Oxidative Stress Is Involved In The Activation of NF-κB Signal Pathway And Immune Inflammatory Response In Grass Carp Gill Induced By Cypermethrin And/Or Sulfamethoxazole Exposure

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Abstract

At present, the concentration of environmental pollutants, such as pesticides and antibiotics exposed in environment, especially in aquatic environment is increasing. These contaminants are exposed through aquatic environment to fish and ultimately accumulate in humans. Research on environmental pollutants has exploded in the past two years. However, there are still few studies on the combined effects of pesticides and antibiotics on fish, especially on fish gills. A separate analysis of the toxic effects caused by an environmental pollutant cannot fully show the real situation. In this paper, cypermethrin (CMN) and sulfamethoxazole (SMZ) were analyzed and found that there was a strong correlation between the pathways affected by the first 30 genes regulated by CMN and SMZ respectively. Therefore, the toxic effects of CMN (0.651 μg/L) and/or SMZ (0.3 μg/L) on grass carp gill were studied in this paper. Histopathology, quantitative real-time PCR and other methods were used to detect the tissue morphology, oxidative stress level, inflammation and apoptosis-related indicators of the fish gills after exposure 42 days. It was found that compared with the single exposure (SMZ/CMN) group, the combined exposure (MIX) group had a more pronounced oxidative stress index imbalance. At the same time, nuclear factor-κB (NF-κB) signal pathway was activated and immuno-inflammatory reaction appeared in MIX group. This study reveals the harm of CMN and SMZ to fish, and provides a reference and basis for the rational use of pesticides and antibiotics.

Introduction

The use of antibiotics has been increasing globally in recent years, especially in high-pollution countries and low-and middle-income countries (Klein, et al., 2018). The total amount of antibiotics used in China in 2013 was about 162,000 tons, of which about 50% was used in animal-related areas (Ying, et al., 2017). As food demand and agricultural production have increased, the use of pesticides has increased, reaching 170,000 tons in Argentina alone in 2018 (http://www.fao.org/faostat/en/). After entering the ecological cycle, these excessive organic substances became organic pollutants, entering rivers and oceans through rainwater and atmosphere, eventually accumulating in aquatic organisms (Mahboob, et al., 2015). Studies have shown that antibiotics can congest in tissues such as plasma, bile and liver of grass carp (Chen, et al., 2018), and pesticide residues have also been detected in fish (Omwenga, et al., 2016). Antibiotics are widely used in production and life (Chen, et al., 2015). These used antibiotics are often released into the environment through a variety of wastewater without any treatment (Kummerer, 2009). Sulfamethoxazole (SMZ) is the main antibiotic contaminant in the waters of the Huangpu River and which has a moderate hazard to the aquatic ecological environment (Chen and Zhou, 2014). SMZ is widely distributed in the Vietnamese rivers, polluting the water body, and promoting the growth of drug-resistant bacteria (Harada, 2018). Long-term exposure to polluted water environment will have a great impact on the health of fish, and fish are highly sensitive to antibiotic exposure (de Lemos, et al., 2007). Studies have shown that SMZ exposure to environmental concentrations inhibits antioxidant capacity and leads to apoptosis and damage in Nile tilapia (Oreochromis niloticus) (Limbu, et al., 2018). Cypermethrin (CMN) is a kind of pyrethroid insecticide with broad spectrum and high effective

insecticidal performance (Sparks, et al., 2021). CMN residues are widely found in fruits, vegetables, air, and water. It has even been reported that CMN has been detected in human breast milk (Bedi, et al., 2015; Bedi, et al., 2013). Long-term exposure to large amounts of pesticides can inhibit the normal development of organisms, threaten reproductive health, and damage the digestive system (Guo, et al., 2021; Martin, et al., 2018). The gills are the first organ of fish in contact with the external environment. Some substances (such as antibiotics and pesticides) can enter fish through the gills, causing fish poisoning, inflammation and apoptosis (Soltanian and Fereidouni, 2017; Zhao, et al., 2020c).

Numerous studies have suggested that oxidative stress is a potential way to antibiotics and pesticide induced toxicity (Lushchak, et al., 2009; Qiu, et al., 2020). Under normal circumstances, the redox in the body is in a dynamic balance, but when the body is stimulated by the outside, the level of reactive oxygen species (ROS) rises, leading to the occurrence of oxidative stress (Chen and Maltagliati, 2018). SMZ of environmental concentrations can damage intestinal epithelial cells through oxidative stress and destroy tight junction proteins (Wang, et al., 2021b). Organophosphorus pesticides can cause neurotoxicity through oxidative stress (Farkhondeh, et al., 2020). Excessive pesticides can affect the growth and metabolism of non-target plants by increasing the content of ROS, reducing the content of soluble sugar and protein in plants and causing cell damage (Shakir, et al., 2018). There are few studies on the effects of pesticides and antibiotics in environmentally relevant concentrations on organisms.

NF-E2-related factor 2 (Nrf2) signaling pathway is one of the main ways to protect cells from oxidative damage. Nrf2 gains activity by dissociating from inhibitory cytoplasmic protein Kelch-like ECH-associated protein 1 (Keap1) to activate antioxidant-related genes in the nucleus (Lu, et al., 2016). Studies have displayed that, when the body experiences oxidative stress, Nrf2-Keap1 signaling pathway is activated (Bellezza, et al., 2018). SMZ produces cytotoxicity by stimulating the production of reactive oxygen species and aryl hydroxylamine metabolites. It will irritate the sea urchin to produce oxidative stress and eventually destroy the sea urchin's defense mechanism (Ragusa, et al., 2017). CMN can activate the Nrf2 signaling pathway to protect the central nervous system. However, the effect of SMZ and CMN on the Nrf2 pathway of grass carp gills is still unclear (Zhou, et al., 2020).

Nuclear factor-κB (NF-κB) signaling pathway plays a key role in inducing gene expression. NF-κB participates in a variety of biological processes including inflammation and apoptosis (Wang, et al., 2021a). Interleukins (IL-6/8/10, etc.) push forward a immense influence on immune inflammation and can mediate the transmission of inflammatory signals (Li, et al., 2021). They are common indicators of inflammation (Liu, et al., 2021). At present, it is generally believed that apoptosis is caused by the highly regulated cysteine protease caspase cascade, and Caspase-3 is just downstream of the entire cascade and is the executor of apoptotic process (Guerin, et al., 2021). The legally farmed dose of SMZ can enhance the oxidative stress, inflammation, and apoptosis levels of largemouth bass, and even remain in the fish (Xie, et al., 2020). CMN can inhibit the activity of cholinesterase and induce the decomposition of nerve cells (Raszewski, et al., 2015). However, in aquatic organisms, there is still a lack of corresponding research on how the combined use of SMZ and CMN can cause inflammation and apoptosis.

In this study, we studied the toxicity and toxicological mechanism of environmental concentrations of CMN and/or SMZ from a comprehensive point of view. The effects of CMN and/or SMZ on gene-gene interaction and phenotype were studied, and the changes of gill tissue structure and various physiological indexes were detected by experimental model. Strive to truly reflect the effects of CMN and/or SMZ on aquatic organisms in the water environment, and provide a new basis for the standardized use of antibiotics and pesticides, and the healthy culture of freshwater fish.

Materials And Methods

2.1 Chemicals and animals

MS-222 and CMN (No. 52315-07-8, purity of 98%), SMZ (No. 1196157-90-0, purity of 99.8%) was purchased from Sigma Chemical Co. (St Louis, Missouri, USA). CMN and SMZ were dissolved in 99% pure dimethyl sulfoxide (DMSO) and store in dark at 4℃.

A total of 120 juvenile grass carps with an average body weight of 105.45 ± 5.68g (Harbin Aquaculture Farm) were adapted in the laboratory for two weeks (Wang, et al., 2021c). Grass carp were reared with dechlorinated tap water in a 500L indoor circulation tank. The whole experiment lasted for 42d with 12h light/dark period. The water temperature was controlled at (27.0 ± 1.5) ℃, the dissolved oxygen content in water was greater than 6.0 mg/L, the ammonia nitrogen content was lower than 0.05 mg/L, the nitrite nitrogen content was lower than 0.06 mg/L, and the pH value was 7.0–8.0.

2.2 Experimental design

The study was conducted under the supervision of the Animal Protection and Utilization Management Committee of Northeast Forestry University. 120 Healthy grass carps were randomly divided into 4 groups, namely control (C) group, CMN group, SMZ group and CMN + SMZ (Mix) group, with 3 replicates in each group. According to some current environmental-related concentration studies(Chen and Zhou, 2014; Zhao, et al., 2020c) and environmental surveys(Wang, et al., 2018; Zhou, et al., 2016), the concentrations of CMN and SMZ are set to 0.651 μg/L and 0.30 μg/L respectively in this study.

During the entire experiment, dechlorinated tap water with the same exposure concentration was replaced every 48h. Water samples were collected at exposure times of 1, 14, 28, and 42 days, and the concentrations of CMN (Enzyme-linked Biotechnology, China) and SMZ (REAGENLLC, USA) were detected with ELISA kit. During the whole experiment, the deviation of the actual average concentration of CMN and SMZ from the nominal concentration is less than 10%, which is relatively stable. After 42 days of exposure, the grass carp was anesthetized with MS-222 (10 mg/L) and the gill tissue were separated and stored at -80°C. No death of grass carp was found throughout the experiment.

2.3 Histopathological observation

After 42d exposure, the extracted gill tissue of each group were fixed with 4% paraformaldehyde and made into paraffin sections with a thickness of 4mm.The tissue sections were stained with hematoxylin-

eosin (H&E) and observed under a microscope (Olympus, Japan) for histological analysis.

2.4 oxidative stress index detection

Use Nanjing Jiancheng Biological Company (NJJCBIO, China) testing kits, and follow the instructions to process the tissue for testing, including superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione (GSH) content.

2.5 Real-time fluorescence quantitative PCR

Using Trizol reagent (Invitrogen, USA) to extract total RNA from the gill tissue, total RNA was measured (260 nm) spectrophotometer nano concentration, and RNA quality was detected (260/280 nm, 260/230 nm). qPCR was performed with HiScript II Q Select RT Super Mix kit (Vazyme Biotech Co., Ltd.) to obtain cDNA. Use FastStart Universal SYBR Green Master reagent (Roche) and Light Cycler® 480 (Roche, Switzerland) system to complete the experiment. Using $2^{-\Delta\Delta CT}$ method of data analysis, the primers shown in Table 1.

Table 1
A list of primers in qPCR analysis of mRNA expression of the target genes.

Genes	GenBank accession	Primer sequence (5'-3')	Product size
IL-1β	MK942107.1	Forward: TCCAAGCACGTCGTGTGACT	126bp
		Reverse: GACGAGGAGATCCGCTGCTT	
IL-6	KC535507.1	Forward: GTCAGGATCAGCACGCCTCT	86bp
		Reverse: TAAATGCGCCCCAGACACCT	
IL-8	JN255694.1	Forward: ACCCTCCTAGCCCTCACTGT	136bp
		Reverse: CATGGTGCTTTGTTGGCAAGG	
IL-10	HQ388294.1	Forward: CGCTTCTACTTGGAGACCATTC	112bp
		Reverse: CCATATCCCGCTTGAGATCTTG	
TNF-α	JQ670916.1	Forward: GGCGCTTTTCAGCGCTATCC	98bp
		Reverse: GTAATTGGAAGGCCGCACCG	
iNOS	HQ589354.1	Forward: GGCTATGACACGCTGTCCCT	142bp
		Reverse: AGCGACTTCCTCCACACGAG	
Nrf2	XM_019123954.1	Forward: GTGAATGAGGAGGAGGTGAAAG	118bp
		Reverse: GGGTACTACTTCCCAGCAAATC	
Keap1	XM_019071157.1	Forward: CGAGTGGAGGAGCATCGTGT	105bp
		Reverse: TGATGGGTGCCGTCGTATCC	
NQO1	NM_001204272.2	Forward: GCTACTGCGGAGGATATTAAGG	100bp
		Reverse: CGTCACTTAATCGACCCTCTTT	
HO-1	MH578354.1	Forward: CAGCATCCCAAAGTCAATCAAG	103bp
		Reverse: GCATAAACTCCCATTCCAACAG	

2.6 Immunoblotting analyses

The nucleoprotein and plasma protein extraction kit (Wanleibio, China) was used to extract NF-κB proteins from the gill tissue. The remaining proteins were extracted from the gill tissue using RIPA lysate (add 100mg tissue to a mixture of 1ml RIPA lysis buffer and 10μl PMSF), and the supernatant was collected, quantified with a BCA kit (Beyotime, China), and stored at -80°C. Western blot and digital imaging equipment (General Electric, USA) were used to detect the signal, and Image-J software was used for quantitative analysis. Primary antibodies include: Caspase-3, Caspase-9, Bcl-2-Associated X

(Bax), B-cell lymphoma-2 (Bcl-2), inhibitory protein of nuclear factor of kappa B-alpha (IκB-α), p-iκBα, Histone H3 (H3), NF-κB (nucleus), NF-κB (cytosol) (Wanleibio, China), β -actin (Abclonal, China).

2.7 Selection and enrichment analysis of differential genes

Using DTC database (http://ctdbase.org), the differential genes under the action of SMZ and CMN were selected, and the genes of top 30 (if there are less than 30 genes, select all of them) were selected for follow-up analysis according to the order of p value from large to small. The selected genes were enriched and analyzed by gene ontology (GO), Kyoto gene and genome encyclopedia (KEGG), Metascape (http://metascape.org/gp/index.html#/main/step1). According to the reference (Sendra, et al., 2021), the GO results were divided into three categories according to biological process (BP), cellular component (CC) and molecular functional (MF).

The top 50 genes (if there are less than 50 genes, select all of them) affected by SMZ and CMN were mixed, and the duplicate genes were removed to get a new gene list. After the mixed gene list, the online software Metascape was used for term enrichment analysis, and the interaction network between genes was generated. The online software STRING 11.0 (http://string-db.org/) was used to generate the interaction network between the proteins corresponding to the mixed gene list, and the k-means algorithm was used to mark them as different paths. Specific reference (Zhao, et al., 2020b).

2.8 Statistical analysis

IBM SPSS 22.0 (Version 22.0, Inc., Chicago, IL, USA) software was used to analyze the experimental data. One-way analysis of variance was adopted, and the data results passed the Tukey test. All the data in this article $F < 0.05$, $P < 0.05$ are statistically significant, and all values are expressed as the mean ± SD value. Graphpad Prism 5.0 (version 5.01, Inc., La Jolla, USA) was used to draw statistics.

Result

3.1 Results of data analysis

According to the results of GO analysis (Fig. 1), it is found that the gene changes caused by CMN and SMZ have something in common in cell process, cell composition and molecular function, indicating that the physiological changes caused by CMN and SMZ may be similar.

In order to determine whether there are similarities between them in the mechanism of action, we carried out signal pathway enrichment analysis (Fig. 2) of differential genes. All the results clearly point out that both CMN and SMZ can cause significant changes in oxidative stress. And found that compared with SMZ, CMN can cause obvious apoptosis, while the effect of SMZ on inflammation is more intense. This may be due to the different types of the two and the different functions they perform. CMN is a kind of insecticide, which aims to destroy the target organisms, so it has a stronger toxic effect. SMZ is an antibiotic, which may protect the body from injury by causing immune inflammatory response and protective apoptosis.

3.2 Interaction analysis

From Fig. 3, it is found that the changes that may be caused by a mixture of CMN and SMZ are not the same as when using CMN or SMZ alone. In the protein-protein interaction analysis (Fig. 3A), the proteins related to oxidative stress, inflammation, and apoptosis are at the center. After enriching the differential genes (Fig. 3C), it was found that the P values of oxidative stress, apoptosis and inflammation-related pathways were low, indicating that the combined use of CMN and SMZ is very likely to affect these three physiological processes.

3.3 Histological observation of gills

As shown in Fig. 4, in the case of CMN and/or SMZ exposure, the gill tissue have obvious pathological changes, and the gill tissue damage under the combined exposure is clearly more serious than the single exposure.

The control group (Fig. 4A) has normal morphology, clear structure, and neatly arranged gill lamella. Gill epithelial hyperplasia, cell swelling and epithelial cell shedding occurred in the CMN single exposure group (Fig. 4B), and even local hyperemia and inconspicuous gill lamella structure (gill lamella fusion). The SMZ single exposure group (Fig. 4C) showed cell necrosis, edema, hyperplasia, hyperemia, and curved gill lamella. The necrosis of the gill epithelium was more serious in the MIX group (Fig. 4D), with more vacuoles in the middle of the gill lamella, and the overall structure gradually deviated from the normal shape.

3.4 The effect of CMN and/or SMZ on the oxidative stress of gills

As shown in the Fig. 5, compared with the control group, the use of CMN and/or SMZ significantly reduced the activity of SOD and the content of GSH, and significantly increased the content of MDA. And compared with CMN or SMZ alone, the MIX group has a stronger ability to induce MDA production, which is 1.9 times that of the control group. It is suggested that the mixed use of CMN and SMZ reduces the antioxidant capacity of grass carp gill tissue and aggravates oxidative damage.

3.5 The effect of CMN and/or SMZ on the Nrf2-Keap1 pathway and inflammatory factors

Tested the mRNA expression level of the Nrf2-Keap1 pathway (Fig. 6) to study the mechanism of oxidative stress induced by CMN and SMZ. According to the test results, the MIX group significantly increased the expression level of Keap1 and decreased the expression level of Nrf2. At the same time, the expression levels of HO-1 and NQO1 in the MIX group were also significantly decreased compared with the individual exposure group. It is suggested that the combined use of CMN and SMZ can inhibit the Nrf2-Keap1 signaling pathway and decrease the antioxidant capacity. The expression level of NQO1 has not been suppressed very seriously.

Studies have described those pesticides and/or antibiotics can induce inflammatory effects in the body (Wang, et al., 2020a; Zhao, et al., 2020c). In order to verify whether the combined use of the two can

cause inflammation in the gill tissue of grass carp, we performed fluorescent quantitative detection of related inflammatory factors. Compared with group C, the combined use of CMN and SMZ made inducible nitric oxide synthase (iNOS) (5.27 times), tumor necrosis factor (TNF-α) (2.94 times) and (IL-1β) (9.84 times), (IL-6) (3.41 times), (IL-8) (32.67 times) mRNA levels increased, and the anti-inflammatory factor interleukin 10 (IL-10) (0.21 times) expression decreased (Fig. 6), indicating that the gill tissue of the grass carp had an inflammatory response.

3.6 The effect of CMN and/or SMZ on NF-κB

Through the changes in protein levels (Fig. 7), compared with the control group, the expression of IκB-α (0.76 times) was significantly reduced in the MIX group, and the expression of p-IκBα (2.26 times) was increased. These results indicate that IκB-α is activated. The level of NF-κB protein increases in the nucleus and decreases in the cytoplasm. These phenomena indicate that NF-κB is activated and transferred to the nucleus to activate the NF-κB signaling pathway. The transcription level of inflammatory factors (Fig. 6A-F) is consistent with the expression of NF-κB, and the trend is identical, suggesting that the combined use of CMN and SMZ may trigger an inflammatory response by activating the NF-κB signaling pathway.

3.7 The influence of CMN and/or SMZ on apoptosis-related signals

To investigate the relationship between the exposure (CMN and/or SMZ) and apoptosis, we examined the expression of apoptosis related gene at the protein level, and the results were shown in Fig. 8. Compared with the control group, whether SMZ or CMN exposure is alone or joint, the expression levels of apoptosis-related proteins (Bax, Caspase-3, and Caspase-9) increased significantly. It was also observed that Bcl-2 was significantly inhibited at the level of transcription and translation. It is suggested that the apoptotic pathway plays a role in the exposure of CMN and/or SMZ. According to the experimental results, the change trend of Bax/Bcl-2 protein ratio is consistent with the expression trend of Caspase-3 and Caspase-9 protein. The expression level of the experimental group is higher than that of the control group, and the MIX group is much higher than the single exposure group. It suggests that the MIX group is more toxic to the tissues and causes more serious damage. The change trend of apoptosis-related proteins is the same as that of nuclear factor NF-κB (Fig. 7, C-D), suggesting that apoptosis may be triggered by the activation of NF-κB signaling pathway.

Discussion

Gills are the respiratory organs of fish, and they complete gas exchange when blood flows through here. The morphological changes of gills are an indicator of early toxicity (Fiedler, et al., 2020). However, it is still unclear whether SMZ and CMN at environmental concentrations can damage fish gills. From the point of view of big data, after analyzing the possible mechanism and results of SMZ and/or CMN, we found that the combined use of SMZ and CMN could cause strong oxidative stress, inflammation, and apoptosis at the same time. After that, we found that long-term exposure to environmental concentrations of SMZ and CMN in fish gills will cause tissue damage, and antioxidant system will also be inhibited.

Compared with the control group, the MIX group caused more severe oxidative damage, inflammation, and apoptosis. These experimental results support the conclusion drawn by big data and prove that the combined use of pesticides and antibiotics at environmental concentrations is harmful to organisms. Furthermore, it is inferred that the organisms living in the polluted water environment are damaged by environmental pollutants.

Liu et al. found that zebrafish exposed to an environment containing SMZ can consume SMZ through daily activities. SMZ entering the body can cause oxidative stress and inflammation in healthy fish (Liu, et al., 2020). After observing the results of the oxidative stress indicators detection in this study, it was found that the exposure of CMN and/or SMZ caused oxidative stress in the tissues. GSH is commonly found in various organisms and is the most important antioxidant. GSH scavenges free radicals in the body through the oxidative dehydrogenation of sulfhydryl (-SH) in the molecular structure (Aldini, et al., 2018). In this study, the content of glutathione was significantly reduced, indicating that the gill tissue contains a lot of free radicals and peroxidation products. These oxidation products exceed the regulating ability of GSH, and GSH forms GSSG after the oxidative dehydrogenation of the -SH group. Suggesting that the overall content of GSH has decreased (Moreno-Sanchez, et al., 2018). Superoxide dismutase (SOD) maintains the oxidation balance in the body by converting superoxide free radicals into hydrogen peroxide, and is a ubiquitous antioxidant enzyme (Sakamoto and Imai, 2017). Generally speaking, strength of the antioxidant ability in the organism can be judged by the content of SOD (Qu, et al., 2019). MDA is the final product of lipid peroxidation. It can not only affect the function of mitochondria but also aggravate the damage of cell membrane (Tsikas, 2017). MDA accumulates in a time-dependent manner under oxidative stress (Wang, et al., 2020c). According to the results of Fig. 5, the MDA content caused by the MIX group increased significantly. Combined with Fig. 4, it can be concluded that the MIX group has a synergistic effect to some extent, causing more serious damage. Similar results have been found in some literature (Aderemi, et al., 2018). Zhao et al. found that the combined use of antibiotics and pesticides aggravated the oxidative stress of the carp spleen and damaged the immune system (Zhao, et al., 2020c). In addition to fish, pesticides and antibiotics can also affect the behavior of shrimp, inhibit neuro enzyme activity and induce oxidative stress (Huynh, et al., 2010). CMN can cause oxidative stress in zebrafish gills and produce a dose-dependent DNA damage response (Paravani, et al., 2019). The oxidative stress index of zebrafish increased 14 days after exposure to SMZ, and the cell membrane was damaged (Tokanova, et al., 2021). These studies confirm that our evidence is reliable.

The Nrf2-Keap1 signaling pathway is the main antioxidant pathway in the body, which can resist the damage caused by toxic substances to the body (Casalino, et al., 2007). And maintain the body's oxidative balance (Copple, et al., 2008). Normally, when the body is subjected to oxidative stress, the Nrf2-Keap1 signaling pathway is activated and Nrf2 is released into the nucleus (Qiu, et al., 2020). Further promote the production of antioxidant products and induce antioxidant reactions (Kaspar, et al., 2009; Zhong, et al., 2015). Studies have shown that when Nrf2 is activated, it can promote the expression of NQO1 and HO-1 (Chen, et al., 2020b; Kaspar and Jaiswal, 2010). However, in the results of this study, it was found that the Nrf2-Keap1 signaling pathway was inhibited (Fig. 6G). This may be due to the combined toxicity of SMZ and CMN beyond the body's own regulatory capacity. The Nrf2 pathway has a

certain protective ability to the human body, but this protective effect is very limited. The protective effect of the Nrf2 pathway depends on the dose of toxic substances and the exposure time to organisms (Zhou, et al., 2020). The gill is the first organ of the fish that encounters the external environment. Long-term exposure to poisons puts the gill tissue in a continuous stress state, produces excessive free radicals and inhibits the Nrf2-Keap1 signaling pathway (David, et al., 2017). In the study of Wang et al. also found that antibiotics can inhibit the expression of zebrafish gills antioxidant-related genes (Wang, et al., 2020a). When the antioxidant products in the body try to restore the oxidative balance and fail, inflammation and apoptosis are triggered (Chi, et al., 2021; Wang, et al., 2020b).

TNF-α is an inflammatory cytokine produced by macrophages or monocytes during acute inflammation. It can induce tissue damage by inducing the production of ROS, and induce cell necrosis and apoptosis(Balkwill, 2006). Studies have demonstrated a critical role in NF-κB signaling pathway in mice induced inflammation (Peng-Yu, et al., 2019). From our test results, it was found that the protein expression level of NF-κB related molecules increased (Fig. 7) and the high expression of TNF-α (Fig. 6D), which proved that the combined exposure of SMZ and CMN can activate the NF-κB signaling pathway and Cause inflammation. Many studies have shown that antibiotics or pesticides can cause oxidative stress and inflammation in healthy organisms. Gentamicin can cause severe nephrotoxicity by inducing oxidative stress and inflammation (Ince, et al., 2020). Dietary exposure to CMN changes the lipid homeostasis and energy metabolism in the salmon liver (Fuller, et al., 2021). Oxidative stress can have a cascade reaction with NF-κB, which in turn triggers inflammation (Wang, et al., 2017). Pesticides can amplify the inflammatory response by increasing the levels of iNOS and Cyclooxygenase-2 (COX-2) (Cupic Miladinovic, et al., 2021). The iNOS/NF-κB cascade reaction produces peroxynitrate, which in turn increases the toxicity of pesticides (Chi, et al., 2018). Combined with the results of this study, the content of iNOS in the MIX group was significantly increased (Fig. 6E), and the combined toxicity enhancement of SMZ and CMN may be achieved through the cascade reaction of iNOS and NF-κB.

Studies have shown that environmental pollutants can activate cell apoptosis through the NF-κB signaling pathway (Arab-Nozari, et al., 2020; Chen, et al., 2020a). Bcl-2 family of proteins are key regulators of apoptosis, which plays a crucial role in maintaining the homeostasis (Siddiqui, et al., 2015). Among them, the pro-apoptotic proteins Bax and Bak can initiate the caspase cascade reaction, which is the programmed cell death (Edlich, 2018). The caspase cascade can activate the mitochondrial stress pathway (Guo, et al., 2019). When cytochrome c in the mitochondria is released and interacts with Apaf-1, Caspase-9 will be activated and works. Caspase-3 is located downstream of the entire caspase cascade, acting as an effector of the reaction and a target for cell lysis (Fan, et al., 2005). Therefore, in apoptosis caused by common environmental pollutants, the up-regulation of caspase family proteins and the down-regulation of Bcl-2 proteins can be seen (Wang, et al., 2017). Similar results can be discovered in this study (Fig. 8), indicating that CMN and/or SMZ exposure caused cell apoptosis in the gill tissue. We have observed that when SMZ and CMN are used in combination, the degree of change in apoptosis indicators is more pronounced than when used alone. Studies have shown that environmental toxins can also activate cell apoptosis through oxidative stress (Zhang, et al., 2021). It is known that sulfa drugs can induce the production of ROS by accumulating corresponding metabolites, and then activate cell

apoptosis and necrosis (Elzagallaai, et al., 2020). These indicate that SMZ may induce apoptosis through oxidative stress. CMN can cause apoptosis and affect the cell cycle through the selective toxicity of enantiomers (Ji, et al., 2021). In summary, the increase in the expression level of apoptosis indicators in the MIX group was accomplished in different ways. Apoptosis caused by CMN and SMZ may be induced through different pathways, and the results are finally presented in the increased apoptosis data.

A large number of studies have proved that pesticides and antibiotics can induce cell apoptosis through the mitochondrial pathway (Javed, et al., 2020; Zhao, et al., 2020a; Zhao, et al., 2020b). The mitochondrial pathway is one of the classic apoptosis pathways. Maintaining the permeability of the mitochondrial membrane requires maintaining the dynamic balance between Bax and Bcl-2 (Zhao, et al., 2018). In this study, the dynamic balance of Bax and Bcl-2 in the combination group was severely disrupted, and Bax/Bcl-2 expression level was 5.02 times that of the control group. In summary, the combined exposure of SMZ and CMN may induce apoptosis by activating the mitochondrial pathway, and after activation, the caspase family executes apoptosis signals. This conclusion is consistent with some reports in the literature (Arslan, et al., 2017).

Conclusion

In general, our research demonstrated that long-term exposure to SMZ (0.651 μg/L) and/or CMN (0.3 μg/L) can cause redox imbalance in fish gills, trigger inflammation and apoptosis, and cause tissue damage. At the same time, the combined exposure of CMN and SMZ caused more serious damage than single exposure, and this damage may be achieved by activating the NF-κB signaling pathway and inhibiting the Nrf2 signaling pathway. Our research showed for the first time that SMZ and CMN at environmentally relevant concentrations can damage gill tissue and provide a new basis for the combined toxicity of pesticides and antibiotics.

Declarations

Ethics approval and consent to participate

Ethics approval and consent to participate this study was approved by the Animal Protection and Utilization Management Committee of Northeast Forestry University.

Availability of data and materials

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

Consent for publication

Not applicable.

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Author information

Baoying Li and Yu Wang contributed equally to this work.

Conflicts of 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.

Authors' contributions

Baoying Li: Writing - Original Draft, Visualization; Yu Wang: Methodology, Conceptualization; Hongjing Zhao: Data curation, Investigation; Kai Yin: Project administration; Yachen Liu: Resources; Dongxu Wang: Visualization; Hui Zong: Supervision; Mingwei Xing: Funding acquisition.

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Figures

Figure 1

GO analysis results. A-C: GO analysis of the top 30 differential genes of CMN. D-F: GO analysis of the top 30 differential genes of SMZ. BP: biological process. CC: cell components. MF: molecular function.

Figure 2

The results of pathway enrichment analysis. A: the results of KEGG pathway enrichment analysis of the top 30 differential genes affected by CMN. B: the results of KEGG pathway enrichment analysis of the top 30 differential genes affected by SMZ. C: the rich term bar graph of the top 30 differential genes affected by CMN was colored by P value. D: the rich term bar graph of the top 30 differential genes affected by SMZ was colored by P value.

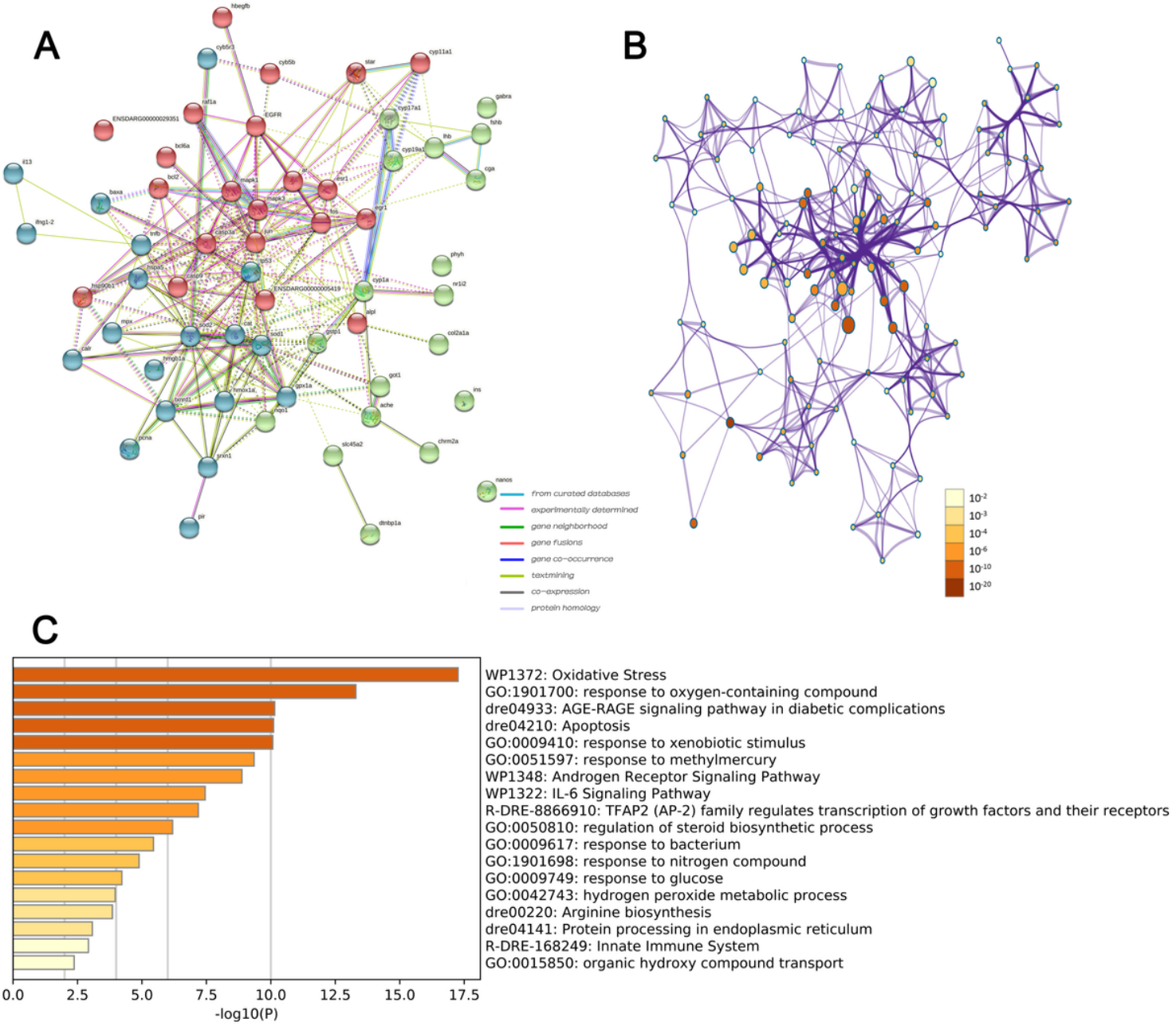

Figure 3

Interaction analysis and enrichment analysis of the list of the top 50 differential genes affected by CMN and SMZ. A: analysis of protein-protein interaction corresponding to genes. B: the interaction between genes was analyzed by P-value staining. C: rich term bar chart, colored by P value.

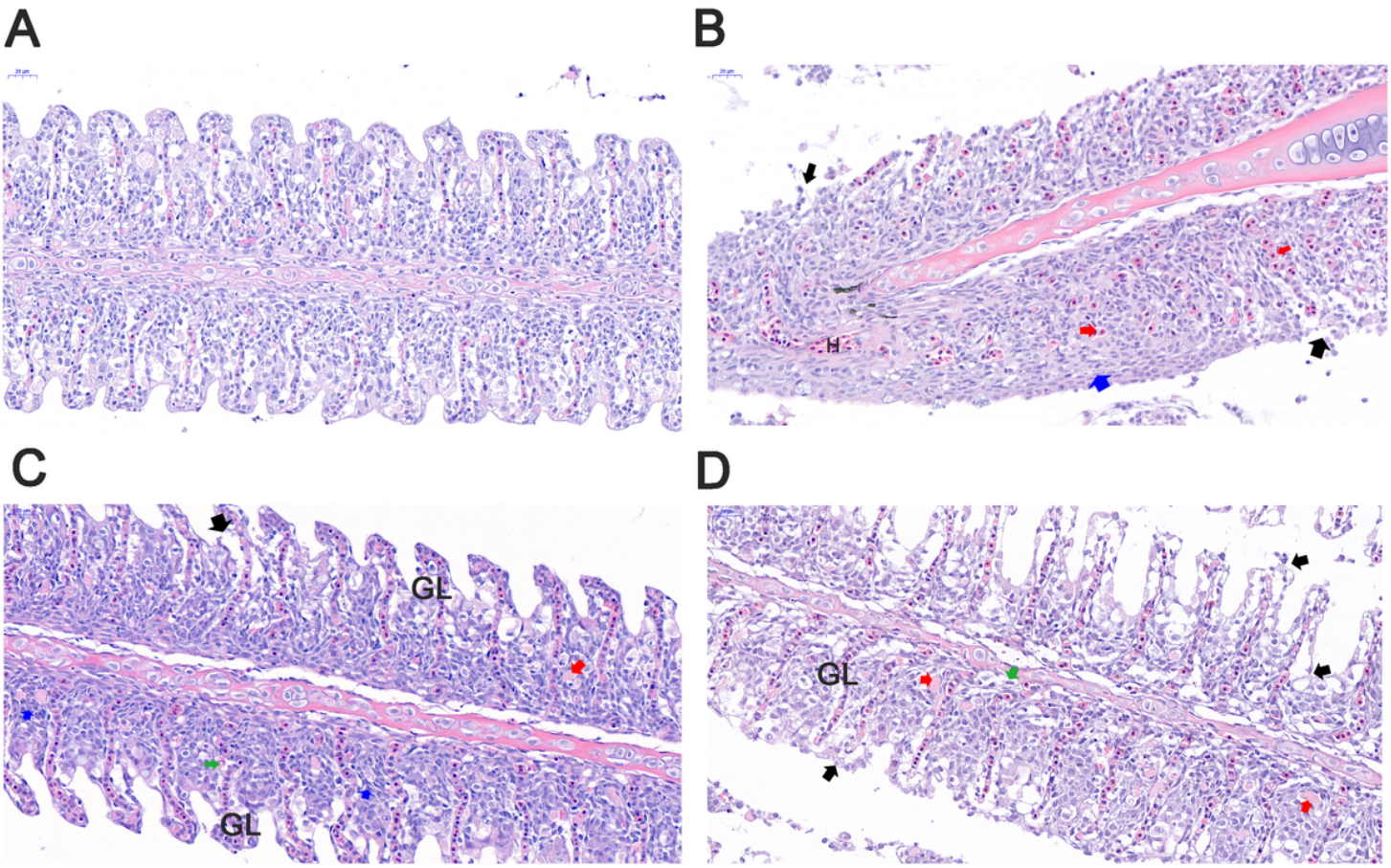

Figure 4

The effect of CMN and/or SMZ on the histological structure of grass carp gills. 41.2x; red arrow: cell swelling; blue arrow: epithelial hyperplasia; black arrow: cell shedding; green arrow: edema; H: hyperemia; GL: curved gill lamella.

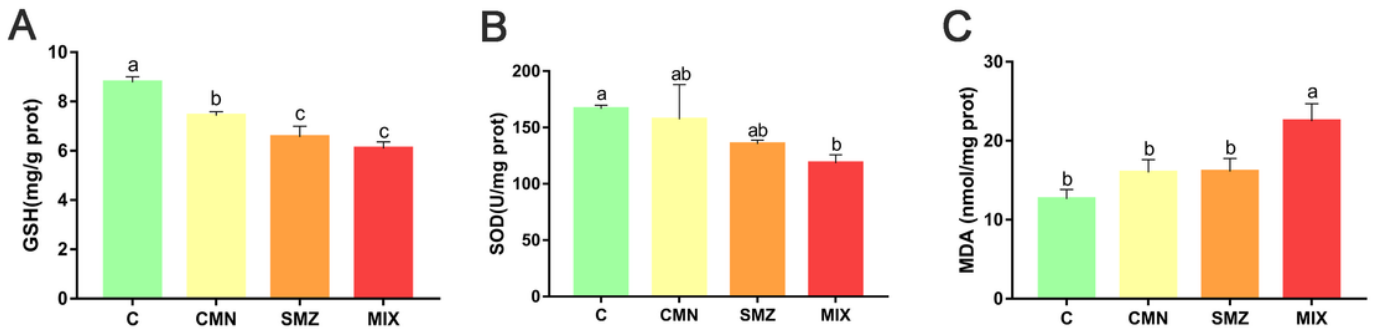

Figure 5

The effect of CMN and/or SMZ on the activity of SOD (B) and the content of GSH (A) and MDA (C). Set up 3 independent repeats, each with 3 fish, the same below. Different letters indicate significant differences between groups ($F<0.05$, $P<0.05$)

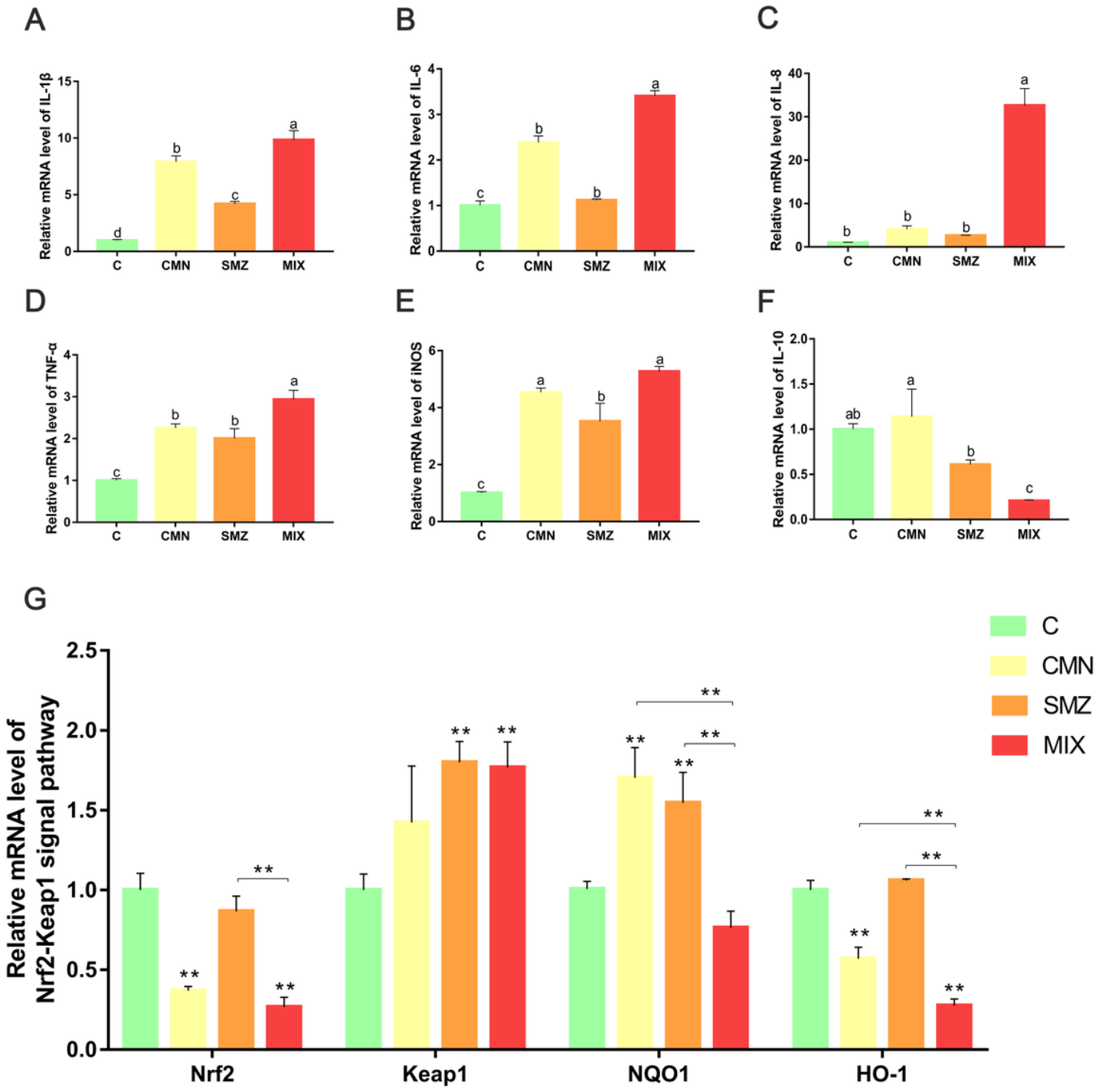

Figure 6

A-F: The effect of CMN and/or SMZ on the transcription of inflammatory factors. Different letters indicate significant differences between groups (F<0.05, P<0.05). G: The effect of CMN and/or SMZ exposure on the transcription of Nrf2 and its downstream target genes (F<0.05, *: 0.01

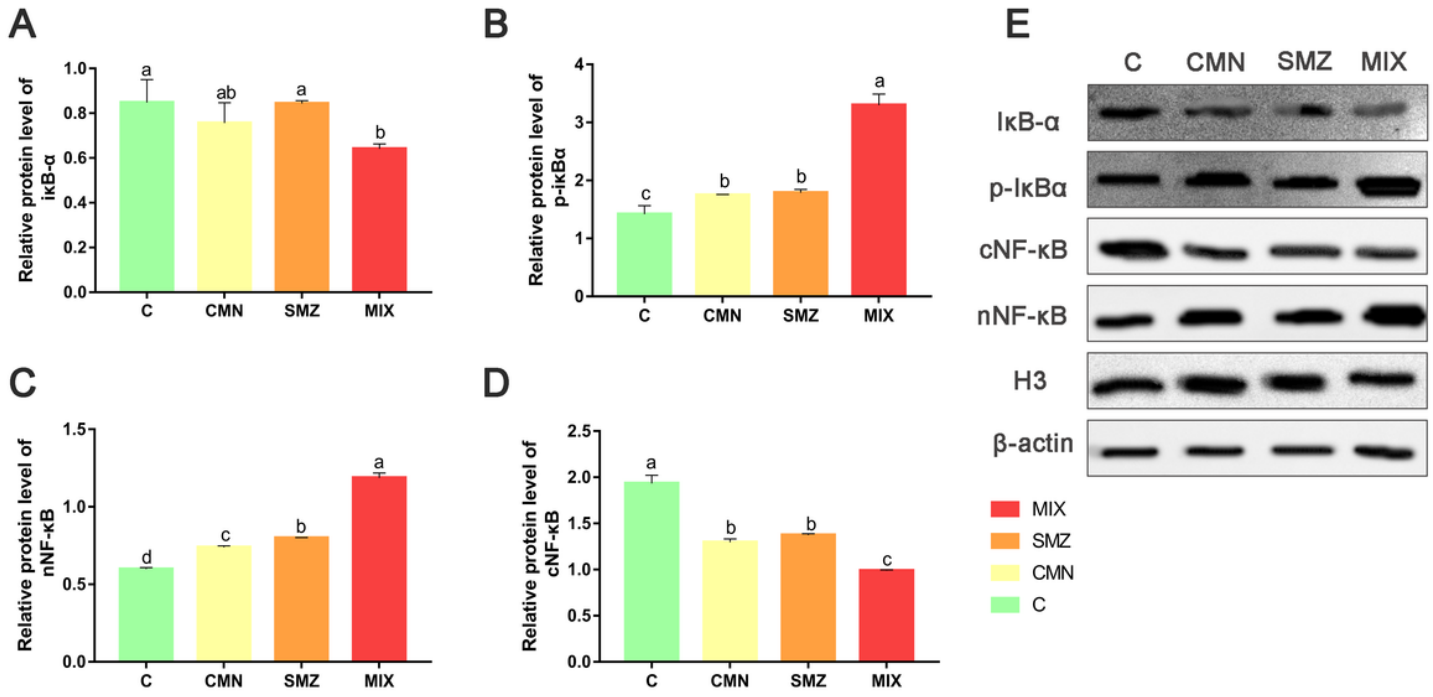

Figure 7

The effect of CMN and/or SMZ on the translation of NF-κB and its inhibitors. Different letters indicate significant differences between groups (F<0.05, P<0.05)

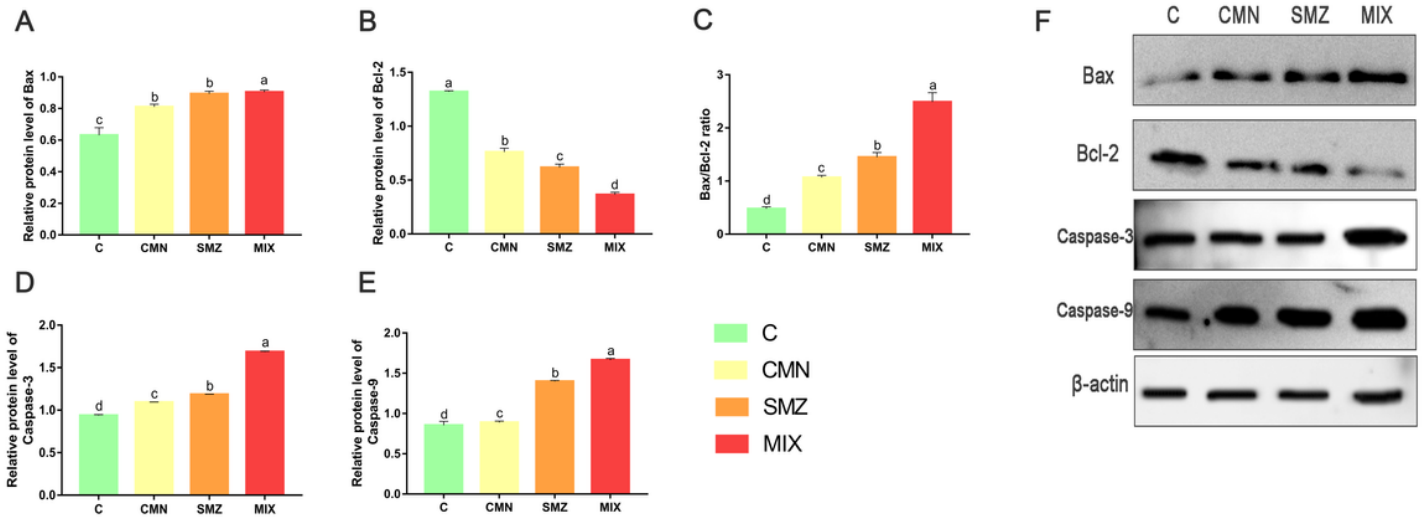

Figure 8

The effect of CMN and/or SMZ on apoptotic pathway. Protein levels of apoptosis-related genes. Different letters indicate significant differences between groups (F<0.05, P<0.05)