Nitrite exposure on gills of *Oreochromis niloticus*: structure change, immune response, and apoptosis

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Nitrite is one of the most common pollutants in high-density aquaculture systems and has a potentially serious impact on the health of fish. The gill, as the organ directly in contact with the environment, performs several vital physiological functions, including gas exchange, osmotic pressure regulation, and mucosal immunity. Using a series of nitrite concentration gradients, we investigated the impact of nitrite on tilapia gills, with a focus on its immune responses. The results indicated that nitrite exposure could significantly increase the number of mucus cells and change the subtypes of chloride cells. Nitrite exposure significantly elevated serum catalase (CAT) activity and decreased superoxide dismutase (SOD) activity. After nitrite treatment, the expression of ion channel protein genes was markedly affected, with a substantial increase in Na^+/Cl^- cotransporter (*ncc*) expression. Inflammatory gene expression in the gill was also significantly altered. Both *in vitro* and *in vivo* experiments revealed that key genes in the IL-17 signaling pathway were considerably altered after nitrite exposure. Mitochondrial damage and apoptosis in the gill tissues were also found after nitrite exposure. In conclusion, nitrite exposure altered immune function and ion transport, especially the activation of the IL-17 signaling pathway, damage to mitochondrial structure, and apoptosis in the gill. This study enhances our understanding of the mechanisms underlying nitrite-induced damage to the gill.

KEYWORDS

gill, nitrite, aquaculture pollution, ion transport, IL-17 signaling pathway

1 Introduction

Nitrite is an intermediate nitrogen compound in aquatic environments, produced during the nitrification of ammonia and the denitrification of nitrate (Svobodova et al., 2005). Normally, nitrite concentrations remain low (Madison and Wang, 2006). However, several factors can lead to nitrite accumulation, including 1) insufficient phytoplankton in the water, 2) an imbalance between ammonifying and nitrifying bacteria, and 3) low dissolved oxygen levels, which inhibit complete nitrification and promote denitrification (Jensen, 2003). Therefore, nitrite levels in complex aquatic environments often exceed regulatory standards. In poorly managed overfed aquaculture, nitrite concentrations can reach as high as 50 mg/L (Kroupova et al., 2005). In real-world aquaculture environments, water is exposed to direct sunlight, and the ultraviolet (UV) rays can induce the photodecomposition of water and the activation of nitrogen-containing groups (Qu et al., 2024). As a result, under the same nitrite concentration, natural aquaculture environments may exhibit greater toxicity. In waters with elevated nitrite concentrations, nitrite is enriched in different tissues of the fish, including the serum, gill, liver, and muscle (Kroupova et al., 2005).

Nitrite affects many physiological functions in fish. First, nitrite exposure damages the structural integrity of the gill. Acute nitrite exposure in *Ctenopharyngodon idella* leads to gill filament swelling, the fusion of second lamellae, and hyperplasia (Liu et al., 2022). Similarly, in acute nitrite exposure in *Larimichthys crocea* after 48 hours, secondary lamellae were distorted, slightly shrunken, and disorganized (Xu et al., 2022). Nitrite exposure induces pathological damage to gill tissue. Second, nitrite exposure disrupts the ion transport mechanisms in fish gills. In grass carp, nitrite significantly increases extracellular potassium ion concentrations (Jensen et al., 1987). Nitrite enters the gill by competing with chloride ions for the same ion channel proteins (Jensen et al., 2000) and subsequently converts functional hemoglobin into methemoglobin, reducing its oxygen-binding capacity. Similarly, in *Cyprinus carpio*, nitrite exposure causes arterial oxygen levels to drop to values matching the environmental oxygen content (Williams and Eddy, 1988), resulting in metabolic imbalance. Third, the gill is one of the most important mucosal immune tissues in fish. The mucosal epithelium activates a variety of innate and adaptive immune responses that function together to provide immune protection (Cabillon and Lazado, 2019). Innate immunity is supported by antimicrobial peptides, interleukins, and interferons, while adaptive immunity is mediated by B and T lymphocytes (Gomez et al., 2013). In *L. crocea*, nitrite stress significantly reduced lysozyme levels and increased immunoglobulin levels (Xu et al., 2022). Similarly, in *Takifugu rubripes*, nitrite exposure significantly increased the expression of *tnf-α*, *il-6*, and *il-12* (Gao et al., 2022).

The interleukin-17 (IL-17) family is an ancient pro-inflammatory cytokine. It plays a critical role in host defense against pathogenic microbial infections and in mediating inflammatory responses. IL-17 cytokines activate key signaling molecules in the IL-17 signaling pathway, such as NF-κB, MAPK, and C/EBP. These molecules, in turn, initiate immune responses or promote cell proliferation (Gu et al., 2013; Zhong et al., 2023). In fish, the gills are one of the most important mucosal immune and barrier organs (Lazado and Caipang, 2014). Notably, IL-17 functions as a tissue-signaling cytokine that contributes to the protection of barrier organs such as the skin and lungs (Eyerich et al., 2017). The IL-17 signaling pathway plays a protective role in mucosal tissues by enhancing host defense against bacterial infections (Gaffen and Moutsopoulos, 2020). Thus, the IL-17 signaling pathway plays a central role in the immune responses of the gills. Studies have demonstrated that nitrite stress disrupts metabolic homeostasis (Yang et al., 2024; Ha et al., 2019) and induces oxidative damage in fish (Zhang et al., 2022; Guo et al., 2020). Prolonged exposure to harmful aquatic environmental conditions may lead to a state of chronic inflammation (Agostini et al., 2018). Meanwhile, the IL-17 signaling pathway has been identified as a key regulator of chronic inflammation and immune responses (Ritzmann et al., 2022). It also plays an important role in the regulation of cellular and systemic metabolic processes (Bechara et al., 2021). For example, transcriptomic and proteomic studies have shown elevated IL-17 signaling pathway gene and protein expression in the gills of *Pseudobagrus fulvidraco* exposed to ammonia (Zhong et al., 2023). In *Scophthalmus maximus* infected with *Vibrio anguillarum*, the mRNA expression levels of *il-17c* and its receptor were also significantly upregulated (Xue et al., 2021). Taken together, current evidence reveals a knowledge gap in understanding the role of the IL-17 signaling pathway in fish gills under nitrite stress.

Meanwhile, studies have reported that the toxicity of harmful environmental conditions is closely associated with mitochondrial damage and apoptosis. In *Danio rerio*, arsenic exposure induced mitochondrial autophagy, leading to mitochondrial damage (Zhang et al., 2023a). Similarly, abamectin exposure in grass carp caused structural damage and a decrease in membrane potential (Wu et al., 2023). The expression of the pro-apoptotic gene *bax* was significantly upregulated, while the anti-apoptotic gene *bcl-2* was significantly downregulated (Xie et al., 2019). In the study of nitrite exposure in *T. rubripes*, the expression of apoptosis-related genes, such as *caspase-3*, *caspase-8*, *caspase-9*, and *p53*, was significantly upregulated. In contrast, the expression of *Bcl-2* was considerably decreased (Gao et al., 2020a).

The gill is a vital organ for gas exchange and the regulation of osmotic pressure in fish. It is also in direct contact with aquaculture water, serving as a crucial chemoreceptor. However, research on the effects of nitrite stress on the gill and its underlying mechanisms remains limited. This study aims to investigate the effects of nitrite exposure on tissue damage, ion transport, immune functions, and apoptosis in the gill.

2 Materials and methods

2.1 Experimental animals

The Genetically Improved Farmed Tilapia (*Oreochromis niloticus*) used in the experiment, weighing 100 ± 10 g, was

purchased from Huadu, Guangzhou, China. Following the acquisition, the fish were kept in water with a temperature maintained at approximately 28 °C, dissolved oxygen levels between 9 and 10 mg/L, and a pH range of 7.1–7.6. The fish were fed a commercial diet (crude protein 32%, crude fat 3.9%, starch 26.4%, and ash content 7.5%) at a rate of 1% of their body weight, twice daily. The experiments were conducted after a 2-week domestication period.

2.2 Experimental design

The domesticated tilapia were divided into two weight classes based on their body weight: Class 1 (90–100 g) and Class 2 (100–110 g). Within each class, the fish were assigned to eight experimental groups using a simple randomization method, with each group comprising 12 fish. Each group was assigned to a separate 120-L tank in a random order to avoid any potential bias related to tank placement. A pre-laboratory study was conducted to determine the lethal nitrite concentration for tilapia. In this study, tilapia were exposed to various nitrite concentrations (ranging from 0 to 90 mg/L) over 14 days. The results showed that tilapia began to die when the nitrite concentration exceeded 60 mg/L. Accordingly, eight nitrite treatment groups at concentrations of 0, 30, 40, 50, 60, 70, 80, and 90 mg/L were established by dissolving the corresponding amount of sodium nitrite (FuChen Chemistry, Tianjin) in 100 L of water. Two-thirds of the water in the tanks was replaced every 24 hours. The nitrite concentrations were corrected and measured using a nitrite kit (EasyBox, CircleCare Biologicals, Beijing), keeping the temperature and pH levels in the tank stabilized.

The study adhered to the ethical guidelines for experimental animals set forth by the Ethics Committee of Sun Yat-sen University (Approval No. SYSU-IACUC-2023-B0453) in June 2023.

2.3 Isolation and culture of gill cells

To perform cardiac perfusion using phosphate-buffered saline (PBS) solution, the connection between the heart and the vein was dissected by making a precise incision. Subsequently, a 1-mL syringe was inserted into the arterial bulb of the fish. PBS was then injected into the arterial bulb, which facilitated the displacement of blood from the gill. Gill filaments were excised using sharp scissors, and mucus was removed through multiple washes using PBS. Gill filaments were digested with 0.25% trypsin (Gibco, Massachusetts) following mechanical trituration using a tissue chopper (The Mickle Laboratory Engineering Co., Ltd., HongKong). The viability of gill cells isolated from fish was assessed using propidium iodide (PI; YuanYe, Shanghai) staining. In each experimental instance, the viability of the cells obtained was consistently above 95%. The cells were plated onto a 24-well plate (NEST, Wuxi) pre-coated with poly-D-lysine (MCE, Shanghai). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Cytiva, Hangzhou) supplemented with 2% penicillin/

streptomycin/amphotericin (YuanYe) and 10% fetal bovine serum (Gibco). The culture was maintained in an incubator at 28 °C with a CO_2 atmosphere.

2.4 Histological and ultrastructure studies

After 7 days of nitrite exposure, gill filaments were collected from four to five randomly selected fish per group. The tissues were fixed, dehydrated, and sectioned at 5-μm thickness using a microtome (Leica, Wetzlar). The gill section was stained with hematoxylin and eosin (H&E). Three representative sections from each group were randomly selected and examined under a Nikon orthoptic microscope.

Additionally, gill filaments from three randomly selected fish per group were dissected, and 1-mm^3 samples of filament ends were fixed in 3% glutaraldehyde. The gill filaments were dehydrated through a graded ethanol and subsequently observed using a focused ion and electron dual-beam scanning electron microscope (Carl Zeiss, Oberkochen).

2.5 Serum collection and oxidative stress parameter analysis

Twelve serum samples were collected from each group. Tilapia were anesthetized by immersion in an ice-water mixture for 10 seconds. Blood was drawn from the vein. A 1-mL blood sample was incubated at room temperature for 2 hours and subsequently centrifuged at 7,500 rpm for 10 min to separate the serum. The serum was used to assess the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) using commercial assay kits (Beyotime, Shanghai).

2.6 Gene expression analysis

Twelve samples were collected from each group for quantitative real-time PCR (qPCR) analysis. Total RNA was extracted using TRIzol reagent (Omega, Cambridge), and its concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Bio-Rad, California). Reverse transcription was performed using the MLV Reverse Transcriptase Kit (Thermo Fisher, Massachusetts). qPCR was conducted using the SYBR green kit (Accurate Biology, Changsha) on a Roche real-time PCR system with a reaction volume of 10 μL.

All the primers used in this study were designed using the NCBI Primer Design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences for the internal control gene (*β-actin*) and genes related to inflammation and immunity (*tnf-α*, *il-1β*, and *ifn-γ*), ion transportation (*ncc*, *nhe*, and *nka1*), and the IL-17 signaling pathway (*mapk*, *fosb*, *traf4a*, *il-1β*, *il-8*, and *il17ra*) are provided in the Supplementary Material (Supplementary Table S1). All primers were verified for specificity using a melting curve analysis in qPCR. The real-time PCR system was 10 μL, and the

reaction group was divided into 5 µL 2× SYBR green mix (Accurate Biology), 0.3 µL each of the primers (10 µM stock), 0.5 µL of the template (100 ng/µL), and 3.4 µL of ultrapure water. There was one technical replicate for each reaction. The reaction conditions were initial denaturation at 95 °C for 1 min and amplification at 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s, 40 cycles. The relative expression levels of the target genes were calculated using the formula $2^{-\Delta\Delta CT}$.

2.7 Analysis of mitochondria in gill tissues

After exposure to 60 mg/L of nitrite for 7 days, gill filaments were collected from tilapia for transmission electron microscopy (TEM), with three fish sampled per group. The gill filaments were initially fixed in 3% glutaraldehyde for 24 hours. They were dehydrated through a gradient of ethanol concentrations. Sectioning was performed at the electron microscopy platform of the School of Life Sciences, Sun Yat-sen University. Images were captured using a 120-kV transmission electron microscope (Jeol Ltd, Tokyo).

2.8 Analysis of apoptosis in gill tissues

After 7 days of exposure to 60 mg/L nitrite, gill filaments were randomly sampled from three fish in each group. The fish were euthanized using eugenol, and tissues were fixed in 4% paraformaldehyde for 24 hours. The samples were then dehydrated through gradient concentrations of ethanol and xylene, embedded in paraffin, and sectioned into 5-µm slices using an automatic microtome (Leica). The sections were incubated overnight at 37°C and subjected to apoptosis staining using a TUNEL Apoptosis Assay Kit (Beyotime).

2.9 Statistical analyses

Statistical analyses were performed using SPSS version 27.0 (SPSS Inc., Chicago, IL, USA), and data visualization was conducted using GraphPad Prism (GraphPad Software, USA). Data were first tested for normality using the Shapiro–Wilk test and for homogeneity of variance using Levene's test. Statistical comparisons between two groups were performed using Student's t-test or a non-parametric test, depending on the result of the normality test. For datasets involving more than three groups, statistical analyses were conducted based on the distribution characteristics and variance homogeneity of the data. Specifically, when the data were confirmed to follow a normal distribution and exhibit homogeneity of variance, one-way ANOVA was employed to examine the differences among groups. Subsequently, Dunnett's test was applied for *post-hoc* comparisons to assess differences between the control group and each experimental group ($p < 0.05$). In cases where the data did not meet the assumptions of normality or homogeneity of variance, the Kruskal–Wallis test was used. *Post-hoc* comparisons were adjusted using the Bonferroni correction ($p < 0.05$).

3 Results

3.1 Nitrite exposure caused histological damage to the gill filament

A significant number of mucus cells (as shown in the black arrow in Figure 1) were observed at the end of the gill filaments after nitrite stress. The most significant increase in mucus cells was observed in the 60 mg/L and 90 mg/L treatment groups.

3.2 Change in chloride cell subtypes and ion transport function

Chloride cells were observed using scanning electron microscopy (Figure 2), with the majority in the control group exhibiting a deep-hole morphology. However, the number of shallow-basin and wave-convex chloride cells increased after exposure to nitrite concentrations of 60 and 90 mg/L.

Nitrite treatment also induced an increasing trend of *ncc* expression in the gill, with significant increases observed at concentrations of 70, 80, and 90 mg/L (Figure 3A). Nitrite exposure significantly reduced *nhe* expression in the gill, with a significant decrease at 50, 60, 70, and 80 mg/L (Figure 3B). Similarly, the expression of *nka1* was significantly reduced at 50, 60, 70, and 90 mg/L (Figure 3C).

3.3 Nitrite exposure caused oxidative stress in fish

As shown in Figure 4, CAT activity in tilapia serum was significantly increased in the 40, 50, 70, and 90 mg/L nitrite-treated groups. In contrast, SOD activity was significantly reduced in the 30, 50, and 80 mg/L nitrite-treated groups, while no significant change was observed in the level of GSH-Px.

3.4 Effects of nitrite exposure on the gill immune gene

As shown in Figure 5, *ifn-γ* expression exhibited a decreasing trend following nitrite stress, with significant downregulation at 40 and 90 mg/L concentrations (Figure 5C). The expression of *tnf-α* showed an increasing then decreasing trend, showing a significant increase at 60 mg/L (Figure 5A). After nitrite exposure, *il-1β* expression significantly increased only at 90 mg/L, with no notable changes at other concentrations (Figure 5B). The expression of *hsp70* tended to increase after nitrite exposure, with significant increases observed at the 60, 70, 80, and 90 mg/L treatment groups (Figure 5D).
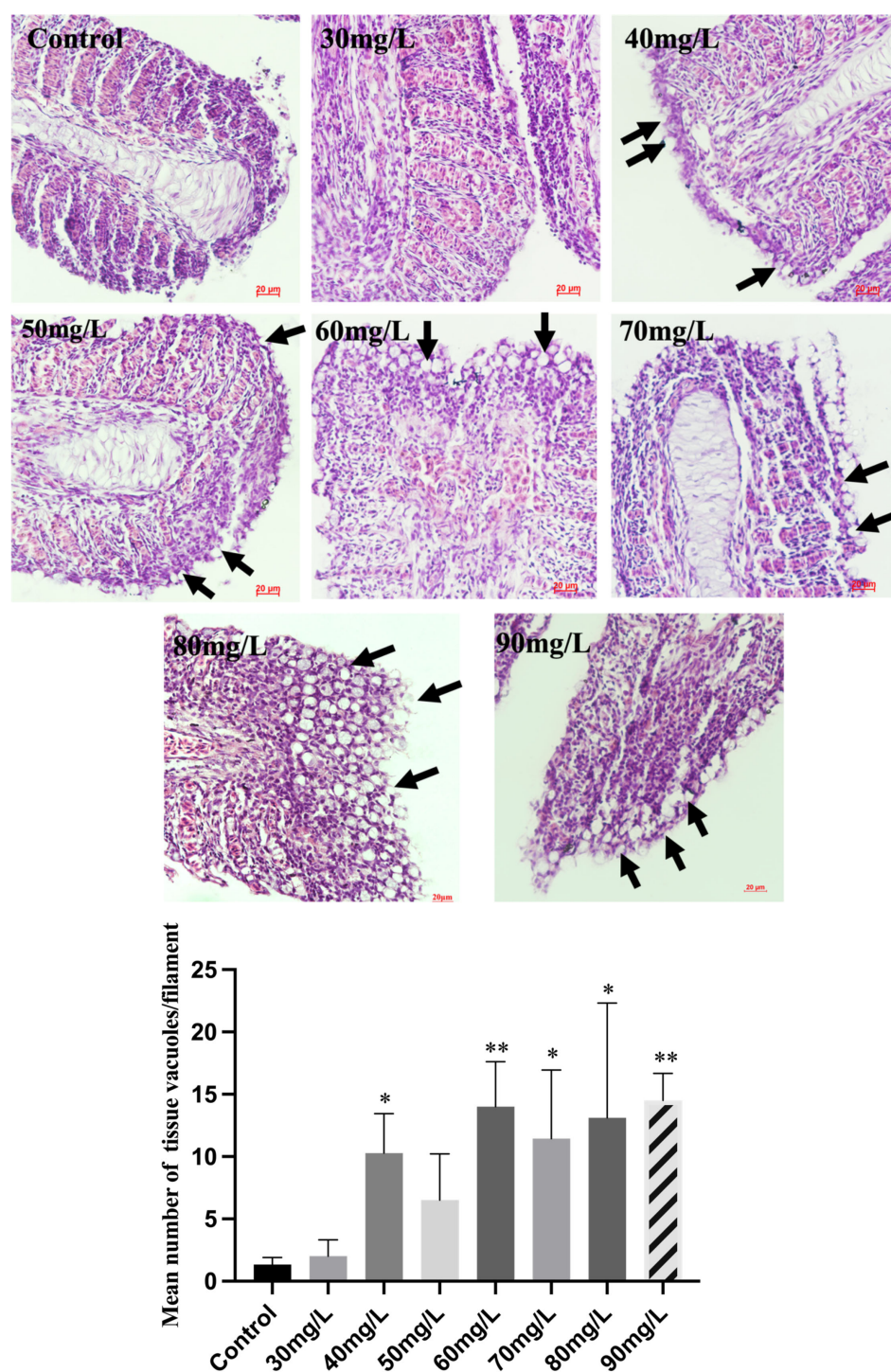
FIGURE 1
Hematoxylin and eosin (H&E) staining of gills under different concentrations of nitrite treatment. Black arrows indicate mucus cells. The magnification of the microscope was ×60. Number of tissue vacuoles in filaments following exposure to different concentrations of nitrite. Values indicate the mean ± SEM (n = 3). *$p < 0.05$; **$p < 0.01$.

3.5 Nitrite exposure activated the IL-17 signaling pathway

After exposing gill cells to 60 mg/L nitrite for 3 and 6 hours, the expression of key genes in the IL-17 signaling pathway was assessed (Figure 6). The expression of *mapk* was significantly increased, while *fosb* mRNA abundance was significantly downregulated after 6 hours of 60 mg/L nitrite treatment. However, *traf4a* expression showed an increasing trend. In addition, exposure to 30 and 60 mg/L nitrite significantly upregulated *il-1β* expression, while *il-8*
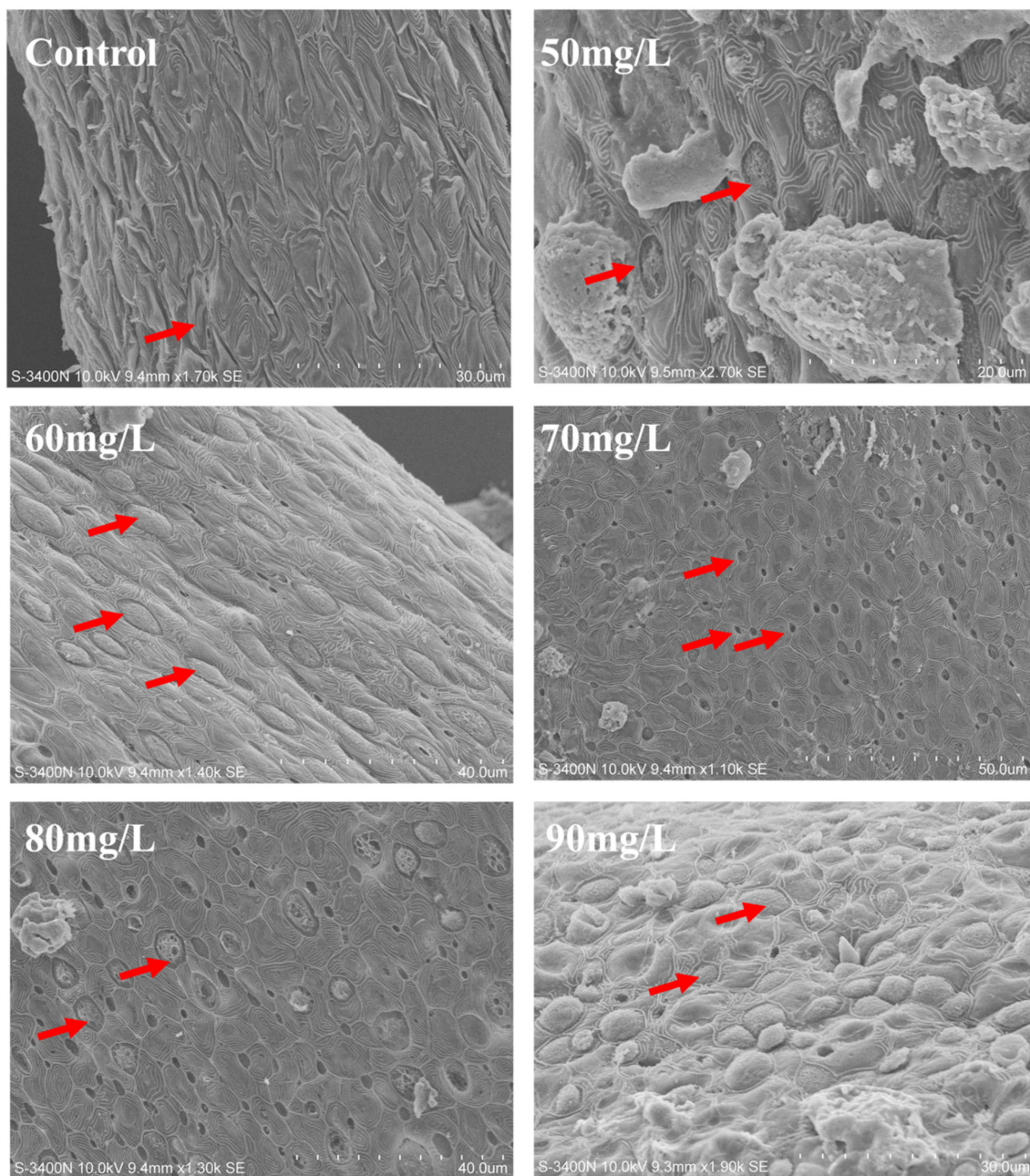
FIGURE 2
Scanning electron micrographs of the number and morphological changes of chlorine cells on gill filaments under different concentrations of nitrite treatment. The red arrows are the chlorine cells.

expression also tended to increase. The expression of *il17ra*, the receptor gene of IL-17, was significantly increased after 6 hours of 60 mg/L exposure.

In a parallel experiment, tilapia were exposed to 60 mg/L nitrite for 7 days to examine the *in vivo* expression of IL-17 pathway-related genes (Figure 7). The results showed that the expression of *traf4a* and *mapk* was significantly upregulated, while that of *fosb* was significantly downregulated. *il-1β* expression showed an upward trend, and *il17ra* was significantly upregulated.

3.6 Nitrite exposure led to mitochondrial structural damage and apoptosis

Transmission electron microscopy showed clear differences between the control and nitrite-treated groups (Figure 8E). In the control group, the gill mitochondria had long and well-organized cristae (Figures 8A, B). In contrast, the nitrite-treated group showed fragmented, punctate, and disorganized cristae (indicated by red arrows in Figures 8C, D), indicating mitochondrial damage.
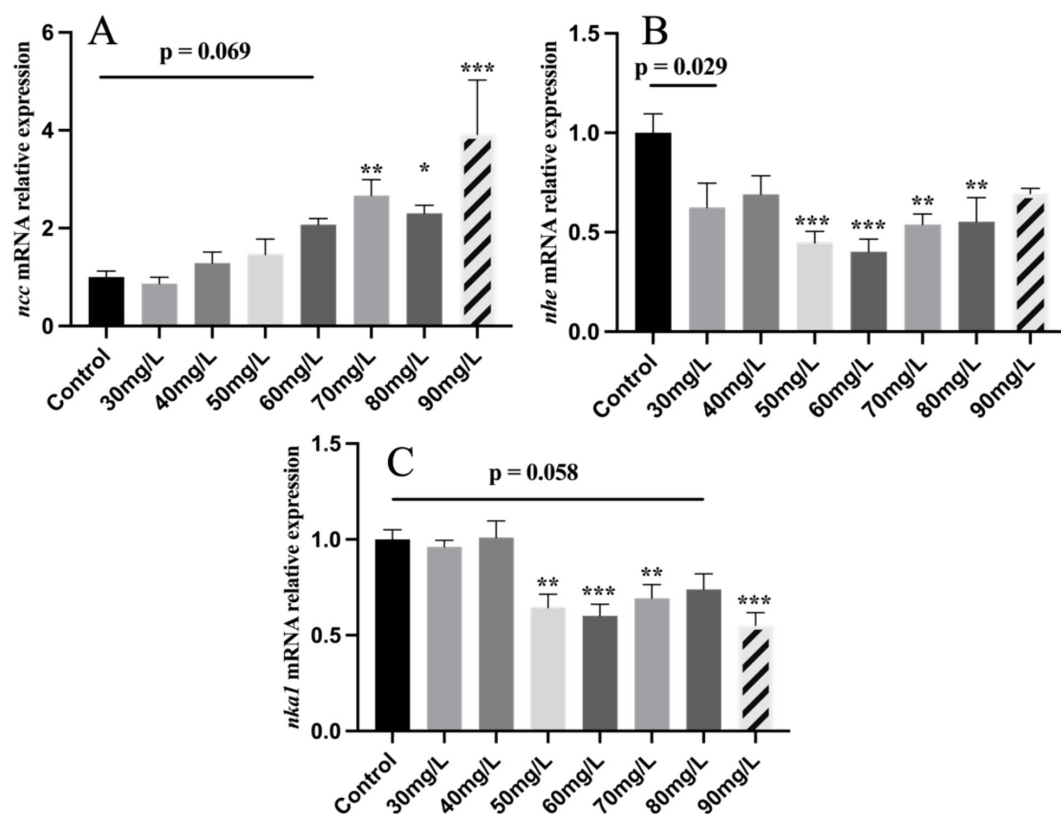
FIGURE 3
The mRNA levels of ion transportation genes in Nile tilapia treated with different concentrations of NO_2^-, including ncc **(A)**, nhe **(B)**, nka1 **(C)**. Values indicate the mean $\pm$ SEM (n = 6-12). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

A TUNEL apoptosis assay was performed after 7 days of nitrite exposure. As shown in Figures 9D–F, numerous Fluorescein isothiocyanate (FITC) positive green fluorescent cells were detected in the interlamellar at the end of gill filaments, in contrast to the control group (Figures 9A–C), indicating the presence of apoptotic cells in this region (Figure 9G).

4 Discussion

Nitrite is a common pollutant in aquaculture environments and is toxic to aquatic organisms, impairing various physiological functions. In this study, we exposed tilapia to graded concentrations of nitrite for 7 days to investigate the effects of nitrite on gill structure and immune function. We investigated the histological and ultrastructural changes of the gill after nitrite treatment. H&E staining of the sections revealed a significant increase in the number of mucus cells after nitrite treatment. These findings are in line with those of other studies on nitrite exposure (Dos Santos Silva et al., 2018). Increased mucus production is also considered a biomarker of harmful environmental conditions affecting the gills (Valdenegro-Vega et al., 2014; Muthukumaravel et al., 2023). In our study, high

levels of nitrite stress significantly increased the number of mucus cells, suggesting that the mucosal immune function of the gills was "challenged", damaging the gill histology.

Chloride cells with different morphologies of apical surfaces— deep-hole, shallow-basin, and wavy-convex—were observed on the gill filament. The wavy-convex subtypes express the ncc ion channel protein (Dymowska et al., 2012). The wavy-convex and shallow-basin subtypes are responsible mainly for the uptake of Cl^- and Ca^{2+}, respectively, in freshwater fish (Chang et al., 2001). Scanning electron microscopy revealed an increase in the number of wave-convex-subtype chloride cells after nitrite exposure. A similar change was observed under chloride-deficient conditions (Lin and Hwang, 2004). Previous research has shown that nitrite and chloride ions competitively inhibit each other's entry into the gills (Williams and Eddy, 1986), both entering the fish through ncc ion channel proteins (Chang et al., 2001). This suggests that increased nitrite levels impair chloride uptake, resulting in a shift in chloride cell subtypes, and that nitrite exposure may alter the ion transport function of fish gills. Meanwhile, nitrite at concentrations of 70, 80, and 90 mg/L significantly increased the expression of the *ncc* in the gill. Notably, ncc is the most important pathway for chloride ion uptake in tilapia (Horng et al., 2009). Na+/H+ exchanger (NHE) plays a key role in sodium ion uptake
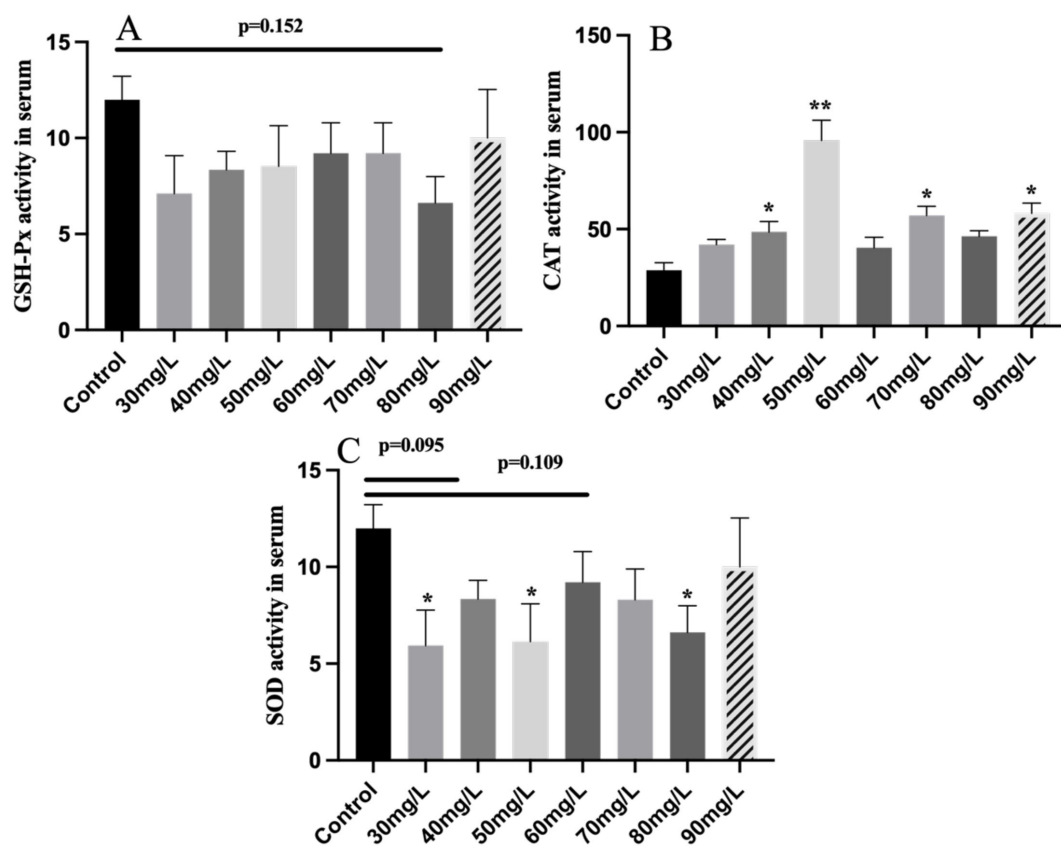
FIGURE 4
The antioxidant enzyme activity of serum in Nile tilapia treated with different concentrations of NO_2^-, including GSH-Px **(A)**, SOD **(B)**, and CAT **(C)**. Values indicate the mean $\pm$ SEM (n = 6−12). *$p < 0.05$; **$p < 0.01$. GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase.

(Edwards et al., 2001). It has been found that nitrite and chloride ions competitively inhibit each other's entry into the gills through ncc ion channel proteins. These findings suggest that elevated nitrite levels reduce chloride ion availability in fish, thereby inducing the upregulation of *ncc* expression in the gill. This is consistent with the results of scanning electron microscopy and further demonstrates that nitrite stress reduces chloride ion uptake and alters ion transport function in fish. Meanwhile, nitrite exposure at concentrations of 50, 60, 70, and 80 mg/L significantly downregulated *nhe* expression in the gill. The significant downregulation of *nhe* may be attributed to the addition of sodium nitrite to the water. Increased sodium ion levels, in turn, induce a compensatory reduction in *nhe* expression. Furthermore, significant reductions in *nka1* mRNA levels were observed in the 50, 60, and 90 mg/L nitrite-treated groups. The downregulation of *nka1* expression likely reflects an adaptive regulatory response to the elevated sodium ion concentration in the water. A limitation of our study is that changes in ion levels and osmolality in fish serum were not assessed. However, this did not affect the validity of our conclusions. Taken together, the alterations in ion transport-related gene expression observed after nitrite stress likely represent compensatory responses. These changes suggest a shift

in ion transport patterns and capacity, reflecting the gill's adaptive mechanisms to cope with nitrite stress.

SOD, CAT, and GSH-Px constitute the primary antioxidant defense system in fish, helping to mitigate oxidative stress. SOD catalyzes the conversion of superoxide radicals into hydrogen peroxide (Korkmaz et al., 2023). In this study, SOD activity was significantly reduced under nitrite concentrations of 30, 50, and 80 mg/L. CAT plays a crucial role in removing hydrogen peroxide and minimizing oxidative damage (Wang et al., 2022). The activity of CAT was significantly increased in the 30, 50, 70, and 90 mg/L nitrite treatments. This response is in line with findings from a study on acute nitrite stress in *L. crocea*, where similar trends in CAT and SOD activities were observed (Xu et al., 2022; Sun et al., 2020). The reduction of SOD activity may be attributed to the gills' limited ability to neutralize external oxidative stimuli, thereby impairing enzymatic function. In contrast, the increased CAT activity indicates that the fish activated compensatory antioxidant responses to mitigate nitrite-induced oxidative stress. GSH-Px activity showed no significant change, suggesting that it may play a less prominent role in the antioxidant response to nitrite stress compared to SOD and CAT.

IL-1β is induced by bacterial lipopolysaccharide (LPS) and acts as a key mediator in the immune response to microbial infections
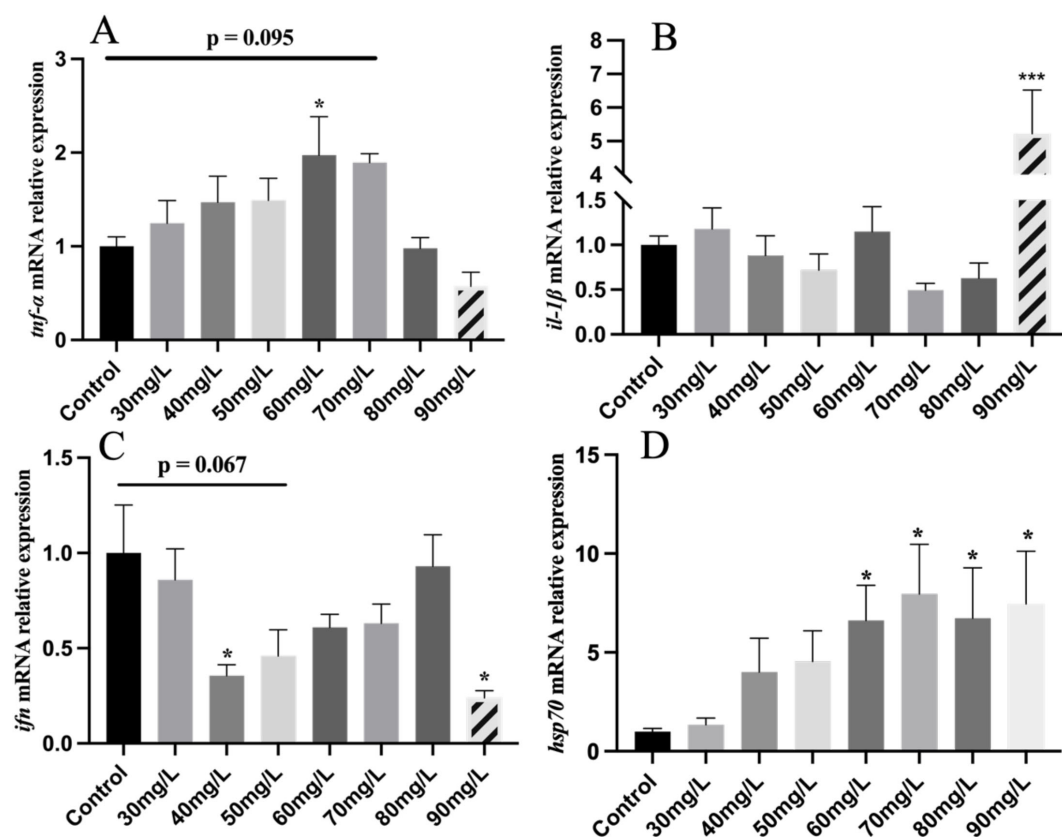
FIGURE 5
The mRNA levels of inflammation and immunity genes in Nile tilapia treated with different concentrations of NO_2^-, including tnf-α **(A)**, il-1β **(B)**, ifn **(C)**, hsp70 **(D)**. Values indicate the mean ± SD (n = 6-12). *$p < 0.05$; ***$p < 0.001$.

and tissue injuries. It activates lymphocytes and stimulates the release of other pro-inflammatory cytokines (Evavold et al., 2018). IFN-γ stimulates the synthesis of antiviral factors that play a crucial role in defending against viral infections (Sieger et al., 2009). Compared to the control group, the relative expression of *il-1β* significantly increased after 90 mg/L nitrite treatment. Similarly, *ifn-γ* expression also declined, with significant reductions observed at 40 mg/L. Similar trends in *il-1β* expression have been reported in studies examining the effects of glyphosate stress in tilapia, suggesting a comparable impact to nitrite stress (Zheng et al., 2021). The decreased levels of *il-1β* in the gills may suggest a weakening of the immune response. The sharp increase at 90 mg/L may indicate a dysregulated immune response resulting from excessive nitrite-induced stress. The significant upregulation of *ifn-γ* expression suggests that nitrite stress may enhance the gill's susceptibility to microbial infection in the aquatic environment. TNF-α is a pro-inflammatory cytokine that works in synergy with IL-1β to mediate a range of inflammatory responses (Sonmez Kaplan et al., 2023). Compared to that in the control group, the expression of *tnf-α* was significantly increased at 60 mg/L nitrite treatments. Acute nitrite exposure also significantly upregulated the expression of *tnf-α* and *hsp70* in *T. rubripes* (Gao et al., 2020b). High levels of nitrite treatment groups significantly increased *hsp70*

expression. Similarly, the expression of *hsp70* was significantly upregulated in the gill during acute nitrite stress in juvenile *S. maximus* (Jia et al., 2016). Changes in *hsp70* expression levels reflect the stability of the fish's physiological state. The upregulation of *hsp70* in response to external stressors suggests that the fish are experiencing chronic stress. In addition, the expression of *tnf-α* was not consistent with that of *il-1β*, implying that a *tnf-α*-specific immune regulatory pathway may be involved in the response to nitrite stress.

The gill is a critical mucosal immune organ in fish and serves as a primary barrier between the fish and the external environment (Zaccone, 2022). IL-17 is a common cytokine secreted by immune cells and plays an essential role in the body's defense against external pathogens. IL-17 is involved in regulating several crucial immune pathways (Saco et al., 2021). In our preliminary experiment, 7-day nitrite exposure significantly increased *il-17* expression. This prompted us to investigate whether the IL-17 signaling pathway plays a critical immune role in the gill.

The IL-17 signaling pathway plays an important role in the body's defense against external pathogens and autoimmune responses (Blauvelt and Chiricozzi, 2018). In this study, we examined changes in key genes of the IL-17 signaling pathway after nitrite exposure both *in vitro* and *in vivo*. The results from both *in*
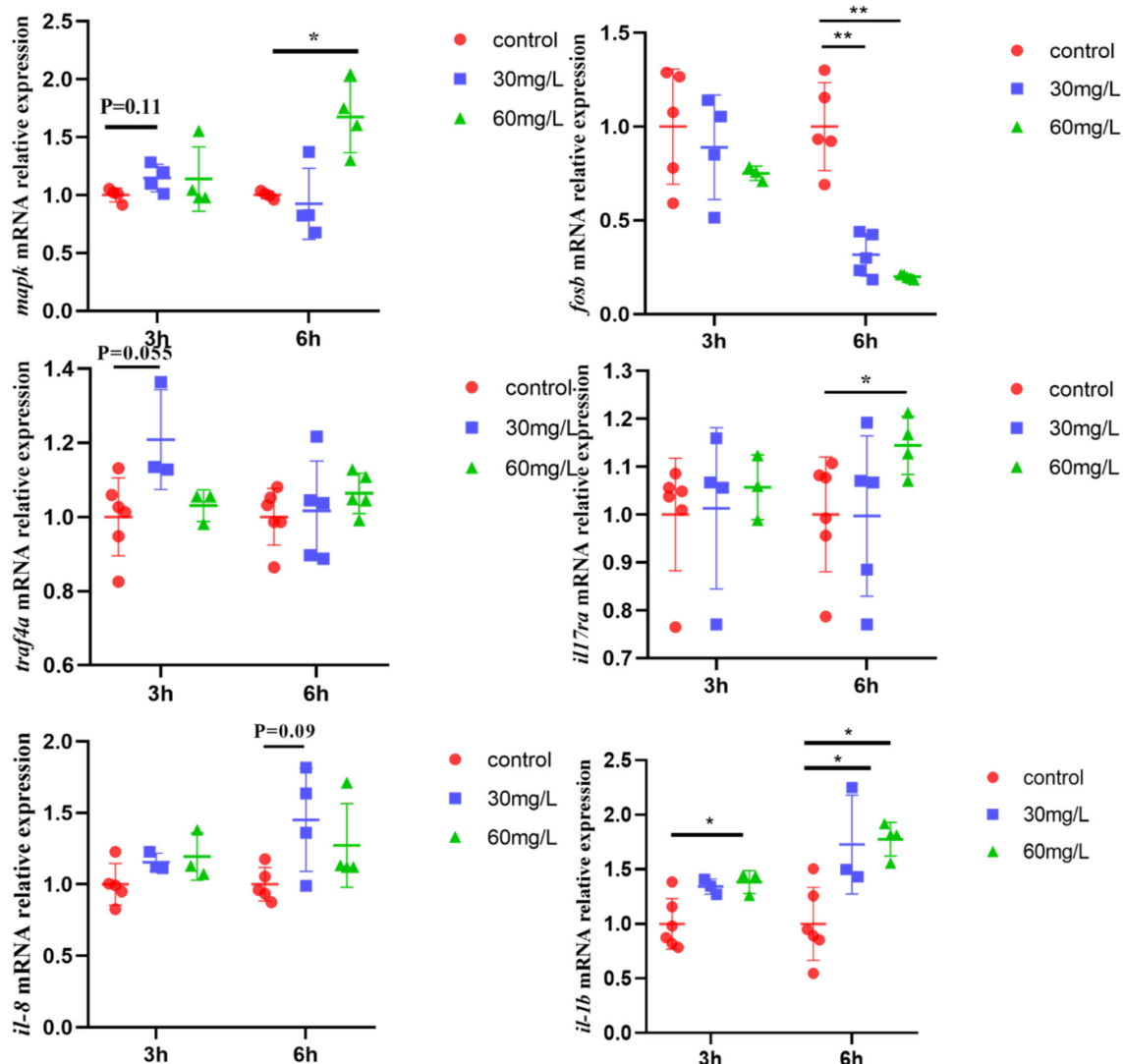
FIGURE 6
The mRNA levels of IL-17 signaling pathway key genes in primary gill cells treated with NO_2^-. Values indicate the mean $\pm$ SEM (n = 4–6). *p < 0.05; **p < 0.01.

vivo and *in vitro* experiments were generally consistent. The expression of key genes in the IL-17 signaling pathway was significantly upregulated in both settings. Interestingly, only *fosb* expression was significantly downregulated. In a study of *S. maximus* infected with *V. anguillarum*, a significant increase in the expression of both *il-17c* and its receptor was found in the skin (Xue et al., 2021; Ni et al., 2023). Our study focused on the expression of the IL-17 gene and receptors and further examined changes in key downstream genes within the IL-17 signaling pathway. IL-17 primarily transmits signals through IL-17RA and IL-17RC heterodimers (Huangfu et al., 2023). In this study, *il17ra* was selected for analysis because it is more broadly expressed in all tissues, while *il17rc* is mainly expressed in non-hematopoietic epithelial cells and mesenchymal cells. Notably, *fosb* expression was significantly downregulated, in contrast to the upregulation observed for other genes in the IL-17 signaling pathway

(Veldhoen, 2017). The protein encoded by *fosb* can dimerize with JUN family proteins to form the AP-1 transcription factor complex, a key component of the IL-17 signaling pathway. Meanwhile, the expression of *traf4a*, a gene crucial for inhibiting IL-17 signaling, was significantly upregulated (Gu et al., 2013). Ammonia stress has been shown to significantly upregulate *fosb* mRNA expression in the gills (Zhong et al., 2023). The difference in *fosb* expression may be attributed to a feedback regulation mechanism, in which increased AP-1 activity induced by 7-day nitrite stress suppresses *fosb* expression. Meanwhile, the increased expression of *traf4a*, a negative regulator of the IL-17 signaling pathway, may serve to prevent excessive inflammatory responses. Nevertheless, *il-1β*, as an inflammatory cytokine downstream of the IL-17 signaling pathway, significantly increased after nitrite exposure, suggesting the induction of an inflammatory response in the gill. Although we further
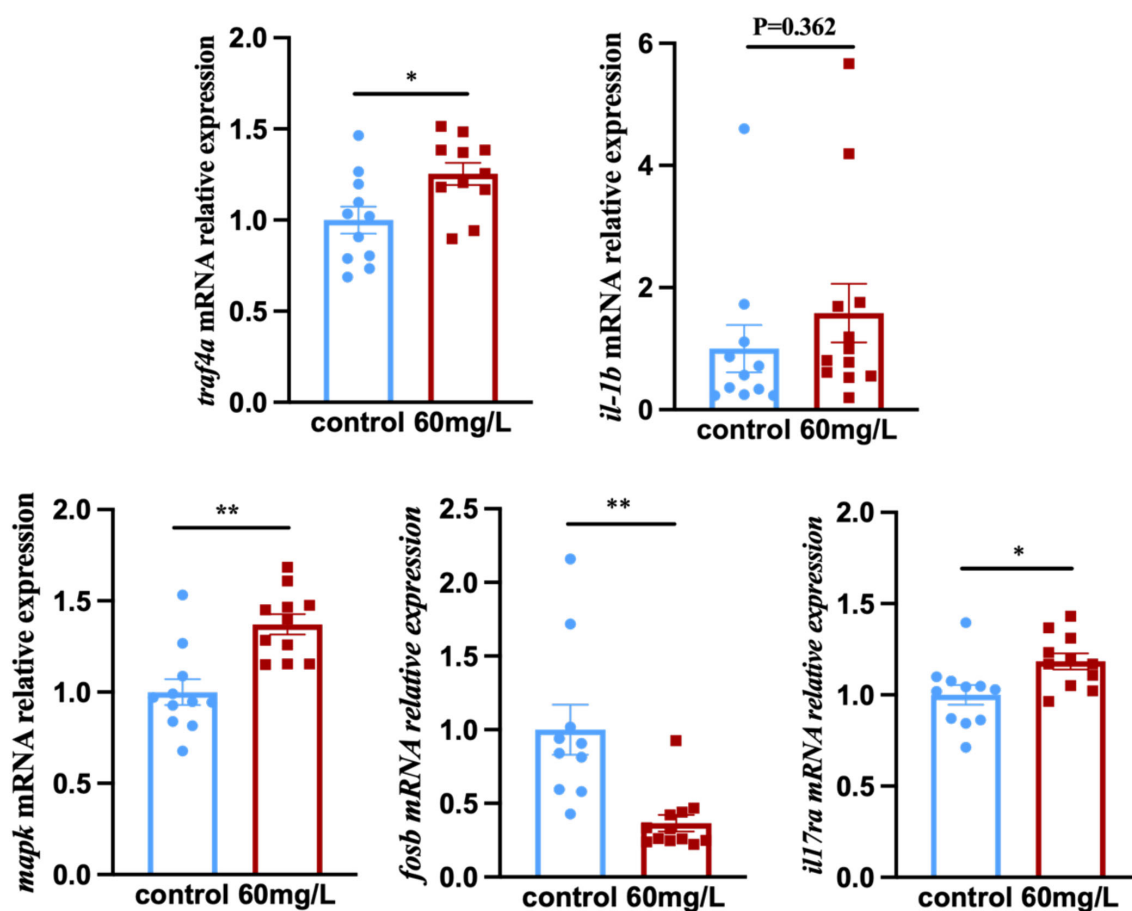
FIGURE 7
The mRNA levels of IL-17 signaling pathway key genes in tilapia treated with NO_2^-. Values indicate the mean $\pm$ SEM (n = 10–12). *$p < 0.05$; **$p < 0.01$.
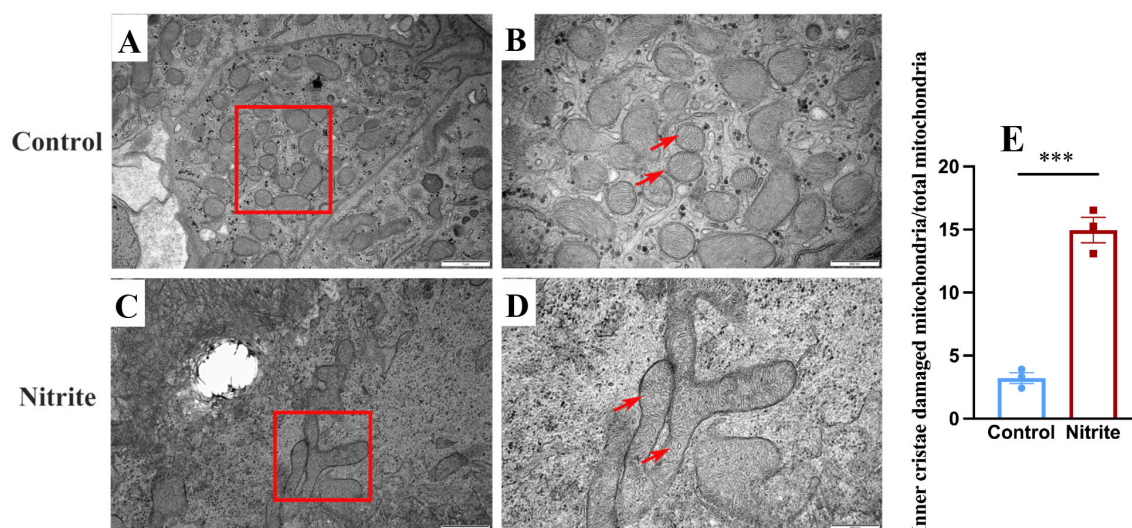

FIGURE 8
Transmission electron micrographs of gill filaments exposed to 60 mg/L nitrite for 7 days. Control group **(A, B)** and nitrite group **(C, D)**. The red arrows are the inner cristae of mitochondria. **(E)** Proportion of mitochondria with damaged inner cristae before and after nitrite exposure. Values indicate the mean $\pm$ SEM (n = 3). ***$p < 0.001$.
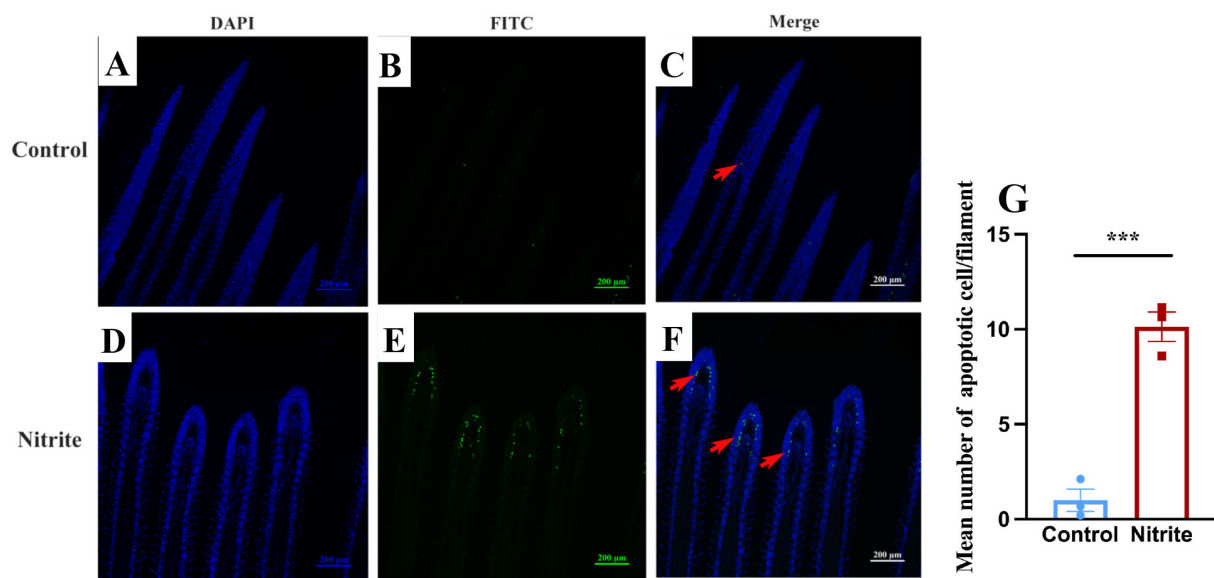
FIGURE 9
TUNEL apoptotic staining was performed on the gill filaments of tilapia exposed to 60 mg/L nitrite for 7 days: **(A, D)** 4'6-Diamidino-2'-phenylindole (DAPI) staining, **(B, E)** FITC staining, and **(C, F)** Merge. **(G)** Average number of apoptotic cells per filament. Values indicate the mean $\pm$ SEM (n = 3). ***p < 0.001.

investigated changes in the transcriptional levels of *mapk* within the IL-17 signaling pathway, we were unable to assess transient MAPK phosphorylation due to the limited availability of fish-specific antibodies. In general, the altered expression of key genes within the IL-17 signaling pathway indicates that the pathway is activated under nitrite-induced stress.

The IL-17 signaling pathway may play an important physiological role in the gill's response to nitrite-induced stress, particularly in modulating immune and inflammatory functions. The activation of the IL-17 signaling pathway can indirectly promote mucosal integrity (Song et al., 2015) and facilitate the differentiation of T cells into T helper 17 (Th17) cells (Miossec and Kolls, 2012). Analysis of the *in vitro* results revealed that nitrite stress activates the IL-17 signaling pathway within 3–6 hours, suggesting a direct regulatory response. However, it is also possible that the activation of the IL-17 signaling pathway in the *in vivo* experiments is an indirect response to tissue damage. After nitrite stress, the activation of this pathway leads to the increased expression of downstream inflammatory genes, such as *il-1β*, thereby enhancing mucosal immune protection in the gills. In parallel, Th17 cell differentiation promotes the upregulation of pro-inflammatory cytokines, including *tnf-α*, which helps defend against pathogens and microbial infections in the aquatic environment. Meanwhile, the IL-17 signaling pathway is modulated by negative regulatory factors to prevent excessive inflammatory responses and maintain immune homeostasis.

In this study, we preliminarily investigated mitochondrial damage and apoptosis in fish gills following nitrite exposure.

Previous research has shown that arsenic disrupts mitochondrial function in zebrafish, leading to the fragmentation and loss of inner mitochondrial cristae (Zhang et al., 2023b). Similarly, our results revealed that nitrite stress disrupted the inner mitochondrial cristae in tilapia gills, suggesting that nitrite may impair mitochondrial function. This mitochondrial damage is likely associated with oxidative stress. Under the same nitrite exposure conditions, we also observed apoptosis in the gill tissue. Consistent with our findings, a previous study on acute nitrite stress in *T. rubripes* reported significant upregulation of apoptosis-related genes (Gao et al., 2020c). A limitation of this study is the lack of investigation into the relationship between mitochondrial damage and apoptosis. The underlying molecular mechanisms can be further investigated in-depth in future studies.

5 Conclusion

In summary, we found that fish gills respond to nitrite stress by altering ion transport patterns, accompanied by corresponding changes in chloride cell subtypes. The activation of the IL-17 signaling pathway in the gills under nitrite exposure was first observed, and its downstream cytokines may enhance inflammatory responses in the gills. The molecular mechanisms by which the IL-17 signaling pathway regulates immune responses in the gill warrant further investigation. Furthermore, nitrite stress induces both apoptosis and mitochondrial damage. The potential correlation between these two phenomena requires further investigation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Animal housing, use and experimentation were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University (Approval No: SYSU-IACUC-2023-B0453) in June 2023. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CS: Data curation, Validation, Conceptualization, Project administration, Investigation, Methodology, Writing – review & editing, Resources, Writing – original draft, Formal analysis, Software. TZ: Methodology, Investigation, Writing – review & editing, Supervision. RC: Writing – review & editing, Investigation, Conceptualization, Validation. YY: Investigation, Data curation, Writing – review & editing. ZL: Supervision, Writing – review & editing, Methodology. JY: Supervision, Writing – review & editing, Investigation. YL: Writing – review & editing, Investigation, Supervision. JL: Conceptualization, Software, Writing – review & editing. WL: Supervision, Writing – review & editing, Investigation, Funding acquisition, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2025.1655930/full#supplementary-material

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