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Grass carps co-exposed to environmentally relevant concentrations of cypermethrin and sulfamethoxazole bear immunodeficiency and are vulnerable to subsequent *Aeromonas hydrophila* infection☆

Hongjing Zhao [a, b, 1], Yu Wang [a, 1], Menghao Guo [a], Mengyao Mu [a], Hongxian Yu [b, **], Mingwei Xing [a, *]

[a] College of Wildlife and Protected Area, Northeast Forestry University, Harbin, 150040, Heilongjiang, PR China
[b] Department of Ecology, College of Wildlife and Protected Area, Northeast Forestry University, Harbin, 150040, Heilongjiang, PR China

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ABSTRACT

The aquatic ecosystem is seriously damaged because of the heavy use of pesticides and antibiotics. Fish is the indispensable link between environmental pollution and human health. However, the toxic effects of environment-related concentrations of pesticides and antibiotics in fish have not been thoroughly studied. In this study, grass carps exposed to cypermethrin (CMN, 0.651 μg/L) or/and sulfamethoxazole (SMZ, 0.3 μg/L) for 42 days caused oxidative stress, apoptosis and immunodeficiency in the spleen of grass carps. CMN or/and SMZ exposure led to oxidative damage (consumption of antioxidant enzymes (superoxide dismutase and catalase)) and lipid peroxidation (accumulation of malondialdehyde), induced apoptosis (increases in TUNEL index, Bax/bcl-2, p53, puma and Caspase family expression). In addition, the levels of immunoglobulin M (IgM), complement 3 (C3) were significantly decreased in all treatment groups, which trend was also found in C-reactive protein in CMN and MIX group, and lysozyme in MIX group. Transcription of almost all genes involved in the Toll-like receptors (TLR) signaling pathway was up-regulated under CMN or/and SMZ exposure. However, when subsequently attacked by *Aeromonas hydrophila* for 2 days, the TLR pathway was inhibited in spleens of all treatment groups accompanied by higher mortality. Overall, the environmentally relevant concentration of CMN and SMZ damages the immune system, triggering oxidative stress and apoptosis in carps. And by affecting the conduction of TLR signaling pathway, CMN or/and SMZ exposure inhibits the innate immune response of fish and reducing their disease resistance. This study highlights the importance of rational and regulated use of these pesticides and antibiotics.

1. Introduction

In agricultural production, in order to control crop diseases and insect pests, the frequency of pesticide use has been increasing, resulting in their widespread presence in air, soil, and aquatic environments (Akhtar et al., 2014). Environmental pollution caused by pesticides, especially in freshwater ecosystems, is the most important environmental problem currently facing developing countries (Mahboob et al., 2015). Cypermethrin (CMN), a widely used type II pyrethroid insecticide, has the advantages of excellent biological activity, broad spectrum and high efficiency (Jin et al., 2011). CMN to mammals and birds has lower toxicity, but extremely high toxicity to aquatic organisms. The insecticidal compound has high hydrophobicity, especially to fish gills adsorption. At the same time, there is a lack of enzyme in fish body that can hydrolyze this kind of insecticide, so it can cause slow metabolism of fish body, eventually leading to fish poisoning (Soltanian and Fereidouni, 2017).

Abbreviations: CMN, cypermethrin; SMZ, sulfamethoxazole; C3, Complement 3; CRP, C-reactive protein; LYS, lysozyme; ROS, reactive oxygen species; TLRs, Toll-like receptors; PAMPs, pathogen-associated molecular patterns; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; GSH, glutathione; ELISA, Enzyme-linked immunosorbent assay; MMC, melanomacrophage centers; *A. hydrophila*, *Aeromonas hydrophil.*

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* Corresponding author.
** Corresponding author.
E-mail addresses: zhaohongjing@nefu.edu.cn (H. Zhao), wangyu2013@nefu.edu.cn (Y. Wang), china.yhx@163.com (H. Yu), xingmingwei@nefu.edu.cn (M. Xing).

[1] Hongjing Zhao and Yu Wang contributed equally to this article.

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Antibiotics are widely used because they have the effect of killing or inhibiting the growth of pathogenic microorganisms. In China, more than half of the antibiotics produced are used in the livestock and aquaculture industry to promote animal growth and treat bacterial diseases (Zhang et al., 2015). Sulfamethoxazole (SMZ) is one of the most widely prescribed antibiotics. In the aquatic ecosystem, it mainly comes from livestock and domestic waste water (Zhou et al., 2016). Because antibiotics have lower bioavailability, higher water solubility, and are not easily degraded in the aquatic environment. During activated sludge treatment, the removal rate of SMZ in waste water is between 20% and 30%. Therefore, the residues of antibiotics have a great impact on the health of aquatic animals, and fish are highly sensitive to their exposure (De Lemos et al., 2007). Grass carp (*Ctenopharyngodon idellus*) is one of the most common species in the freshwater fish breeding market in China with proportion up to 5.9 million tons. However, this specie has stronger response under pressure stress, and it is more likely to be polluted by environmental pollutants (Fenoll et al., 2012).

After pesticides and antibiotics are applied in agricultural and animal husbandry production, they can enter the aquatic ecosystem through a variety of mechanisms, including pollution of groundwater and bottom sediments, urban runoff, industrial plant wastewater discharge, community wastewater treatment, atmospheric rainfall, etc., resulting in frequent detection in the water environment. The concentration of CMN is usually 0.01–9.8 μg/L in the water, which can reach 194 μg/L in the runoff of the farming area after application of pesticides (Marino and Ronco, 2005; Vryzas et al., 2011; Xing et al., 2012; Zhou et al., 2016). The concentration range of SMZ detected in water is 259.60 ng/L to 385.00 ng/L (Chen and Zhou, 2014; Chen et al., 2015). Chemical exposure in these concentrations may lead to reactive oxygen species (ROS) bursting and oxidative stress in fish, posing risks to aquatic species and have significant effects on the nervous, reproductive and immune systems (Koprucu et al., 2010; Prashanth and David, 2010; Limbu et al., 2018; Wang et al., 2018a). Excessive ROS are scavenged by antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Valavanidis et al., 2006; Wang et al., 2020), which levels, however, displayed significant decreases in five fish cell lines after exposure to CMN in 20 ng/ml for 24 h (Taju et al., 2014). Limbu et al. reported that oxidative stress induced by SMZ exposure can also lead to DNA damage and apoptosis in Nile tilapia (Limbu et al., 2018). Previous study demonstrated that sea urchin subjected to acute and sublethal SMZ suffered alters in the transcriptional levels of oxidative stress and apoptosis genes (Ragusa et al., 2017). To analyze CMN and SMZ effects in a system more complex than the *in vitro* analysis and sea urchin, we then designed to evaluate immune-related biochemical, and molecular changes in the spleen of grass carps.

The fish immune system is the material basis of fish immune response. As the major peripheral lymphoid organ, the spleen exerts the functions of implementing immune defense and the maintaining the internal homeostasis (Xing et al., 2015). The Toll-like receptors (TLRs), a well-characterized pattern recognition receptors (PRRs), participated in mediating the immune response activated by a variety of pathogen-associated molecular patterns (PAMPs), including lipopolysaccharides (LPS), β-glycan and other dangerous endogenous molecules (Sasai and Yamamoto, 2013). Although at mg/L antibiotics treatment has been suggested to induce mild inflammation through TLRs regulation in zebrafish and cells (Bode et al., 2014; Grasa et al., 2015), questions remain regarding environmentally relevant concentrations of exposure.

This study was first designed to assess whether the immunotoxicity of environmentally relevant concentrations of CMN and SMZ to fish was related to oxidative stress, DNA damage and apoptosis pathway. The second objective was to investigate whether TLR signaling is involved in the immune response under CMN/SMZ exposure and subsequent *Aeromonas hydrophila* (*A. hydrophila*) infection.

2. Materials and methods

2.1. Chemicals and reagents

CMN (CAS No. 52315-07-8, 98% in purity) and SMZ (CAS No. 1196157-90-0, 99.8% in purity) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). The stock solutions of CMN and SMZ were prepared by DMSO (99% purity) dissolution and stored at 4 °C in the dark. *A. hydrophila* was presented by bacteria Laboratory of Heilongjiang River Fishery Research Institute of Chinese Academy of Fishery Sciences.

2.2. Fish accommodation

The fish for the experiment was purchased from Harbin aquaculture farm and temporarily domesticated for 2 weeks under the laboratory conditions. Then healthy and active grass carps with an average weight of (105.45 ± 5.68) g were selected and randomly divided into 4 groups, each group with 3 repetitions, 20 fish for each repetition. The fishes were raised in the indoor circulating water breeding barrel (500 L). The whole test cycle was manually timed (08:00, 12:00, 17:00), and the actual feeding amount was recorded every day for 42 days. The content of dissolved oxygen in aquaculture water was more than 6.0 mg/L, water temperature (27.0 ± 1.5) °C, pH 7.0–8.0, ammonia nitrogen content < 0.05 mg/L, nitrite nitrogen content < 0.06 mg/L, light cycle is natural light cycle.

2.3. Experimental design

The Animal Care, Use and Ethics Committee at NEFU (approval no. UT-31; June 20, 2014) gave approval and supervision to the present experimental procedures. Carps were randomly divided into 4 groups, namely control-, CMN-, SMZ- and the combine group of CMN and SMZ (MIX). Based on the available literature on the environmental investigation, the concentrations of CMN and SMZ used in the present study were set at 0.651 μg/L and 0.3 μg/L, respectively (Wen, 2003; Chen and Zhou, 2014; Chen et al., 2015). During the test, the contact solution in treated and control groups was renewed every 48 h with dechlorinated tap water to maintain the proper CMN and SMZ concentrations and water quality. Water samples at different exposure time points (1, 14, 28 and 42 days) were collected for concentrations analysis of CMN (Enzyme-linked Biotechnology, China) and SMZ (REAGEN LLC, USA) by using specific ELISA kits (Table S1). During the exposure period, the mean concentrations of actual these chemicals deviated by less than 10% from the nominal concentrations were considered as relatively stable. After 42 days of exposure, 10 individuals were anesthetized with MS-222 (10 mg/L, Sigma, USA) per treatment, and the caudal vein blood was collected with a capillary pipette containing sodium heparin. The spleen was immediately frozen in liquid nitrogen and stored at 80 °C for RNA isolation. There was no death of grass carps in the whole 42 days experimental period.

2.4. Host resistance challenge assay/A. hydrophila

An *A. hydrophila* strain was grown in LB (30 °C) to OD600 = 1, whose suspension was centrifuged (10 min, 4000 rpm, 4 °C) and re-suspended in sterile PBS at 1×10^7 CFU/mL (Zhu et al., 2019). Grass carps were injected intraperitoneally (ip) with 0.2 ml/100 g body

weight of the bacterial suspension, by entering the sterile injection syringe along the inner base of the ventral fin in the direction of the pectoral fin. After injection, the carps were kept as previous. The mortality of grass carps was observed every 2 h and then spleen tissues were collected.

2.5. Histological observation

After 42 days exposure, spleen tissue specimens (1.0 mm^3) were quickly extracted from all four groups. Histological analysis was performed using hematoxylin-eosin (H & E) staining. After ultra-thin sectioning, double staining of uranyl acetate and lead citrate. Pathological observation using a microscope (Olympus, Japan).

2.6. Detection of antioxidant system

Preparation of 10% tissue homogenate: add 9 times cold normal saline (0.9% sodium chloride aqueous solution) according to the proportion of weight (g): Volume (ML) = 1:9. First use a tissue mill to grind grass carps' spleen tissue and centrifuge (2000 rpm, 4 °C) for 15 min, the supernatant was taken to obtain a 10% tissue homogenate. Enzyme detection kits and biochemical indicators were used Nanjing Jiancheng assay kit (NJJCBIO, China) including SOD and CAT activities, malondialdehyde (MDA) and glutathione (GSH) contents and Coomassie Brilliant Blue sequencing protein content. Tests according to kit instructions.

2.7. Quantitative real-time PCR (qPCR) analysis

Total RNA was extracted from the spleen using RNAiso Plus reagent (Invitrogen, USA). NanoPhotometer spectrophotometer was used to estimate total RNA concentration at 260 nm, and RNA quality was evaluated by 260/280 nm, 260/230 nm ratio. Following the manufacturer's instructions, total RNA was reversely transcribed into cDNA using HiScript II Q Select RT SuperMix for qPCR (Vazyme Biotech co., Ltd). Relative mRNA levels were performed at LightCycler® 480 and determined using FastStart Universal SYBR Green Master reagent (Roche, Switzerland). The primer sequence was listed in Table S2. Calculate mRNA expression using the $2^{-\Delta\Delta CT}$ method, as previously described (Wang et al., 2018c; Wang et al., 2018d; Jing et al., 2019).

2.8. Immunohistochemistry

Paraffin sections were dewaxed (xylene and anhydrous ethanol), antigen repair was performed on the sections, and PBS was soaked for 5 min × 3 times. Hydrogen peroxide was incubated and soaked in PBS for 5 min × 3 times. Next, the goat serum was sealed, followed by primary antibody incubation (Bcl-2 and Bax), secondary antibody incubation, brown positive cells were observed with DAB (Boster, China), and then hematoxylin restaining. Finally, the slices were dehydrated, sealed and observed under the microscope (Olympus, Japan).

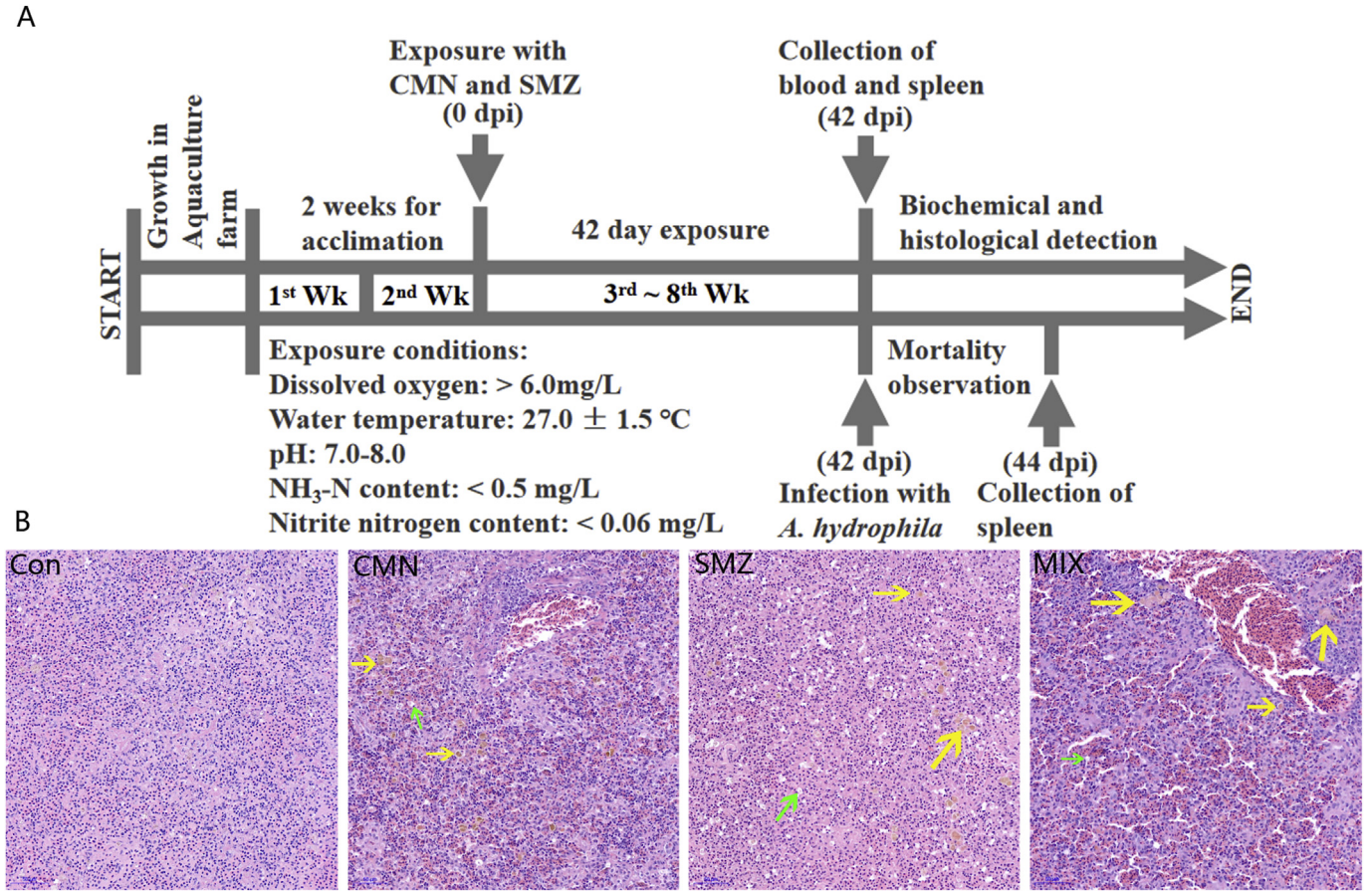

Fig. 1. (A) Timeline of experiment. (B) Histological changes of the spleen. Pathological features including cell vacuolation (green arrows) and melano-macrophage centers (yellow arrows) were indicate in the spleen tissues. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.9. TUNEL assay

According to Wanleibio's TUNEL FITC apoptosis detection kit. The spleen slices were dewaxed with xylene, then hydrated with gradient ethanol, dripped with 0.1% Triton x-100 (0.1% sodium citrate) for 50 μL, and placed at room temperature for 8 min. PBS was rinsed for three times, 50 μL of TUNEL solution was dripped, incubated at 37 °C in dark for 60 min, and then PBS was rinsed. DAPI was re dyed, PBS was rinsed for three times, the sections were taken out, and the fluorescent quenching agent was dripped to seal the sections. The staining effect was observed under the fluorescence microscope.

2.10. Enzyme-linked immunosorbent assay (ELISA)

The supernatant serum (blood samples centrifugation at 3000 rpm, 10 min, 4 °C) was collected and stored at 20 °C for determination of serum immunoglobulin M (IgM), component 3 (C3), C-reactive protein (CRP) content and lysozyme (LYS) activity by using ELISA kits (Shanghai Enzyme Biotechnology Co., Ltd). The amount of enzyme required to convert 1 μmol of substrate per minute at 37 °C was considered as a unit of enzyme activity (U).

2.11. Statistical analysis

Three biological replicates were set for each treatment. SPSS 22.0 (version 22.0, Inc., Chicago, IL) was used to test the homogeneity of variance of data by Levene test, and the differences between groups were analyzed by one-way ANOVA. All statistical graphs were drawn with graphpad software prism 5 (version 5.01, graphpad software, Inc., La Jolla, USA), and the data were expressed as mean ± standard error (SD). $P < 0.05$ was used as the standard for statistical significance. Principal component analysis and heat mapping are done using the free online data analysis platform omicshare tools (www.omicshare.com).

3. Results and discussion

3.1. Experimental process and histological observation

An increasing number of studies have demonstrated that exposure to some environmental pollutants (pesticides, antibiotics, heavy metals) can induce oxidative toxicity and immunotoxicity in organisms (Limbu et al., 2018; Zhao et al., 2019a). However, the molecular effects of these cellular process under these pollutants-combined exposure are unavailable. Therefore, grass carps in the present study were exposed to CMN and SMZ for 42 days, and then challenged with *A. hydrophila* for 2 days post injection (dpi) (Fig. 1A). Histopathologically, the parenchyma of spleen is complete and clearly divided into hematogenous red pulp and the lymphoid white pulp in control group (Fig. 1B). However, an increase cell vacuolation and the activation of melanomacrophage centers (MMC) were showed in the histopathological section of fish spleen in SMN, CMN and MIX group (Fig. 1B, Fig. S1). MMC, also known as macrophage aggregates, increase in the condition of stress and various adverse environmental conditions like chemical pollutants (Agius and Roberts, 2003). These results preliminarily proved that under the exposure of CMN and SMZ in environmentally relevant concentrations, the injury of spleen parenchyma was induced with mechanism and specific pathways to be explored.

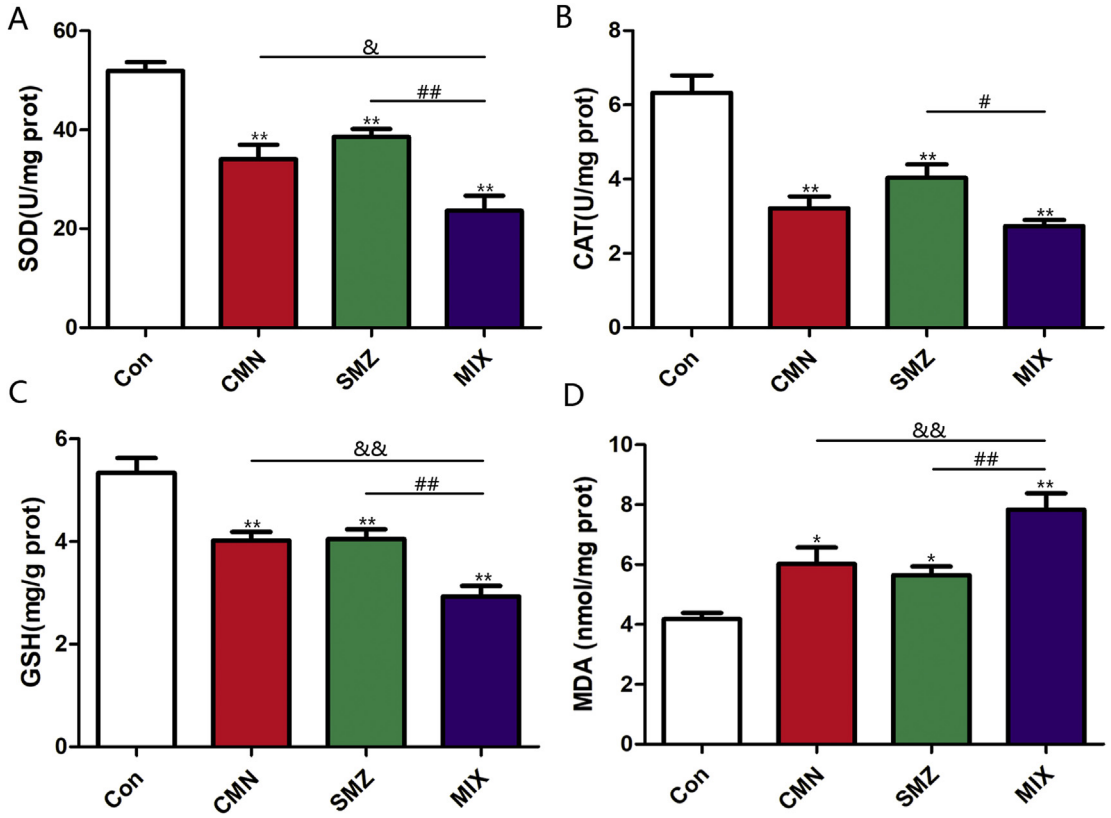

Fig. 2. The activities of SOD (A) and CAT (B), content of GSH (C) and MDA (D) in control and different treated fishes, n = 3. The * indicates significant differences between control group and experience group ($P^{*} < 0.05$, $P^{**} < 0.01$), the & indicates significant differences between CMN and MIX group ($P^{\&}<0.05$, $P^{\&\&}<0.01$), the # indicates significant differences between SMZ and MIX group ($P^{\#} < 0.05$, $P^{\#\#}<0.01$).

3.2. CMN or/and SMZ induce oxidative stress and the following apoptosis in spleen tissues

Studies have shown that environmental chemicals can destroy the balance between endogenous and exogenous ROS, which mainly originated from mitochondria, leading organisms to oxidative damage and possible cell apoptosis (Liu et al., 2016; Limbu et al., 2018). We thus analyzed some important indicators in the antioxidant system and apoptotic pathway in the spleen tissues of grass carps under CMN or/and SMZ treatment. As the first barriers against oxidative stress, GSH, CAT and SOD (Zhao et al., 2015; Sun et al., 2018) were often used as important indicators to monitor and evaluate the toxicity of environmental pollution together with ROS decomposition products (MDA) (Kim and Kang, 2015). In addition, some studies have shown that ROS were also the mediators of P53, a vital indicator of cell apoptosis (Wang et al., 2018b; Zhao et al., 2018). In this experiment, in treated groups, the content of GSH and activities of CAT and SOD were decreased significantly, accompanied by increased content of MDA (Fig. 2). In addition, as key target genes for p53-induced cell cycle arrest and apoptosis, PUMA and the Bax/bcl-2 initiate the apoptosis process by affecting the release of mitochondrial Cyto-c (Li et al., 2004; Wang et al., 2017; Guo et al., 2019). In this study, we found that the transcription levels of genes related to apoptosis (P53 and PUMA) and the Bax/bcl-2 protein ratio were up-regulated, following increased transcription levels of apoptotic executor, caspase-3 and caspase-9 (Figs. 3 and 4). These results showed that CMN or/and SMZ exposure triggered oxidative stress and activated the apoptotic pathway. At the same time, consistent with the degree of apoptosis indicators, TUNEL index also suggested that the gravity of spleen damages at environmentally relevant concentrations displayed a rise tendency of SMZ < CMN < MIX group (Fig. 4E, Fig. S2). It may be because of limited tissue-specific effects of SMZ. In all, these results suggest that CMN or/and SMZ induce oxidative stress in grass carps and the increase of ROS might be a major factor in the apoptosis of splenocytes.

3.3. Grass carps co-exposed to environmentally relevant concentrations of CMN and SMZ bear immunodeficiency through TLR pathway

Non-specific humoral immune factors are mainly present in the blood and mucus of the fish, and are the first line of defense against pathogenic infections, mainly including complement, LYS, CRP, enzyme, interferon, etc. And the specific humoral immune factors, IgM is the main regulatory molecule in humoral immunity. The level of serum immune parameters of grass carps exposed to CMN and SMZ was shown in Fig. 5. After 42 days of exposure in the experimental group, the contents of C3 and IgM in the serum of grass carps were suppressed under treatment groups than that in the control group (Fig. 5A and B, $P < 0.05$). Moreover, the CRP levels displayed significant decreases in the CMN and MIX treatment group and not significantly affected in SMZ group compared to the control (Fig. 5C, $P < 0.05$). No significant fluctuations in LYS activity was observed after CMN and SMZ treatment. However, LYS activity was significantly inhibited at MIX group, as shown in Fig. 5D. Moreover, the levels of C3, IgM, CRP and LYS activity in the MIX group were significantly reduced compared with CMN and SMZ ($P < 0.05$). In all, combined with the results of humoral immune factors, grass carps co-exposed to environmentally relevant concentrations of CMN and SMZ bore immunodeficiency.

What is the mechanism responsible for the immune response of

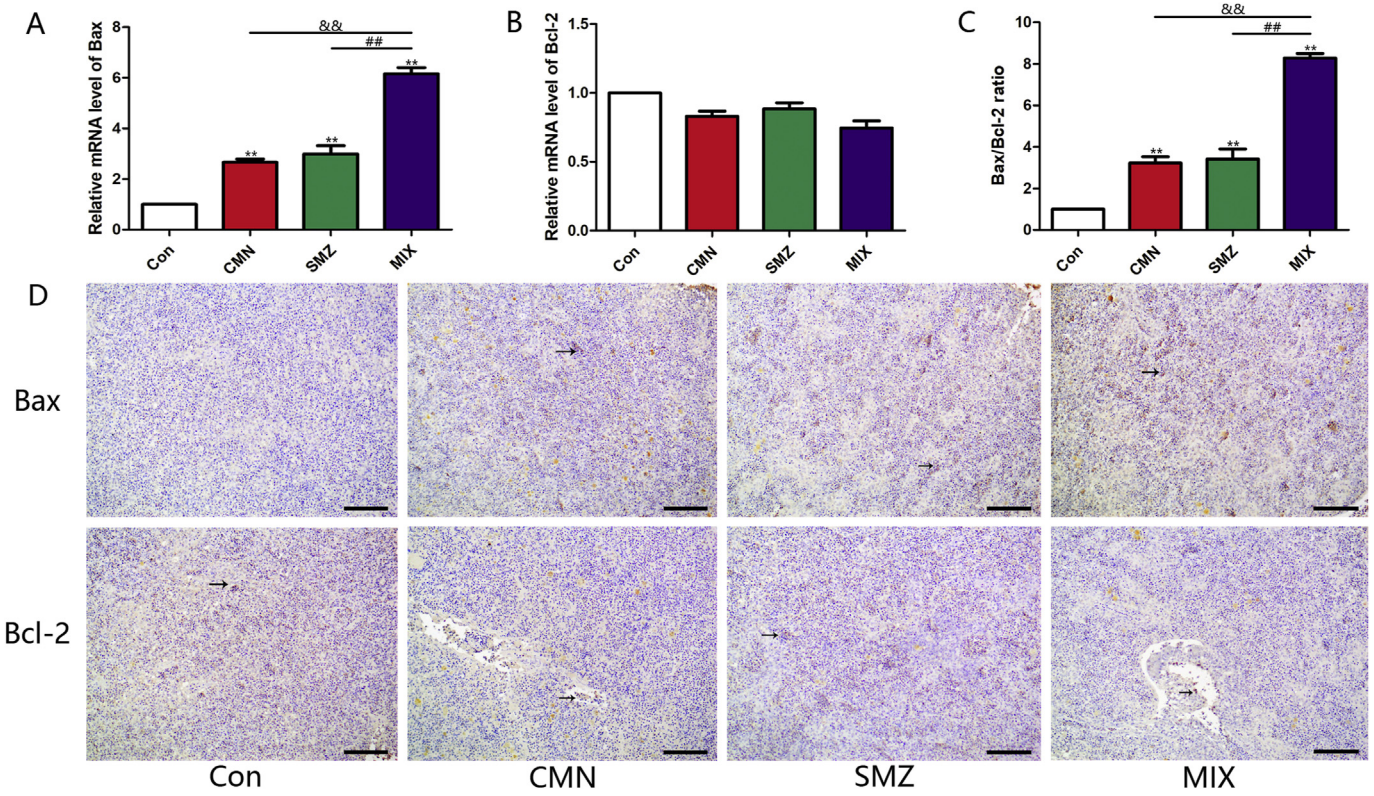

Fig. 3. (A–C) mRNA levels of genes related to apoptosis. (D) Immunohistochemistry for Bax and Bcl-2, 200 ×, n = 3. The * indicates significant differences between control group and experience group ($P^{*} < 0.05$, $P^{**} < 0.01$), the & indicates significant differences between CMN and MIX group ($P^{\&}<0.05$, $P^{\&\&}<0.01$), the # indicates significant differences between SMZ and MIX group ($P^{\#} < 0.05$, $P^{\#\#}<0.01$).

young healthy grass carps to CMN and SMZ exposure observed above? We then asked whether TLR pathway participates in CNM or/and SMZ induced oxidative stress and immune response. Different environmental pollutants caused immune chaos by disturbing the levels of cytokines in fish immune system (Zhao et al., 2019b). More and more studies suggest that TLRs participated in mediating the immune response activated by oxidative stress in fish (Xu et al., 2013) by recognition of oxidative specific epitopes (Wei et al., 2018). As a PAMP recognition receptor, TLR4 can be recognized and bound by oxidative specific epitopes induced by chemical poisons (Liu et al., 2019; Zhang et al., 2019). After which the nuclear transcription factor NF-κB is then activated through the adaptor of MyD88, ultimately leading to the release of inflammatory cytokines (Wei et al., 2018). TNF-α is secreted by activated macrophages and enhances its microbicidal potential reciprocally (Zou and Secombes, 2016). IL-1β activates neutrophils and macrophages, IL-6 promotes the induction of acute phase proteins (Engelsma et al., 2001; Bird et al., 2002). In this study, TLR immune signal was significantly activated under the exposure of environment related concentrations of CMN or/and SMZ, suggested by increased TLR2, TLR4 and MyD88, and significantly upregulated pro-inflammatory genes (TNF-α, IL-1β, IL-6 and IL-8) (Fig. 6). However, there was no significant difference (IL-10) and some decrease (TGF-β) under some anti-inflammatory cytokines (Fig. 6H and I). This anomaly was in agreement with those reported in zebrafish embryos (Xiong et al., 2019), which suggested the neutralization of the over-expressed pro-inflammatory cytokines. Thus, the TGF-β and IL-10 might fail to inhibit the inflammatory response (Chen and Manning, 1996). The above research showed that CMN or/and SMZ induced the overexpression of inflammatory cytokines by interfering with the transduction of TLR signaling pathways, which might cause the occurrence of immunodeficiency and inflammation in fish tissues and organs and damage the fish immune system (Xiong et al., 2019).

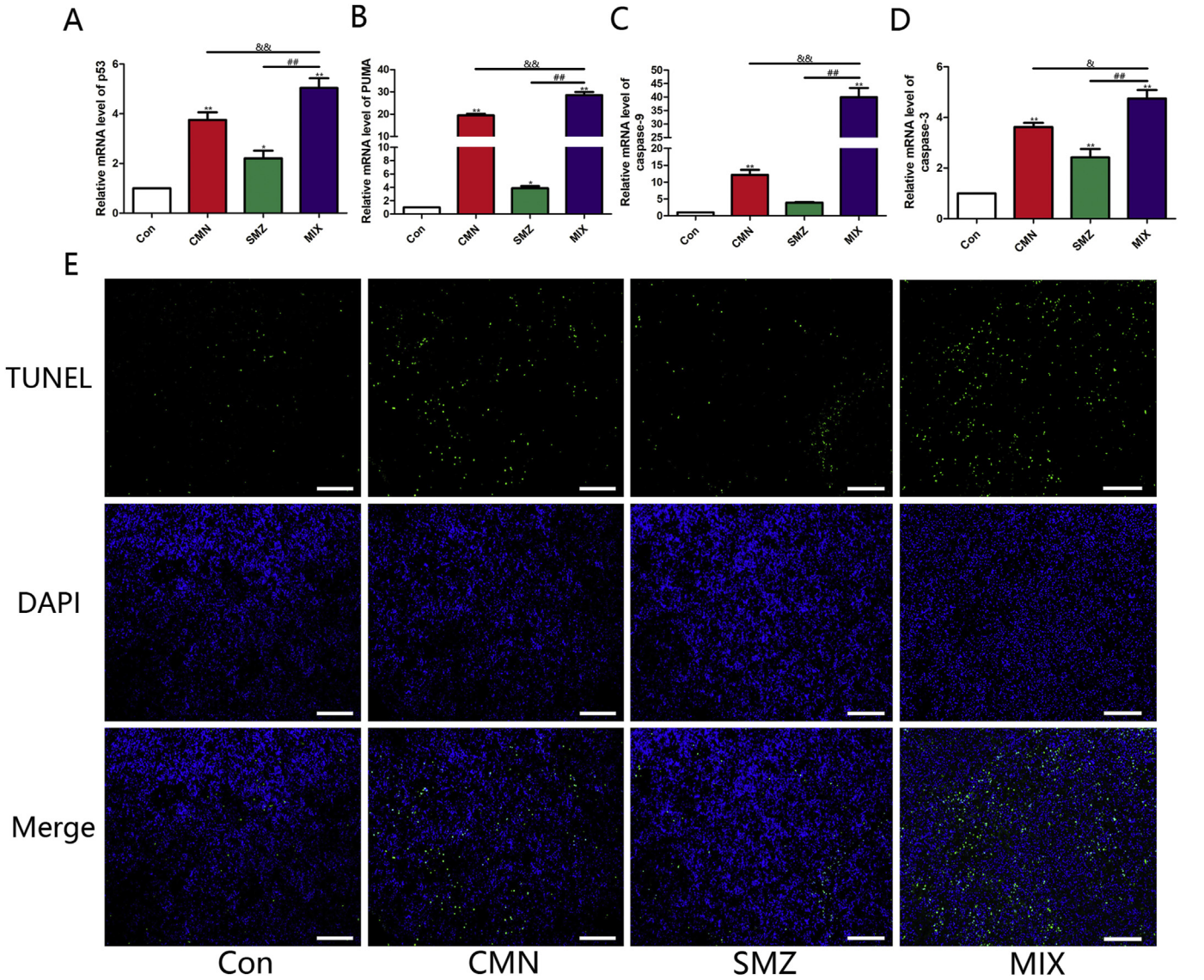

Fig. 4. (A–D) mRNA levels of genes related to apoptosis. (E) Apoptosis assayed with TUNEL staining. Green: TUNEL, blue: DAPI, 200×, n = 3. The * indicates significant differences between control group and experience group ($P^{*} < 0.05$, $P^{**} < 0.01$), the & indicates significant differences between CMN and MIX group ($P^{\&}<0.05$, $P^{\&\&}<0.01$), the # indicates significant differences between SMZ and MIX group ($P^{\#} < 0.05$, $P^{\#\#}<0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Grass carps co-exposed to environmentally relevant concentrations of CMN and SMZ are vulnerable to subsequent A. hydrophila infection

The damage of immune system is considered to be the result of exposure to environmental pollutants. Studies show that the occurrence of various diseases of fish is closely related to the presence of pollutants in the aquatic environment. These chemical pollutants can have a stress effect on fish, resulting in a significant decline in the ability of fish to resist pathogen invasion. Whether the immunosuppression induced by CMN and SMZ would affect the disease resistance ability of fish, resulting in the increase of sensitivity of fish to bacteria and other original microorganisms has not been directly proved. Therefore, in this study, after 42 days of CMN or/and SMZ exposure experiments, host resistance test was performed by *A. hydrophila*, one of the most virulent species resulting in great economic loss and threatening the development of grass carp aquaculture (Liu et al., 2015). The results showed that during 2 dpi under *A. hydrophila* insult, the mortality of grass carps in MIX treatment group was significantly higher than that in the treatment alone group and the control group (Fig. 7A). Interestingly, the 24-h mortality was significantly lower than 48 h overall. With pathogenic bacteria invasion, the anti-inflammatory cytokines were demonstrated to be up-regulated to encounter (Ming et al., 2020). Therefore, low pathogen load, coupled with the immune response activation, made the fish eliminate bacteria effectively, which may be a reason of low mortality in grass carps at 1 dpi. On the contrary, the risen tendency in bacterial loads of *A. hydrophila* and host mortality was disclosed as the infection time by. Studies have shown that long-term exposure to environmental pollutants can change the structure and function of fish's immune system, and any change in immune parameters may increase the sensitivity of fish to pathogenic microorganisms, leading to the occurrence of fish diseases (Nakayama et al., 2017; Zhang et al., 2020). Thus, to unveil the possible involvement of immune factors in response to exogenous bacteria, after CMN or/and SMZ exposure, the changes of TLR-related cytokines were detected after challenge with *A. hydrophila* for 2 days. The results showed that the expression of related regulatory factors in the TLR signaling pathway were inhibited to varying degrees (Fig. 7B–H). Consistent with previous studies (Singh et al., 2017; Zhang et al., 2019), the exposure to pesticide and antibiotic inhibited the recognition of *A. hydrophila* by the TLR receptor, let alone to trigger a normal immune response to effectively eliminate bacteria, ultimately causing an increased mortality (Fig. 7A).

4. Conclusion

Herein, the impact of antibiotics and pesticides on the water environment and aquatic organisms has increasingly become the focus of attention. In the present study, CMN (0.651 μg/L) and SMZ (0.3 μg/L) were used to assess the immunotoxicity of these environmentally relevant xenobiotic pollutants to fishes. The concentration levels are environmentally relevant and already used in other studies to provide results easily comparable with previous data. CMN or/and SMZ induced oxidative stress and apoptosis in grass carps exposed to environment related concentrations, which changed the structure of spleen and caused immunotoxicity. Furthermore, by disturbance in TLR pathway, exposure to such low levels of CMN and SMZ increased the mortality rate of grass carps challenged with *A. hydrophila*. It is worth noting that more deteriorated effects were observed in CMN and SMZ co-administrated

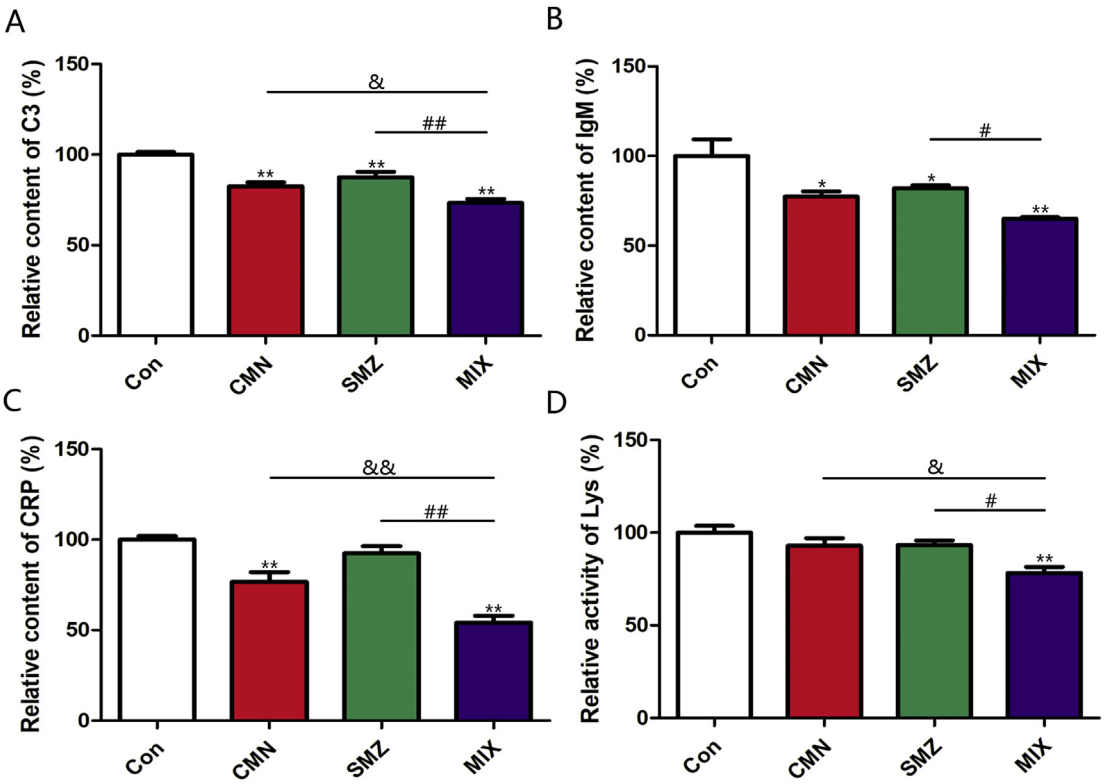

Fig. 5. Alterations in serum C3 (A), IgM (B), CRP (C) and LYS (D) after grass carps were exposed to CMN or/and SMZ for 42 days, n = 3. The * indicates significant differences between control group and experience group ($P^{*} < 0.05$, $P^{**} < 0.01$), the & indicates significant differences between CMN and MIX group ($P^{\&}<0.05$, $P^{\&\&}<0.01$), the # indicates significant differences between SMZ and MIX group ($P^{\#} < 0.05$, $P^{\#\#}<0.01$).

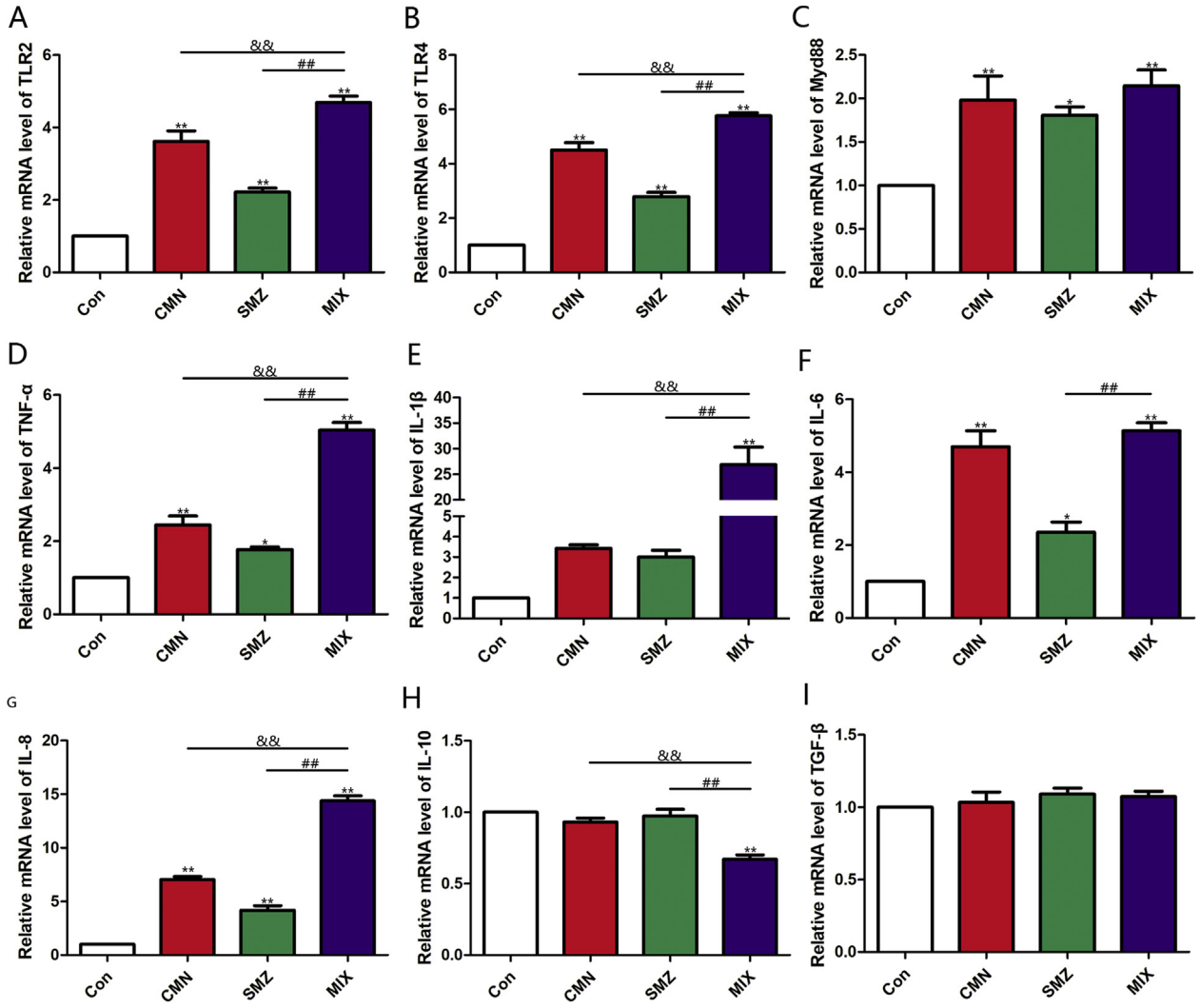

Fig. 6. mRNA levels of genes related to TLR pathways, n = 3. The * indicates significant differences between control group and experience group ($P^{*} < 0.05$, $P^{**} < 0.01$), the & indicates significant differences between CMN and MIX group ($P^{\&}<0.05$, $P^{\&\&}<0.01$), the # indicates significant differences between SMZ and MIX group ($P^{\#} < 0.05$, $P^{\#\#}<0.01$).

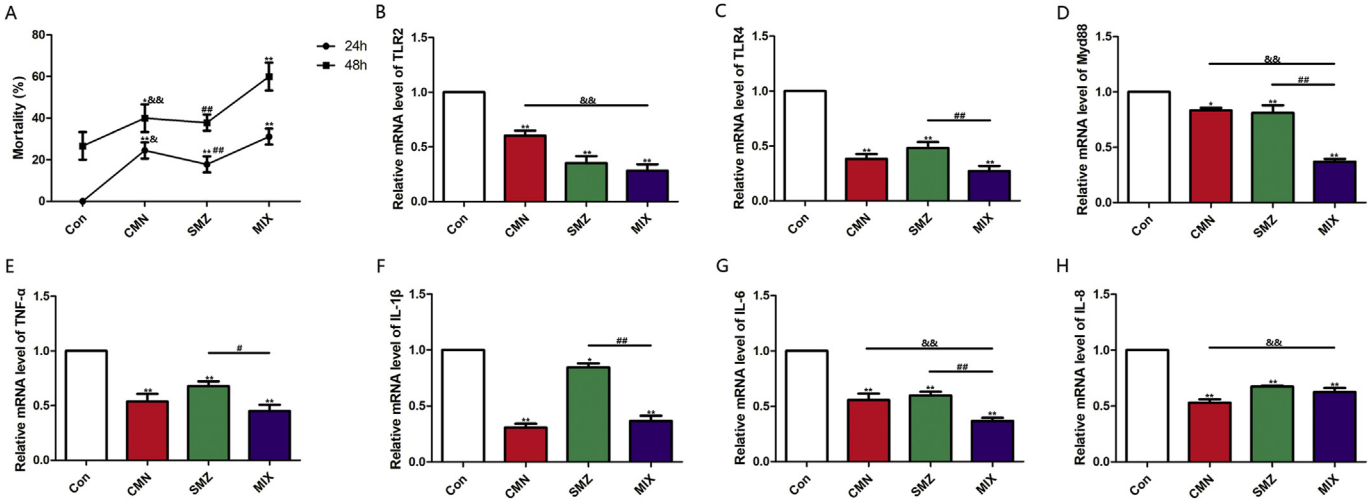

Fig. 7. The effect of CMN or/and SMZ on disease resistance in the grass carps. (A) Mortality percentages in CMN or/and SMZ-exposed grass carps at 48 h after challenged with *A. hydrophila*. (B) mRNA levels of genes related to TLR pathways, n = 3. The * indicates significant differences between control group and experience group ($P^{*} < 0.05$, $P^{**} < 0.01$), the & indicates significant differences between CMN and MIX group ($P^{\&}<0.05$, $P^{\&\&}<0.01$), the # indicates significant differences between SMZ and MIX group ($P^{\#} < 0.05$, $P^{\#\#}<0.01$).

groups compared with their individuals. The results revealed the potential ecological risks of pesticides and antibiotics in the aquatic environment, and highlighted the importance of rational and regulated use of these pesticides and antibiotics.

CRediT authorship contribution statement

Hongjing Zhao: Conceptualization, Data curation, Software, Formal analysis, Writing - original draft, Writing - review & editing. **Yu Wang:** Conceptualization, Software, Formal analysis, Investigation, Visualization. **Menghao Guo:** Investigation, Data curation. **Mengyao Mu:** Methodology. **Hongxian Yu:** Validation, Resources, Funding acquisition, Writing - review & editing. **Mingwei Xing:** Data curation, Validation, Resources, Funding acquisition, Project administration.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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