed to xenobiotics (Adeyemi et al., 2020). It can help integrate all the parameters to show changes that could not be so easily noticed (Mukherjee et al., 2022). In this study, the method was used to integrate biochemical indicators to understand the impact of ENR on grass carp more comprehensively. These results showed that the response of CAT and ACHE activity might imply its high sensitivity to ENR. This is consistent with the analysis results of a single indicator. The results of these two indicators were more representative, which helped us better understand the toxicity of ENR to grass carp. Interestingly, the stress caused by ENR exposure is stronger at 100 μg/L. This may be because high concentrations of ENR trigger defense mechanisms more rapidly, and after a short-time response, the biomarker gradually adjusts and returns to a baseline condition. IBR indicators can be a quantitative and effective tool for monitoring ENR on fish toxicology.

5 Conclusions

This study was a contribution to the assessment of adverse effects of ENR on grass carp. Multi-biomarker methods indicate that exposure to ENR can

cause grass carp oxidative damage and neurotoxicity and affect digestive enzyme activity. In addition, our results indicate that CAT and ACHE activities have a higher response to ENR. CAT and ACHE activities are more sensitive to ENR. Further, it was observed that a concentration of 100 µg/L ENR was the most toxic to grass carp after 21 days of exposure. Histopathological results indicate that ENR may induce cell apoptosis and cause hepatopancreas cell damage in grass carp. Considering the large-scale use of ENR in aquaculture, it may pose a major risk to aquatic organisms. The concentration of ENR in the aquatic environment needs to be controlled to avoid further harm to humans.

Author Contribution Xu-Qian Cao: writing original draft preparation; Xu Wang: methodology and software; Bin Liu: index measurement; Shu-Wen He: index measurement; Zhi-Han Cao: fish culture and sampling; Shao-Ying Xing: fish culture and sampling; Ping Li: writing, reviewing, and editing; Zhi-Hua Li: conceptualization and overall guidance.

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Data Availability The data and materials that support the findings of this study are available from the corresponding authors upon reasonable request.

Code Availability Not applicable.

Declarations

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Approval of Animal Ethics Committee of Shandong University was taken.

Consent to Participate It is not applicable.

Consent for Publication All the authors are in agreement with the publishment.

Conflict of Interest The authors declare no competing interests.

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