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Environmentally relevant concentration of cypermethrin or/and sulfamethoxazole induce neurotoxicity of grass carp: Involvement of blood-brain barrier, oxidative stress and apoptosis

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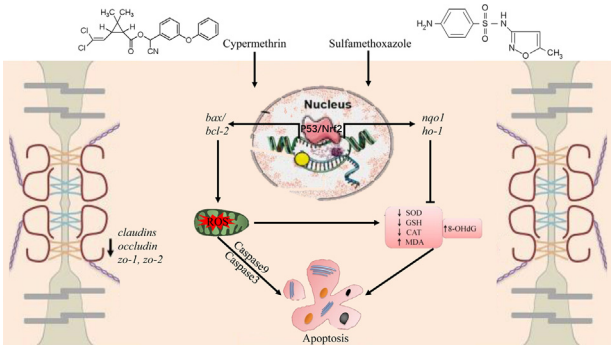
HIGHLIGHTS

- CMN or/and SMZ exposure caused neurotoxicity in grass carp.
- Oxidative stress and apoptosis participated in the neurotoxicity.
- Antioxidant system triggered cellular protection through Nrf2 pathway.

GRAPHICAL ABSTRACT



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ABSTRACT

In water environment, the interaction between environmental pollutants is very complex, among which pesticides and antibiotics are dominant. However, most studies only focus on individual toxic effects, rather combined. In this study, the sub-chronic exposure effect of cypermethrin (CMN, 0.65 μg/L), sulfamethoxazole (SMZ, 0.30 μg/L) and their mixture on grass crap (*Ctenopharyngodon idellus*) was investigated. The brain tight junction, oxidative stress and apoptosis-related indices were determined after 42 days of exposure. In terms of brain function, acetyl cholinesterase (AChE) activity was significantly inhibited by CMN, SMZ and their mixtures during exposure periods. Obvious histological damage from cellular and subcellular levels were also observed, which were further confirmed by a decrease in tight junction protein levels. Malondialdehyde (MDA) and 8-hydroxy-2-deoxyguanosine (8-OHdG) contents were significantly increased by individual compounds and mixtures, in which the content of glutathione (GSH) displayed the opposite trend. In mechanism, nuclear factor (erythrocyte derived 2) like 2(Nrf2) pathway was activated, which may trigger cellular protection to cope with CMN and SMZ exposure. However, apoptosis was also detected from the level of mRNA and histochemistry. In general, these two exogenous induced similar biological responses. The neurotoxicity of CMN was strengthened by SMZ with regard to these indices in most cases and *vice versa*. This study will reveal the potential co-ecological risks of pesticide and antibiotic in the aquatic organism, and provide basic data for their safety and risk assessment.

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1. Introduction

Antibiotics and pesticides are indispensable for maintaining public health and improving agricultural efficiency (Hamscher and Bachour, 2018; Sun et al., 2020). However, from 2000 to 2015, global antibiotic

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consumption increased from 21.2 billion to 34.8 billion (Klein et al., 2018). In 2017, the global annual use of agricultural pesticides exceeded by 4.1 million tons (FAO, 2020). These organic pollutants can undergo geo-biochemical cycle through river-ocean-aquatic organisms (Zhang et al., 2015). In addition, as the top consumer in aquatic ecosystem, fish consumption could have a serious impact on human health. Pesticide and antibiotics residues had been detected in many wild fishes and fish products consumed by humans (Omwenga et al., 2016).

As a type II pyrethroid insecticide, cypermethrin (CMN) has the characteristics of high efficiency and low toxicity. CMN often hovers between 0.01 and 9.8 μg/L in water, while can reach up to 194 μg/L in farmland runoff after its application (Marino and Ronco, 2005; Vryzas et al., 2011; Xing et al., 2012). As one of the most widely used antibiotic, SMZ ranges from ng/L to mg/L in various aquatic environments, including rivers and wastewater treatment plants (Hughes et al., 2013; Rivera-Jaimes et al., 2018; Munro et al., 2019). SMZ has long been considered as a persistent pollutant in aquatic environment due to its wide use and low biodegradability (Al-Ahmad et al., 1999). The environmental relevant CMN and SMZ can be deleterious to both human and fish, in particular, the nervous system. In general, pesticides and antibiotics can cross the blood-brain barrier (BBB), exhibiting neurotoxicity property (Thyagarajan and Deshpande, 2014; Ali et al., 2020). Our hypothesis is that BBB breakdown can be involved in the CMN and SMZ-induced neurotoxic effects. Thus, relevant biomarkers in the brain tissue were monitored to explore the exact mechanism.

Oxidative stress plays an indispensable role in neurotoxicity (Garza-Lombo et al., 2018; Al et al., 2020; Shaw et al., 2020), leading to peroxidation of neuro-lipids. Nuclear factor (erythrocyte derived 2) like 2 (Nrf2) signaling pathway is one of the most crucial ways to protect cells from oxidative damage (Kaspar et al., 2009). After the separation from its inhibitory cytoplasmic protein Kelch-like ECH-associated protein 1 (Keap1) (Ge et al., 2017), Nrf2 is activated and binds to the intra-nuclear antioxidant response element, resulting in transcriptional activation of genes encoding antioxidants and/or phase II enzymes, such as heme oxygenase-1 (HO-1) and NADPH quinine oxidoreductase-1 (NQO1) (Wei et al., 2018). Thus, Nrf2-Keap1-ARE system has long been reported to be a biomarker of neuronal stress under various exogenous (Lee et al., 2014).

Generally, reactive oxygen species (ROS) has two functions: first, to promote hemostasis as message molecule; second, to promote apoptosis if exceed. Environmental pollutants, including heavy metals, pesticides, organophosphate flame retardants, have been reported to lead to neuronal apoptosis mainly through extensive ROS (Zhao et al., 2018; Jiao et al., 2019; Chang et al., 2020; Shaw et al., 2020). The main protein dominating apoptosis is p53, a tumor suppressor protein, which also acts as a transcription factor for the induction of pro-apoptotic genes (such as Bax) and inhibition of anti-apoptotic genes (such as Bcl-2) (Wu et al., 2001; Liu et al., 2019; Zhao et al., 2019). When this programmed cell death initiates, the Caspases protease family would be cascaded to degrade cytoskeleton as a death executor (Wang et al., 2017). However, how do CMN and SMZ cross-talk *via* ROS-apoptosis in aquatic organisms has not been delineated.

Grass carp (*Ctenopharyngodon idella*) has been received much attention by environmental scientists, primarily due to its potential use for biological control of aquatic vegetation (Chilton and Muoneke, 1992; Zhao et al., 2020). As a common freshwater teleost, the grass crap was reported more easily susceptible to various kinds of diseases than other carp species (https://thefishsite.com/articles/cultured-aquatic-species-grass-carp), thus have been proposed as test organisms in toxicological assays (Fernandezdavila et al., 2012; YC et al., 2020). We thus suggested the grass carp is also a sensitive and effective bio-indicator in environmental science. The aim of this study was to evaluate the effects of low environmental related concentrations of CMN or/and SMZ in the brain tissue of grass carp and to detect the potential mechanism by monitoring oxidative stress and apoptosis-related indices.

2. Materials and methods

2.1. Animals

The present study was carried out on 120 juvenile grass carps weighing 105.45 ± 5.68 g. The animals were obtained from Harbin aquaculture farm, and were acclimated in laboratory for 2 weeks. Pre-aerated tap water was used in the experiment, and grass carps were raised in indoor circulating tanks (500 L). The water temperature was maintained at (27.0 ± 1.5) °C, the content of dissolved oxygen >6.0 mg/L, pH = 7.0–8.0, ammonia nitrogen <0.05 mg/L, nitrite nitrogen <0.06 mg/L, and photoperiod was natural photoperiod. Carps were fed the commercial base diet. This study was carried out following the Guidelines of the Animal Protection and Use Committee of Northeast Forestry University.

2.2. Chemicals

CMN (No. 52315-07-8, 98% pure), SMZ (No. 1196157-90-0, 99.8% pure) and MS-222 were purchased from Sigma Chemical Co. (Missouri, USA). CMN and SMZ reserves were prepared by dimethyl sulfoxide (99% purity) dissolution method and stored at 4 °C in dark.

2.3. Experimental design

After two weeks of acclimatization, carps were randomly divided into four groups, 3 repetitions for each group and 10 fish for each repetition. The settings were as follows: Control (Con) group: carps exposed to aerated tap water. CMN group: carps exposed to 0.65 μg/L CMN aqueous solution for 42 days. SMZ group: carp exposed to 0.30 μg/L SMZ aqueous solution for 42 days. CMN + (SMZ) group (MIX): carps exposed to 0.65 μg/L CMN + 0.30 μg/L SMZ aqueous solution for 42 days.

The predicted environmental concentration of CMN in surface waters, assumed to be caused by spray drift over 0.25 m deep water, has been estimated to 0.02–3 μg/L (Linders et al., 1994). Moreover, the 96 h median lethal concentration of CMN to grass carp was detected as 6.51 μg/L (Ma, 2003). In the Huangpu River, SMZ in water samples reached 259.6 ng/L as average concentrations (Chen and Zhou, 2014). According to the related reports of environmental pollution in the existing literature above, the CMN for 0.65 μg/L and SMZ for 0.30 μg/L were used in this study. During the experiment, the water was renewed every 48 h with de-chlorinated tap water, which contained the same concentration of CMN and SMZ as before. The concentration detection kit of CMN (Enzyme-linked Biotechnology, China) and SMZ (REAGEN LLC, USA) were analyzed at different exposure time points (1, 2nd, 4th and 6th week), during which the actual average concentration of these chemicals were deviated from the nominal concentration by less than 10%, which was considered to be relatively stable (Table S1). The behavior changes of grass carps were observed at fixed points every day (8:00, 10:00, 12:00, 14:00, 16:00, 18:00, 20:00, 22:00) and recorded. After 42 days, grass carp were anesthetized with MS-222 (10 mg/L) and venous blood was collected by capillary pipette containing heparin sodium. Brain tissues were separated immediately for morphological observation, and the rest were frozen in liquid nitrogen and stored at −80 °C. During the 42-day experiment, grass carps were not recorded death.

2.4. Histopathological observation

The fresh cerebellum tissues of grass carp were fixed with 4% paraformaldehyde, rehydrated and dehydrated in concentrations gradient of ethanol (80%, 95% and 100%), transparent in xylene, embedded in paraffin, sliced, and operated according to HE staining kit. Finally, the morphological changes of the sections were observed under the light microscope (Nikon DS- F12) and scored the brain tissue injury as the criteria listed (Table S2).

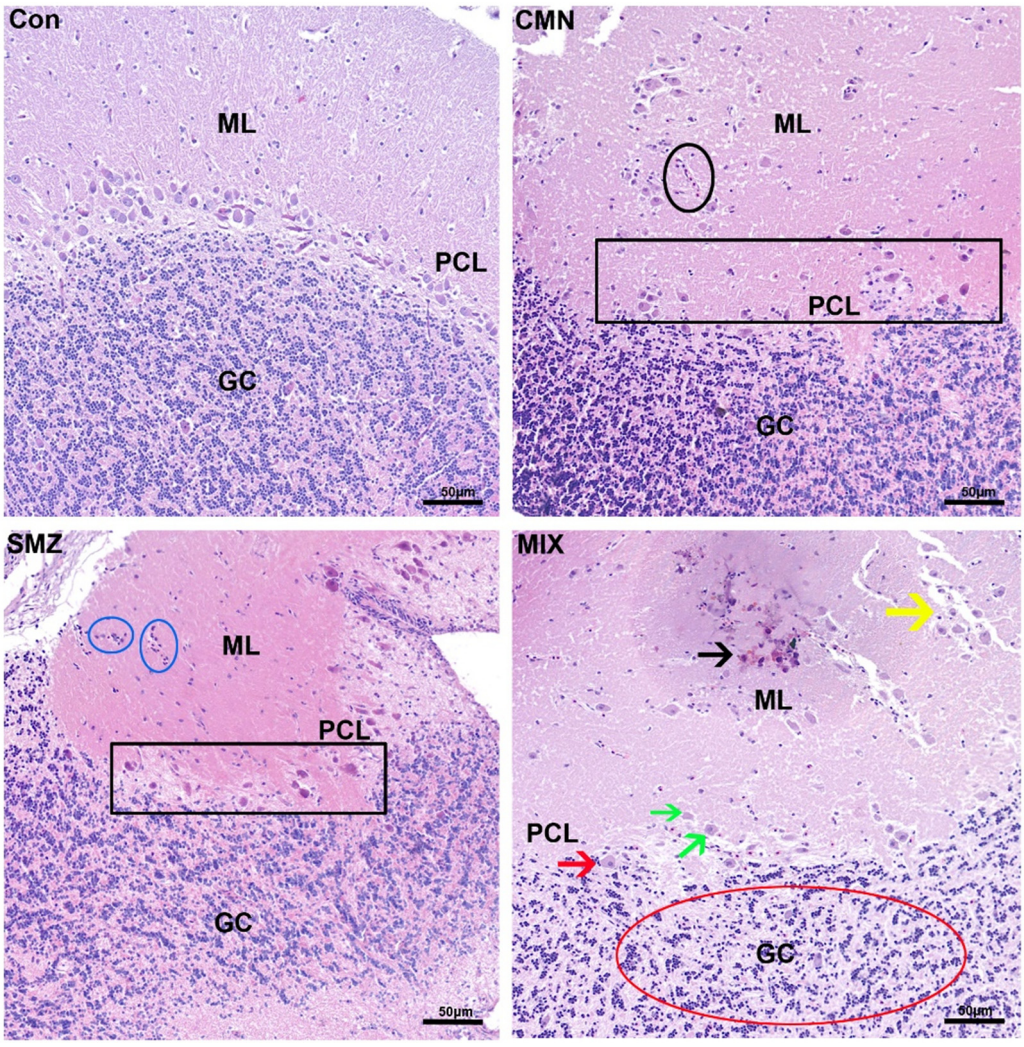

Fig. 1. Effects of CMN or/and SMZ induced on pathological structure. Histological changes of the cerebellum, 200×. ML – Molecular Layer; PL – Purkinje Layer; GL – Granular Layer. Microthrombus (black circle); purkinje cells were disorderly arranged (black rectangle); glial nodules (blue circle); hyperemia (black arrow); loose structure (yellow arrow); nucleus dissolved, and disappeared (green arrow); nucleus heterotopic (red arrow); the number of granulosa layer cells decreased in general (red circle). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. Ultrastructural observation

After the grass carps were anesthetized and planed, the brain tissue was quickly peeled off on ice, and cut into small pieces of about $1 \times 1 \times 1$ mm^3. It was then fixed with 2.5% glutaraldehyde and 1% osmium acid in turn, dehydrated by gradient of ethanol and acetone, embedded in epoxy resin, cut into 90 nm ultrathin sections, stained with uranyl acetate and lead citrate, and finally observed by transmission electron microscope (TEM) (GEM-1200ES, Japan).

2.6. Detection of acetyl cholinesterase (AChE) activity, oxidative stress related indicators and 8-OHdG content

The brain tissue samples were weighed, and 9 times volume of normal saline was added according to the proportion of weight (g): volume (mL) = 1:9, and then ground with tissue grinding, 2500 rpm, centrifugation for 10 min, and the supernatant was taken for testing. The levels of ROS and organic 8-OHdG were detected according to the instructions of the kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., China). After washing and incubation with the primary antibody, HRP-linked secondary antibody and chromogen, absorbances were read at a 450 nm microplate reader. The contents of glutathione (GSH) and malondialdehyde (MDA), the activities of acetyl cholinesterase (AChE), superoxide dismutase (SOD) and catalase (CAT) were detected according to the instructions of the kit (NJJCBIO, China). Finally, the absorbance was detected at 420 nm, 532 nm, 412 nm, 450 nm and 405 nm.

2.7. qPCR analysis

Total brain RNA was extracted using TRIZOL reagent (Invitrogen, USA). UV spectrophotometer was used to detect OD260/OD280 to verify RNA concentration and purity, which integrity was detected by 1.0% agarose gel electrophoresis. cDNA was synthesized according to HiScript II Q Select RT SuperMix kit (Vazyme Biotech co., Ltd) and then stored at −20 °C. The SYBR qPCR Mix kit (Roche, Switzerland) was referred to qPCR detection (Light Cycler® 480 System (Roche, Switzerland)). The primer sequences were listed in Table S3. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of mRNA, as previously described (Chen et al., 2020a; Jing et al., 2019).

2.8. Caspase9/3 activity assay

According to the different substrates, the activity of Caspase 9/3 Can Be Detected by measuring the absorbance (Beyotime, China). 10 mg brain tissue and 100 μL lysate mixture were homogenized with glass

homogenizer on ice bath. Then the homogenate was transferred to a 1.5 mL centrifuge tube, and then cracked in ice bath for 5 min. Centrifugation was set at 16000–20000 *g* at 4 °C for 10–15 min, after which the supernatant was transferred to the centrifugal tube of ice bath precooling.

2.9. TUNEL fluorescence staining to evaluate the rate of apoptosis

According to Wanleibio's TUNEL FITC apoptosis detection kit (China), the DNA apoptosis rates in paraffin-embedded sections of brain were observed. The normal cell nucleus showed blue and the apoptotic cell nucleus showed green under a fluorescent microscope. The positive cells (apoptosis rate) were calculated using Image Pro-plus 6.0 image analysis software.

2.10. Statistical analysis

SPSS (version 22.0, Chicago, IL) was used for statistical analysis and one-way ANOVA was used for comparison. The data were expressed as mean ± standard deviation (SD). Those values significantly different from each other are indicated by $P < 0.05$ or $P < 0.01$. GraphPad prism (version 5.01, La Jolla, USA) was used for image processing.

3. Results

3.1. Effects of CMN or/and SMZ exposure on behavior of grass carp

On days 1–7 of the experiment, all grass carps had normal behaviors without obvious symptoms of maladaptation. For 8–14 days, grass carps in exposed group had vigorous activities, manifested as quick swimming and occasionally hitting the container wall, in which the reaction of the MIX group was the most obvious. With the prolonged exposure time (15–42 days), the grass carp in the CMN, SMZ and MIX group appeared to move slowly, and occasionally moved quickly. The bodies of the CMN and MIX groups can be poor in balance with the phenomenon of side-swimming and sinking to the bottom. (Data not shown).

3.2. Histopathological observation of cerebellar tissue under the exposure of CMN or/and SMZ

As shown in Fig. 1, in the Con group, the cerebellar tissue structure was clear, and the molecular layer (ML), the purkinje cell layer (PL) and granular layer (GL) were intact. In CMN group, there was a few micro-thrombosis composed of aggregated and dissolved red blood cells (black circle), and purkinje cells were disorderly arranged (black rectangle). In SMZ group, the focal proliferation of glial cells in the molecular layer was aggregated into glial nodules (blue circle); purkinje cells arranged in disorder (black rectangle). In MIX group, the molecular layer displayed hyperemia (black arrow), loose structure (yellow arrow); purkinje's cell structure was vague with dissolved disappeared nucleus (green arrow), nucleus heterotopic (red arrow); the number of granulosa layer cells decreased in general (red circle). After the statistical data of histological score of brain tissue staining, the combined group received the highest injury score, after which following CMN, SMZ and Con (Table S2 and Fig. S1).

3.3. Ultrastructural observation of cerebellar tissue under the exposure of CMN or/and SMZ

Brain tissue was collected for TEM to accurately evaluate the neuronal cells and subcellular structures (Fig. 2). In Con group, the structure of neurons was intact with complete mitochondria, myelin sheath (red rectangle) and abundant synapses (black circle). In CMN, SMZ and MIX groups, mitochondria displayed obvious swelling (green arrow), vacuolization (yellow arrow), damaged myelin sheath layers (red rectangle); and decreased synapses (black circle), which suggested the decline of nerve conduction function. At the same time, in CMN and MIX groups, the colour of neurons was relatively light. In SMZ group, microglia on the right side were close to neurons, which may cause neurological damage (white rectangle).

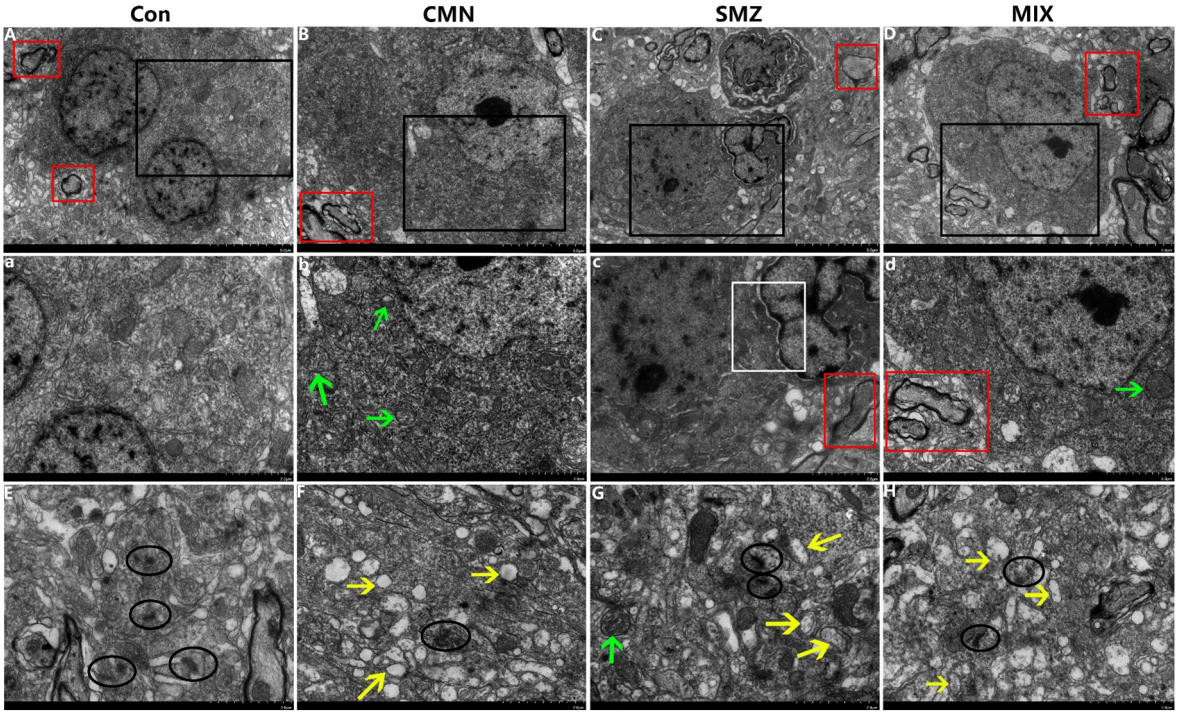

Fig. 2. Ultrastructural observation. The black boxes in Fig. 2 A, B, and C correspond to Fig. 2 a, b, and c, which were high-magnification pictures. Synapses (black circle); myelin sheath structures (red rectangle); mitochondria swelled (green arrow); mitochondria vacuoles (yellow arrow); microglia on the right side were close to neurons (white box). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Effect of CMN or/and SMZ exposure on tight junction

In this study, the mRNA expression levels of BBB tight junction proteins (Claudins, Occludin and ZOs) were investigated and displayed in Fig. 3. In CMN, SMZ and MIX groups, the mRNA expression levels of Claudin 1/3/4, Occludin, ZO-1 and ZO-2 were significantly lower than the Con group ($P < 0.05$ or $P < 0.01$). Moreover, compared with CMN or SMZ groups, some genes including Occludin, ZO-1 and ZO-2, displayed more decrements in MIX group ($P < 0.01$).

3.5. Effect of CMN or/and SMZ exposure on AChE activity and oxidative stress parameters

After CMN and SMZ exposure, AChE activities decreased in CMN (0.75 fold), SMZ (0.87 fold) and MIX (0.67 fold) group ($P < 0.05$ or $P < 0.01$) (Fig. 4A). These results indicated that CMN and SMZ can weaken the decomposition of acetylcholine (ACh), and lead to abnormal aggregation of ACh *in vivo*, which affected the cholinergic mechanism. Moreover, the combined exposure group (MIX group) had a weaker effect on ACh decomposition compared with other treatment (compared to CMN, $P > 0.05$; compared to SMZ, $P < 0.01$). The same tendency was also found in GSH, which content was significantly decreased in CMN (0.72 fold, $P < 0.05$), SMZ (0.75 fold, $P < 0.05$) and MIX group (0.60 fold, $P < 0.01$) (Fig. 4D). However, the ROS levels, MDA and 8-OHdG contents as well as SOD and CAT activities displayed rises in varying degrees, in which MIX group increased most significantly ($P < 0.01$) (Fig. 4B, C, E-G).

3.6. Effects of CMN or/and SMZ exposure on Nrf2 and its downstream target gene transcription

The mRNA level of Nrf2 was obviously increased in the CMN (34.74 fold), SMZ (17.26 fold) and MIX (96.78 fold) groups compared with that in the Con group ($P < 0.01$). However, the mRNA expression of Keap1 was remarkably reduced in CMN (0.67 fold), SMZ (0.66 fold) and MIX (0.38 fold) groups ($P < 0.01$). Compared with CMN or SMZ, these two indicators reached the highest value (for Nrf2, $P < 0.01$) and the lowest value (for Keap1, $P < 0.05$) in the MIX group. Moreover, the mRNA levels of NQO1 and HO-1 were obviously upregulated in the CMN, SMZ and MIX exposure groups ($P < 0.05$ or $P < 0.01$) (Fig. 5C and D). Also, compared with the SMZ group, the MIX group displayed significant increase when it referred to NQO1 ($P < 0.01$) (Fig. 5C).

3.7. Effects of CMN or/and SMZ exposure on apoptosis pathway

The gene expression of p53 in carp brain was upregulated in CMN group (2.74 fold), SMZ group (4.00 fold) and MIX group (6.25 fold) (Fig. 6A) ($P < 0.01$). The expression of Bax follows a similar pattern to that of p53, which displayed increases in CMN group (5.00 fold), SMZ group (4.35 fold) and MIX group (6.00 fold). Bcl-2 showed marked downregulation in CMN group (0.66 fold), SMZ group (0.8 fold) and MIX group (0.54 fold) (Fig. 6C). These results led to up-regulated Bax/Bcl-2 ratio ($P < 0.01$) in treatment groups. Moreover, the mRNA levels and activities of Caspase-9 and Caspase-3 were found to be upregulated after CMN and SMZ exposure (Fig. 6E-H), in which the MIX group increased the most ($P < 0.01$). As shown by TUNEL staining in Fig. 7 and Fig. S2, compared with the Con group, significant increases of the apoptosis rate were counted in CMN (4.33 fold, $P < 0.01$), SMZ (2.54 fold, $P < 0.01$) and the MIX group (6.43 fold, $P < 0.01$), respectively.

4. Discussion

Developmental eco-neurotoxicity must be framed under the regulatory framework for ecotoxicological perspective (Basu, 2015). However, guidelines for neurotoxicity assessment consider only mammals and

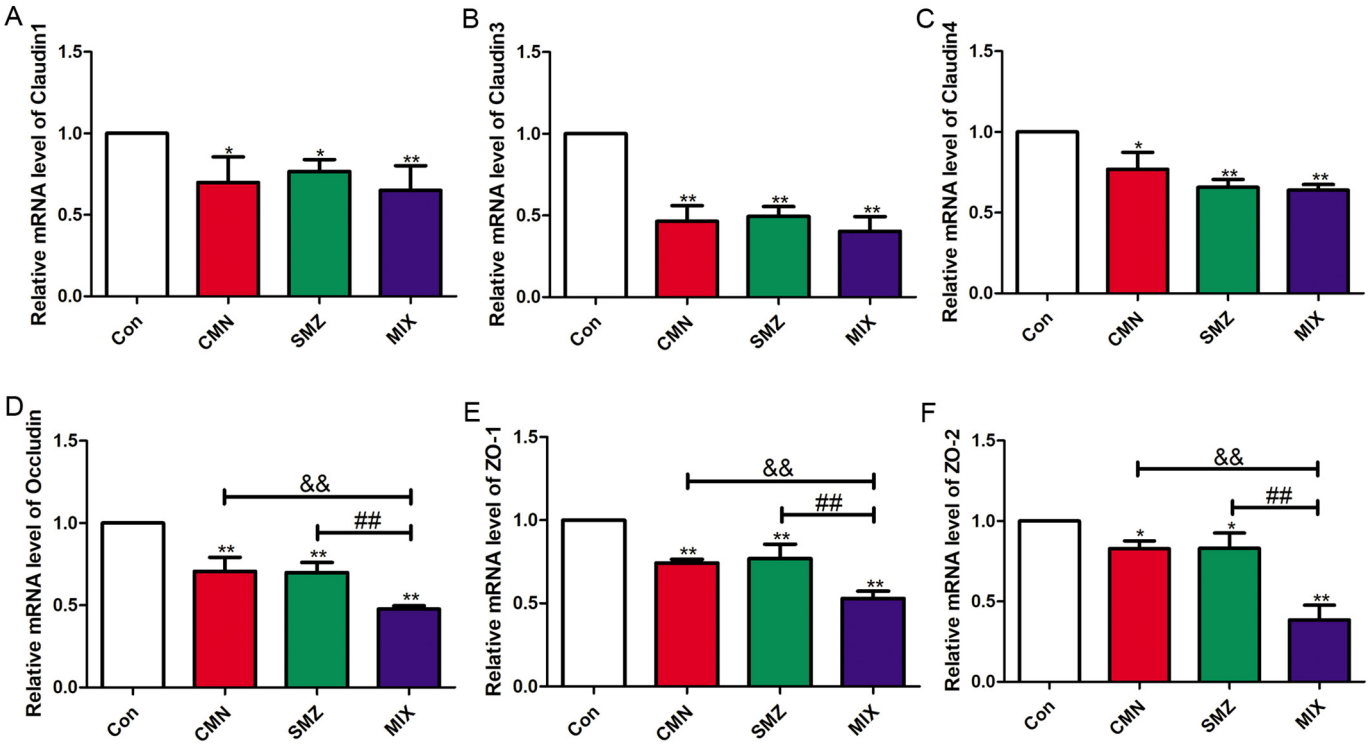

Fig. 3. Effect of CMN or/and SMZ exposure on mRNA expression levels of BBB tight junction proteins. (A-C) mRNA levels of Claudin-1/3/4. (D) mRNA levels of occluding. (*E*-F) mRNA levels of ZO-1/2. Three independent repetitions were set and each repetition includes 3 fish. The * indicates significant differences between control group and experience group (P * < 0.05, P ** < 0.01), the # indicates significant differences between SMZ and MIX group (P # < 0.05, P ## < 0.01), the & indicates significant differences between CMN and MIX group (P & < 0.05, P && < 0.01).

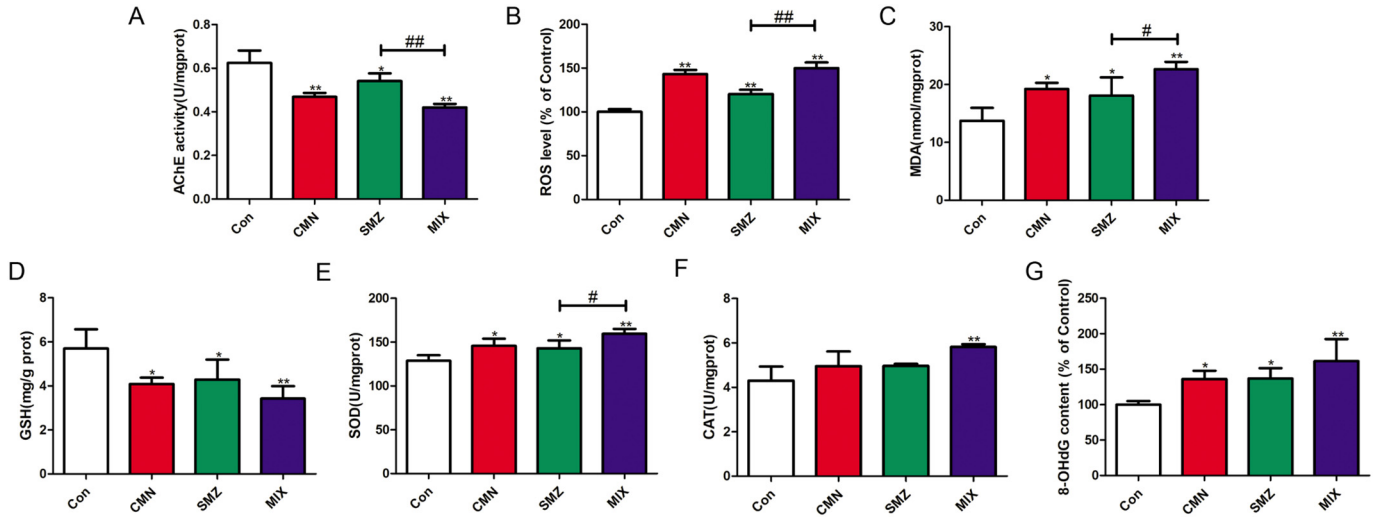

Fig. 4. Effects of CMN or/and SMZ exposure on AChE activity and oxidative stress parameters. The activity of AChE (A), levels of ROS (B), contents of MDA (C) and GSH (D), activities of SOD (E) and CAT (F), content of 8-OHdG (G). Three independent repetitions were set and each repetition includes 2 fish. The * indicates significant differences between control group and experience group (P * < 0.05, P ** < 0.01), the # indicates significant differences between SMZ and MIX group (P # < 0.05, P ## < 0.01), the & indicates significant differences between CMN and MIX group (P & < 0.05, P && < 0.01).

birds, not in aquatic systems. In this sense, it is of great advantage to benefit from the knowledge obtained with aquatic model organisms. Fish models has been used in both (developmental) neurotoxicity as well as in ecotoxicological studies, with investigations considering the involved mechanisms of toxicity. For the fish species grass carp and medaka, changes in global gene expression profile (Shi et al., 2020) and whole mount antibody staining (Padilla et al., 2009) have been suggested as methodological approaches to characterize respective neurotoxic effects and mechanisms involved. Grass carp have also been used as a system toxicology model in a screening protocol to investigate environmental neurotoxicants considering various nervous system endpoints (Fu et al., 2019).

BBB is critical to the self-stabilization of the central nervous system, which functions as transfer of nutrients, and blocker of harmful substances. Therefore, the toxicity of the poison depends to a certain extent on the permeability of the BBB to the specific toxin (Van Dyk and

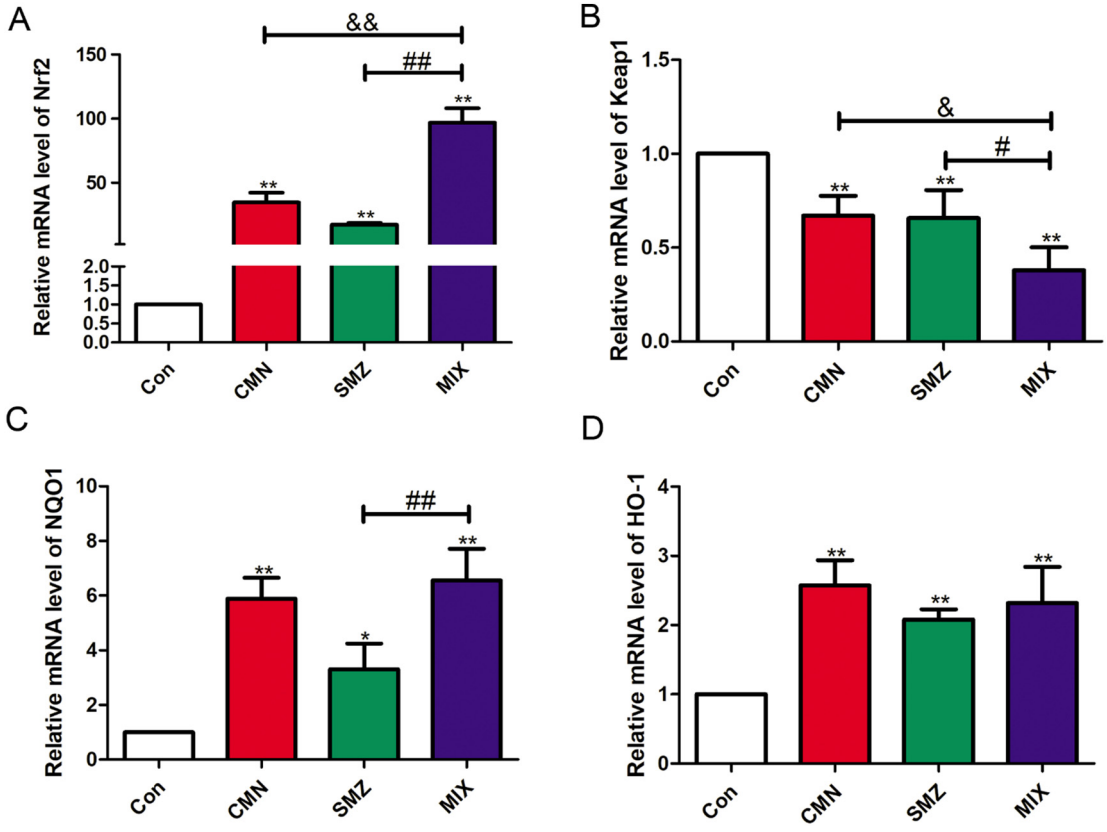

Fig. 5. Effects of CMN or/and SMZ exposure on Nrf2 and its downstream target gene transcription. (A) mRNA levels of Nrf2. (B) Keap1. (C) NQO1. (D) HO-1. Three independent repetitions were set and each repetition includes 3 fish. The * indicates significant differences between control group and experience group (P * < 0.05, P ** < 0.01), the # indicates significant differences between SMZ and MIX group (P # < 0.05, P ## < 0.01), the & indicates significant differences between CMN and MIX group (P & < 0.05, P && < 0.01).

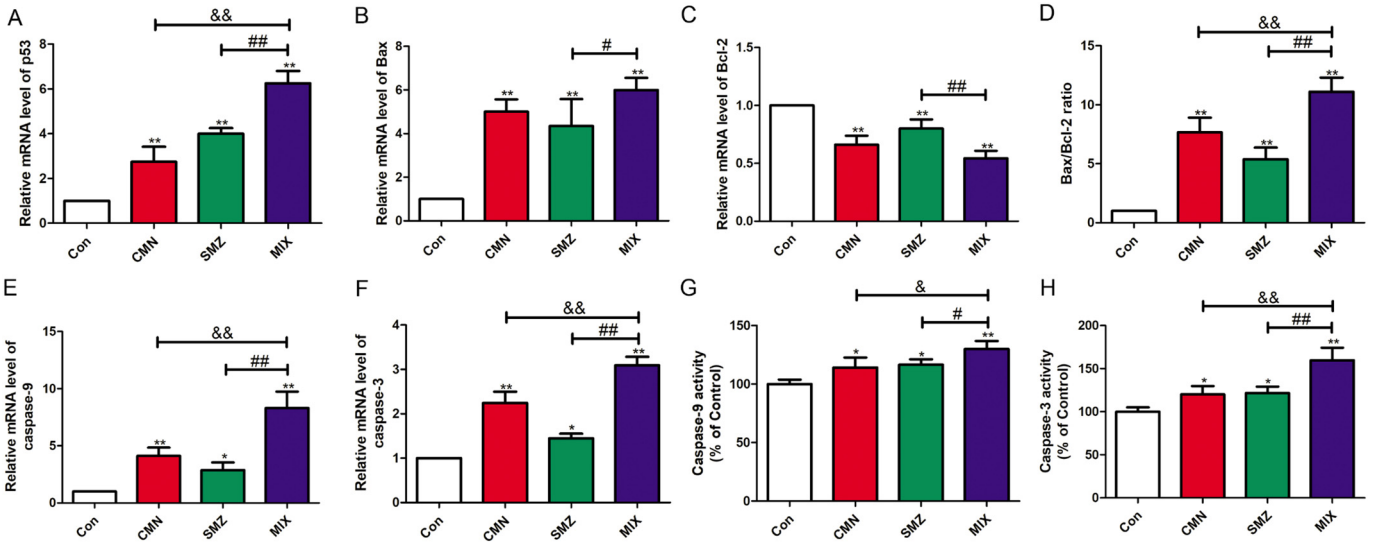

Fig. 6. Effects of CMN or/and SMZ exposure on apoptosis pathway. mRNA levels of apoptosis-related genes. Three independent repetitions were set and each repetition includes 3 fish. The * indicates significant differences between control group and experience group (P * < 0.05, P ** < 0.01), the # indicates significant differences between SMZ and MIX group (P # < 0.05, P ## <0.01), the & indicates significant differences between CMN and MIX group (P & < 0.05, P && < 0.01).

Pletschke, 2011). Moreover, the brain is a vulnerable organ due to its characteristics of richness in lipid and low expression of antioxidant enzymes (Thyagarajan and Deshpande, 2014; Ali et al., 2020). It is well known that CMN plays a neurotoxic role by crossing the BBB through the change of sodium ion channel (Ali et al., 2020). SMZ is also considered to increase the level of plasmatic bilirubin by shifting it from its albumin binding site, resulting in kernicterus and associated brain damage in the same way (Wadsworth and Suh, 1988; Thyagarajan and Deshpande, 2014). However, literature evidence to correlate the incidences of brain damage specifically with CMN-SMZ treatment is scarce. After the present 42-day exposure, CMN and SMZ provoked a rupture on BBB, suggested by down-regulated tight junction proteins (occludin, claudins and ZOs), which may lead to the increase of BBB permeability. This is consistent with previous studies by Maurya et al., who demonstrated that CMN down-regulates the expression of Claudin5, leading to the transmission of pesticides from blood to central nervous system (Maurya et al., 2014). Similarly, Balbuena et al. also showed that exposure to malathion resulted in decreased levels of Occludin, Claudin5, ZO-1 and ZO-2, which may lead to deprivation of the BBB's characteristics of low permeability and high transendothelial electrical resistance (Balbuena et al., 2011). In summary, CMN and SMZ can induce grass carp neurotoxicity *via* changing the permeability of BBB, causing abnormal behavior, histopathological changes, and ultrastructural damage (nerve cell damage, mitochondrial dysfunction, synapse reduction).

In aquatic ecosystems, AChE activity is generally considered to be the main marker of pollutant exposure, especially in the effects of neurotoxic compounds and heavy metal exposure (Cajaraville et al., 2000;

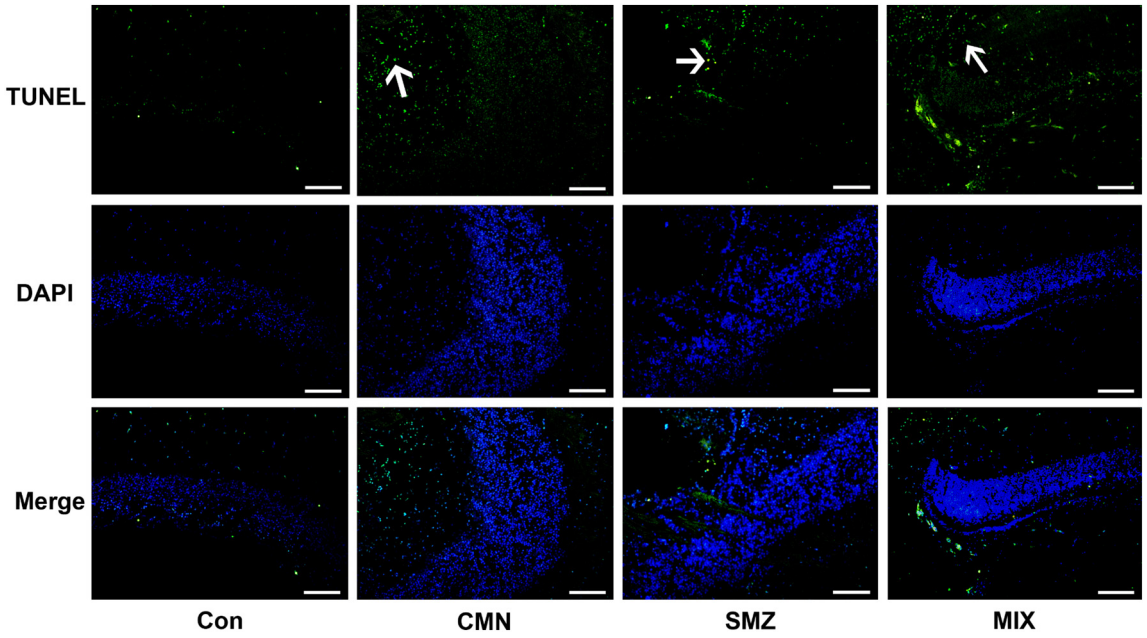

Fig. 7. Apoptosis assayed with TUNEL staining. Green: TUNEL, blue: DAPI, 200 × . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Junejo et al., 2019). In the model of fishes, when AChE is inhibited under pollutants (antibiotics, ofloxacin and SMZ), the neurotransmitter ACh in nerve synapses and neuromuscular junctions cannot be hydrolyzed (Erwin et al., 2003), leading to abnormal growth, survival, feeding and reproduction behavior (Dos et al., 2005; Yang et al., 2019). In this study, exposure to CMN and SMZ alone for 42 days significantly inhibited the activity of AChE, and in the MIX group, AChE showed lower activity than the SMZ and CMN groups. These results were partly consistent with previous findings which suggested the reduction of AChE activity in the brain of goldfish and rainbow trout under the exposure of SMZ (Liu et al., 2014) and CMN (Alak et al., 2019), respectively. These results suggest that CMN and SMZ lead to the depletion of AChE, making accumulation of ACh in synapses, disturbing transmission at parasympathetic nerve endings, sympathetic ganglia and the neuromuscular endplates. Moreover, the combined exposure displayed a stronger toxicity.

GSH is a cellular antioxidant and has a wide range of roles in protecting the central nervous system from oxidative stress (Mukhopadhyay et al., 2015). In our study, we found that exposure to CMN and SMZ resulted in the decrease of GSH content in grass carp brain tissue, indicating a destroy of cellular redox homeostasis (Dwivedi et al., 2020). Oxidative stress caused by CMN or SMZ was characterized by the elevated levels of MDA, one of the end products of lipid peroxidation (Wang et al., 2020) and 8-OHdG, a marker for DNA oxidative damage (Kawanishi et al., 2016). The content of GSH, MDA and 8-OHDG reciprocally suggested that the increased ROS levels caused proteomic, lipid and DNA oxidative damage due to CMN and SMZ exposure, in which co-administration reached the most significant. On the other hand, the production of ROS can induce the expression and activity of some cytoprotective enzymes, including SOD and CAT antioxidant enzymes. In this study, SOD and CAT activities displayed a compensatory up-regulation when facing with excessive ROS production under the exposure of CMN and SMZ in the brain. However, this was not an isolated event, CMN was reported to cause an increase in the activity of CAT and SOD in the gills of zebrafish due to a large amount of ROS generation (Paravani et al., 2019), and so do SMZ in the intestine of tilapia (Limbu et al., 2018). The current results clearly showed that CMN and SMZ can cause serious damage to the grass carp brain by disrupting the redox homeostasis even at low environmentally relevant concentrations. In order to uncover the mechanism behind, the Nrf2-Keap1 pathway came into our view (Sun et al., 2020). Under exogenous pollutants, Nrf2 acts as a vital transcriptional factor to regulate cellular redox equilibrium (Jia et al., 2019). In our study, the up-regulation of HO-1 and NQO1 may be related to the activation of the Nrf2 pathway as a response to the neurotoxicity induced by CMN and SMZ. The findings of Gargouri and Zhou et al. well confirmed this point, who suggested the activation of Nrf2 activation under pesticides (CMN) stress, which subsequently induced NQO1 and HO-1 expression in cortical neurons (Gargouri et al., 2018; Zhou et al., 2020). Similarly, Promsan et al. studied that antibiotics (gentamicin) can also induce the expression of Nrf2, HO-1 and NQO1 in rat renal tubular cells (Promsan et al., 2016). Those above changes clearly indicated that low environmentally relevant concentrations of CMN and SMZ can induce mild oxidative stress response, while grass carp exerts its survival mechanism by Nrf2 and its downstream antioxidant genes.

ROS have extensively been recognized to trigger the intrinsic apoptotic pathway (Shaw et al., 2020). Therefore, we studied this phenomenon by monitoring the related key genes. P53 is an important pressoreceptor against ROS, leading to the initiation of apoptotic stage (Liu et al., 2008). In this study, the transcriptional expression of p53 was increased in CMN, SMZ and MIX groups, indicating an initiation of apoptotic damage under exogenous exposure. Bax locates in the outer mitochondrial membrane and is a direct target for p53 transcription activation. The intrinsic apoptosis pathway is mainly activated by p53-Bax cascade, which then induces cell apoptosis by the mitochondria-dependent pathway (Wang et al., 2018a; Li et al., 2020). By the contrary, Bcl-2 functions to prevent Bax from forming Bax pores on the outer membrane of mitochondria, thus preventing the formation of heptamarized apoptotic body complex (Chen et al., 2020b; Guo et al., 2019; Liu et al., 2020; Shaw et al., 2020). In our study, Bcl-2 was observed to be down regulated after chronic exposure and showed a pattern contrary to Bax as expected. The increase of Bax/Bcl-2 ratio indicated that CMN and SMZ exposure can induces mitochondrial dependent apoptosis. Finally, the entire signal cascade performs its work by activating Caspases protease, leading to the hydrolysis of intracellular proteins and skeletons, namely the programmed cell death (Wang et al., 2018b). In this study, the genes for both Caspase-9 (initiator) and Caspase-3 (executioner) were found to be upregulated after CMN and SMZ joint exposure. Our findings corroborate with Arslan et al. who reported that CMN increased the expression of pro-apoptotic Bax and Caspase-3 and downregulated anti-apoptotic Bcl-2 in common carp brain (Arslan et al., 2017). And Xi et al. also demonstrated norfloxacin could trigger apoptosis (Bax/Bcl-2 and Caspase expression) in the brain of zebrafish embryos (Xi et al., 2019).

Many environmental pollutants are well known to act as neurotoxic compounds and commonly occur as complex mixtures with low concentrations in aquatic systems. Populations and communities of different species, however, can be dramatically impacted by these xenobiotics (Scholz et al., 2006; Relyea, 2009). CMN and SMX are pharmaceuticals with different mechanisms of action. In terms of neurotoxicity, teleost fishes are particularly vulnerable to CMN, compared to mammals or birds (Edwards et al., 1986). Particularly, known and even chemicals with unknown neurotoxic potential, occur together in the environment, these may generate toxic effects exceeding the single compounds. Therefore, it is urgent to develop meaningful effect-based methods to screen and investigate the neurotoxicity of chemicals and their combined effect. In the present toxicological detection, especially Occludin, ZOs, Nrf2 and Caspases, indicated that the neurotoxicity of CMN was sometimes amplified when SMZ co-administration, indicating a complex interaction may occur between CMN and SMX. The mutual reinforcement is commonly observed in organic compounds in water environment. In zebrafish (*Danio rerio*), Guo et al. reported an enhanced toxic effect of a mixture of CMN and malathion (Guo et al., 2017) and similar conclusions were obtained following exposure to binary mixture of bisphenol AF and SMZ with thyroid endocrine disruption (Kwon et al., 2016). The present results raise concerns that pharmaceuticals, including pesticides and antibiotics, may elevate the biotoxicity of other pollutants in aquatic environments (Geiger et al., 2016). However, the biomolecular process and their link to behavioural alterations and multi-generation effects, should be investigated in more detail by the usage of multi-omics and epigenetic methods, respectively.

5. Conclusion

This study showed the neurotoxic potential of CMN and SMZ on grass carp brain. CMN and SMZ, even at low environmental-related concentrations, can trigger changes in the permeability of the BBB and decrease the activity of AChE. Antioxidant response, a compensatory regulation under exogenous stress, may trigger cellular protection to cope with CMN and SMZ exposure by Nrf2 pathway. Meanwhile, apoptosis is significantly induced under the exposure of CMN or/and SMZ. Moreover, it seems that a combination of CMN and SMZ for neurotoxicity of grass carp produces worse results than either of the two exogenous exposed alone, in terms of Nrf2- and apoptosis-related indexes. This study will reveal the potential ecological risks of pesticides and antibiotics in the aquatic environment, and provide basic data for their safety and risk assessment.

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. **Yachen Liu:** 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 they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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