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A Preliminary Study on the Effects of Nitrite Exposure on Hematological Parameters, Oxidative Stress, and Immune-Related Responses in Pearl Gentian Grouper

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Abstract: Nitrite represents one of the most typical contaminants in aqueous species. The research was conducted to evaluate the impacts of nitrite exposure on the survival, gill morphology, hematological parameters, immune response, and meat flavor of pearl gentian grouper. The fish were exposed to 0, 5, 10, and 20 mg/L of nitrite for 96 h (note: N-0, N-5, N-10, and N-20 indicate nitrite concentrations of 0, 5, 10, and 20 mg/L, respectively). The blood, gills, and muscles were collected from fish to determine hematological parameters, immune response, oxidative stress, and meat flavor after 0, 12, 24, 36, 48, 60, 72, and 96 h of exposure. The data showed that the aspartate aminotransferase (AST), cortisol (COR), malondialdehyde (MDA), alanine aminotransferase (ALT), alkaline phosphatase (AKP), and free amino acids (FAAs) contents were significantly increased, while the glutathione (GSH), immunoglobulin M (IgM), superoxide dismutase (SOD), and lysozyme (LZM) contents were remarkably declined in the N-20 group after 72 h of exposure. In gills, exposure to the higher concentrations of nitrite resulted in the proliferation and hypertrophy of epithelial cells of gill lamellae, as well as an increase in mucous cells. In addition, all fish in the N-10 and N-20 groups died after 96 h of exposure. Our findings suggested that exposure to higher concentrations of nitrite disrupted blood physiology and oxidative stress, leading to dysfunction in the pearl gentian grouper.

Keywords: pearl gentian grouper; nitrite exposure; hematological parameters



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1. Introduction

Nitrite usually is available within the aquatic environment as a pollutant [1]. It is a toxic intermediate product produced during nitrification and denitrification by bacteria [2]. The levels of nitrite in natural water are low [3]. In contrast, nitrite may accumulate to very high levels in intensive farming due to overfeeding [4].

High nitrite exposure may lead to immune deficiency and tissue damage in fish [5]. The fish immune system consists of acquired and innate immune systems. Acquired immunity is the first line of defense from stress in fish and plays a critical part in resisting stress. Immunoglobulin M (IgM) and lysozyme (LZM) are immune-related factors that are involved in protecting against potentially detrimental conditions [6]. Environmental stress from contaminants appears to be an important determinant of reduced immunity [7]. Early studies have demonstrated that exposure to high concentrations of nitrite may lead to changes in immune function [8]. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are both transaminases that are commonly used to assess the degree

of liver damage in the organisms [9]. Several studies have demonstrated a remarkable increase in serum AST and ALT levels following exposure to high concentrations of nitrite for 96 h compared with the control group (CK) [10,11]. Additionally, nitrite can lead to the lipid peroxidation of the organisms through the enhanced generation of reactive oxygen species (ROS) [12]. Oxidative stress can destroy essential cellular biomolecules, which, in turn, can impair cellular functions [13]. Fish antioxidant mechanisms are effective against oxidative stress caused by nitrite exposure [14]. Some studies have shown that high concentrations of nitrite can change antioxidant enzyme activities in fish [15,16].

The toxic effects of nitrite have been extensively researched in aquatic organisms. The toxicity of nitrite to aquatic species depends on nitrite accumulation levels and detoxification capacity [17]. In freshwater fish, nitrite toxicity to fish stems from competitive inhibition of chloride [18]. High concentrations of nitrite cause partial replacement of Cl^- uptake by NO_2^- in fish [19]. In contrast, the mechanism of nitrite uptake in seawater fish is not known, but a potential pathway for nitrite accumulation has been demonstrated to occur through the gill epithelium [20]. Nitrite exposure can induce nitrite accumulation in the gills, liver, and muscle tissues, exerting effects on osmotic pressure homeostasis, respiration, and excretion of nitrogenous waste products in fish [21]. Additionally, high concentrations of nitrite can cause histopathological changes in the gills, such as swelling of epithelial cells, dilatation or congestion of the gill capillaries, and other lesions [17,22].

The pearl gentian grouper, also known as the tiger spotter, is a hybrid fish obtained by artificial breeding of female brown-marbled grouper and male giant grouper, mainly distributed in Guangdong, Fujian, Zhejiang, and Hainan. Consumers favor pearl gentian grouper for its advantages of strong disease resistance, high protein content, delicious flesh, and fast growth and reproduction rate [23,24]. In recent years, large-scale intensive farming has been carried out due to its tremendous commercial value. However, the intensification of aquaculture causes the overuse of protein feed, thus increasing the nitrite load. In the process of intensive farming, nitrite is a major influence on the growth of fish, and the higher the culture density, the more obvious the effect. Currently, there are limited studies on the toxic effects of nitrite in pearl gentian grouper. Therefore, to explore the possible mechanisms of such toxicity, we evaluated the impact of different nitrite concentrations on survival, tissue damage, stress response, and immune specificity of pearl gentian grouper.

2. Materials and Methods

2.1. Animals

Pearl gentian grouper were purchased from the Pudong New Area Market in Shanghai. The same batch of grouper was selected to be healthy and free of injuries and diseases, with an average body length of (27.50 ± 1.55) cm and an average body mass of (445 ± 50) g, and transported to the laboratory with water and oxygen. The temporary culture pond was disinfected with potassium permanganate solution and temporarily reared in 600 L inflatable seawater flow tank for 24 h, i.e., light for 14 h and night light for 10 h. All fish were fasted over the acclimation period. Experimental seawater was filtered from tap water and sea salt by particulate activated carbon, prepared the day before the experiment, and then continuously aerated for 24 h. The temporarily cultured water had the following parameters: the density of temporary culture was 20 kg/m^3 , the temperature of the water was $23\sim 25^\circ\text{C}$, the salinity was $23\sim 25\%$, the dissolved oxygen was $7\sim 8 \text{ mg/L}$, TAN $< 0.1 \text{ mg/L}$, and the pH was $7.5\sim 8.5$. During the interim culture, the water was recycled through a recirculation filter, with 50% of the water replaced every 12 h [25]. The experiment was approved by the Animal Care and Use Committee of Shanghai Ocean University (SHOU-DW-2021-073).

2.2. Nitrite Exposure and Sampling

NaNO_2 (purity $\geq 99.96\%$, Aladdin Biotechnology Co., Ltd., Xi'an, China) was dissolved in 5 L of distilled water to make a stock solution and diluted with seawater to make nitrite test solutions (10 g/L). The 96 h LC50 concentration of nitrite for grouper croaker was determined to be 24.755 mg/L according to the research method of Kim et al. [14], but

with minor modifications. After 24 h of temporary culture. Based on the 96 h LC₅₀ dose, 120 fish were randomly chosen for 96 h of acute exposure to nitrite at 4 concentrations: 5 (low), 10 (medium), 20 (high), and 0 mg/L (control). There were 30 fish in each group, and the experiment was performed in 6 replicates. The four sample groups were marked as follows: CK (0 mg/L), N-5 (5 mg/L), N-10 (10 mg/L), and N-20 (20 mg/L), respectively. The required nitrite levels were reached by adding nitrite test solution (10 g/L), and the seawater was changed every 12 h. The level of nitrite in the water tanks of each group was determined every 4 h. The waterborne nitrite levels were measured according to the method of Lin et al. [26]. The other water-quality conditions were consistent with the water-environment conditions of grouper during temporary culture.

After exposure for 0, 12, 24, 36, 48, 60, 72, and 96 h, three fish were randomly selected from each group, and the selected fish were put into 300 mg/L of MS-222 anesthetic solution. The blood of grouper was taken from the tail vein, without anticoagulant. The blood was stored at 4 °C for 12 h and then centrifuged at $10,614 \times g$, at 4 °C, for 5 min, and the liquid supernatant (serum) was collected [27]. The serum was stored at −80 °C before use for index determination. The gill filaments on the secondary gill arch were cut from the live grouper and rinsed with physiological saline to remove blood from the surface. Subsequently, the specimens were put into 4% formaldehyde solution and 2.5% glutaraldehyde solution for light microscopy and scanning electron microscopy (SEM), respectively [28,29]. At last, grouper muscles were dissected for free amino acids (FAAs) assay. Meanwhile, the survival status and death number of the fish were recorded.

2.3. Biochemical Analysis of Serum

The serum AST, ALT, alkaline phosphatase (AKP), cortisol (COR), IgM, LZM, glutathione (GSH), superoxide dismutase (SOD), and malondialdehyde (MDA) levels were measured with a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.4. Light Microscopy of Gill Tissues

Gills were fixed in 4% paraformaldehyde and washed with phosphate-buffered saline (PBS, 0.1 mol/L, pH 7.4). Then they were dehydrated with different volume fractions of ethanol for 10 min; treated transparently with xylene; embedded in paraffin; and sectioned continuously, using a microtome (Leica, RM2135, Nussloch, Germany), at a section thickness of 7–10 µm. Gill slices were stained with hematoxylin and eosin (H&E). Light microscopy (Olympus BX-43, JESCO, Tokyo, Japan) was used for observation and photography. The epithelial cell width and lamellae length of grouper gills were measured by using Image J software.

2.5. SEM of Gill Tissues

The fixed gill samples were rinsed with PBS (0.1 mol/L, pH 7.4) 3 times, with each time lasting 15 min. Ethanol concentrations were 30%, 50%, 70%, 80%, 90%, 95%, and 100%, and dehydration treatment was performed with ethanol for 15 min at each concentration. The sample was replaced with isoamyl acetate 2 times, and then it was frozen at −80 °C for 24 h and freeze-dried for 48 h. Gold-plated conductive coatings were applied to the samples. SEM (Hitachi SU5000, Tokyo, Japan) was used to observe and photograph the samples. The lamellar spacing and lamellar surface area of grouper gill were measured by using Image J software.

2.6. FAAs Assay

Briefly, 2 g of grouper muscle and 10 mL of 5% trichloroacetic acid were homogenized and centrifuged at 10,000 rpm for 10 min. The supernatant was diluted to 25 mL after repeated extraction and centrifugation [30]. Subsequently, 1 mL of supernatant was filtered through a 0.22 µm disposable filter. The FAAs contents were determined by an amino acid analyzer (Hitachi L-8800, Tokyo, Japan).

2.7. Statistical Analysis

The data were expressed as the mean $\pm$ SD. According to the method of Xu et al. [31], a two-way ANOVA (listed in Table 1) was used to analyze the independent and interactive effects of exposure time and nitrite concentration. If significant differences of interaction were found, Duncan's multiple range tests were used to determine the differences between means. The significance level was set to $p < 0.05$. The Levene test was used to check the homogeneity of the samples before applying Duncan. All statistical analyses were performed by using SPSS version 26.0 software. Origin software was used to create graphs.

Table 1. The results of the interaction between time and/or nitrite exposure on FAAs, blood biochemical parameter levels, oxidative stress, and immune-related markers are shown below.

Parameters	Source of Variation	df	F	p
AST	Time	7	80.372	<0.01
	Nitrite exposure	3	145.538	<0.01
	Time $\times$ Nitrite exposure	21	15.991	<0.01
ALT	Time	7	64.960	<0.01
	Nitrite exposure	3	18.887	<0.01
	Time $\times$ Nitrite exposure	21	3.571	<0.01
AKP	Time	7	83.231	<0.01
	Nitrite exposure	3	1.892	<0.01
	Time $\times$ Nitrite exposure	21	30.997	<0.01
COR	Time	7	9.960	<0.01
	Nitrite exposure	3	14.020	<0.01
	Time $\times$ Nitrite exposure	21	5.207	<0.01
IgM	Time	7	8.967	<0.01
	Nitrite exposure	3	21.576	<0.01
	Time $\times$ Nitrite exposure	21	8.320	<0.01
LZM	Time	7	5.420	<0.01
	Nitrite exposure	3	22.268	<0.01
	Time $\times$ Nitrite exposure	21	1.910	<0.01
GSH	Time	7	41.299	<0.01
	Nitrite exposure	3	31.318	<0.01
	Time $\times$ Nitrite exposure	21	7.401	<0.01
SOD	Time	7	6.716	<0.01
	Nitrite exposure	3	34.429	<0.01
	Time $\times$ Nitrite exposure	21	3.623	<0.01
MDA	Time	7	8.967	<0.01
	Nitrite exposure	3	21.576	<0.01
	Time $\times$ Nitrite exposure	21	8.320	<0.01
Asp	Time	7	2591.511	<0.01
	Nitrite exposure	3	437.998	<0.01
	Time $\times$ Nitrite exposure	21	232.758	<0.01
Thr	Time	7	3140.926	<0.01
	Nitrite exposure	3	926.126	<0.01
	Time $\times$ Nitrite exposure	21	404.840	<0.01
Ser	Time	7	8349.712	<0.01
	Nitrite exposure	3	5801.992	<0.01
	Time $\times$ Nitrite exposure	21	1601.050	<0.01
Glu	Time	7	29,835.143	<0.01
	Nitrite exposure	3	5147.610	<0.01
	Time $\times$ Nitrite exposure	21	4510.256	<0.01

Table 1. Cont.

Parameters	Source of Variation	df	F	p
Gly	Time	7	656,602.897	<0.01
	Nitrite exposure	3	100,475.191	<0.01
	Time × Nitrite exposure	21	60,079.333	<0.01
Ala	Time	7	38,253.514	<0.01
	Nitrite exposure	3	19,294.190	<0.01
	Time × Nitrite exposure	21	7223.457	<0.01
Cys	Time	7	1561.556	<0.01
	Nitrite exposure	3	138.543	<0.01
	Time × Nitrite exposure	21	68.667	<0.01
Val	Time	7	414.019	<0.01
	Nitrite exposure	3	1882.457	<0.01
	Time × Nitrite exposure	21	577.514	<0.01
Met	Time	7	317.313	<0.01
	Nitrite exposure	3	1050.469	<0.01
	Time × Nitrite exposure	21	252.804	<0.01
Ile	Time	7	1241.423	<0.01
	Nitrite exposure	3	3313.531	<0.01
	Time × Nitrite exposure	21	864.202	<0.01
Leu	Time	7	8808.686	<0.01
	Nitrite exposure	3	30,658.972	<0.01
	Time × Nitrite exposure	21	7723.441	<0.01
Tyr	Time	7	1346.143	<0.01
	Nitrite exposure	3	4115.105	<0.01
	Time × Nitrite exposure	21	1067.422	<0.01
Phe	Time	7	7631.201	<0.01
	Nitrite exposure	3	11,812.878	<0.01
	Time × Nitrite exposure	21	2839.133	<0.01
Lys	Time	7	72,215.842	<0.01
	Nitrite exposure	3	51,218.828	<0.01
	Time × Nitrite exposure	21	28,975.977	<0.01
Nh3	Time	7	3178.977	<0.01
	Nitrite exposure	3	3152.285	<0.01
	Time × Nitrite exposure	21	1027.451	<0.01
His	Time	7	658.735	<0.01
	Nitrite exposure	3	3949.875	<0.01
	Time × Nitrite exposure	21	1337.317	<0.01
Arg	Time	7	1492.122	<0.01
	Nitrite exposure	3	1178.105	<0.01
	Time × Nitrite exposure	21	903.914	<0.01

3. Results

3.1. The Effects of Nitrite Exposure on the Survival of Grouper

Grouper in the N-0, N-5, N-10, and N-20 groups were subjected to exposure for 96 h. In the course of the experiment, the water-quality index was strictly controlled within the prescribed range and did not change. At first, the swimming state of grouper was normal. With the increase of the nitrite level, grouper showed varying degrees of stress behavior, such as mania, shortness of breath, near-death spasm, slow movement, and reduced avoidance. The frequency of gill overturning increased, and local hyperemia and body surface mucus secretion increased. The grouper tended to return to normal after 24 h of adaptation. After 72 h, some grouper turned white, and a small number of dead individuals appeared. Dead fish had erect dorsal fins, pectoral fins, and operculum; the

mouths were open violently; the gills were bright red or dark red; and the body was rigid and curved. The lightening of the body color of the N-20 group was more obvious. Table 2 shows the survival rates observed in the experiment. Grouper in the N-0, N-5, N-10, and N-20 groups showed 100% survival after 72 h. However, all fish in the N-10 and N-20 groups died after 96 h.

Table 2. Changes in survival rates (%) of pearl gentian grouper exposed to various nitrite concentrations.

Nitrite Concentration (mg/L)	Time of Exposure (h)							
	0 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h
0	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100	-
20	100	100	100	100	100	100	100	-

3.2. Effects of Nitrite Exposure on Serum Biochemical Index

As shown in Figure 1, serum AST activities were increased with the increase of exposure concentrations at 12, 24, 36, and 60 h. AST and ALT contents were increased significantly in the N-20 group following exposure for 48 and 72 h, as compared with CK ($p < 0.05$). In addition, AKP activities were significantly elevated in the fish exposed to N-10 and N-20 for 48, 60, and 72 h compared with CK ($p < 0.05$). Similar changes in AKP activities were observed in the N-5 group after exposure for 60 and 72 h.

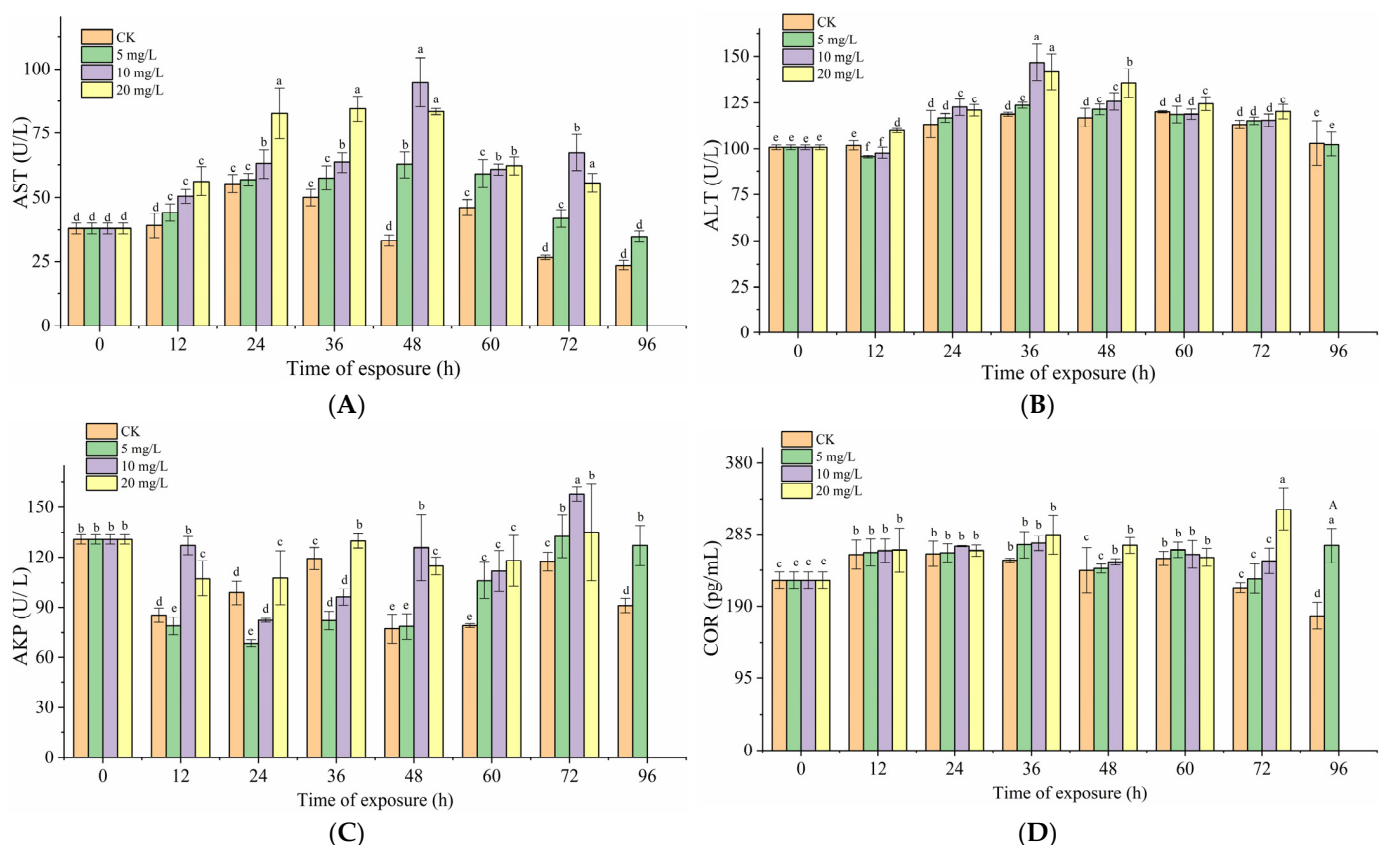


Figure 1. Changes in serum (A) AST, (B) ALT, (C) AKP, and (D) COR levels in pearl gentian grouper exposed to various nitrite concentrations over 96 h. Data are means $\pm$ SD. Different lowercase letters indicate that there are significant differences among groups ($p < 0.05$).

The COR levels were increased significantly after 48 and 72 h of exposure in the N-20 group compared with CK ($p < 0.05$). Meanwhile, the correlation between serum COR levels

and exposure concentrations was positive at 36 and 48 h. The levels of COR in the CK group were significantly lower than that at 0 h after 96 h of exposure ($p < 0.05$).

3.3. Effects of Nitrite Exposure on Immunity in Grouper

Figure 2 shows that, compared with CK, the IgM levels were considerably decreased after 60 and 72 h of exposure in the N-20 group ($p < 0.05$). However, there was little difference in IgM levels between the nitrite-treated groups and CK after exposure to nitrite for 12 h. LZM levels were also significantly decreased in the N-20 group following exposure for 48, 60, and 72 h ($p < 0.05$).

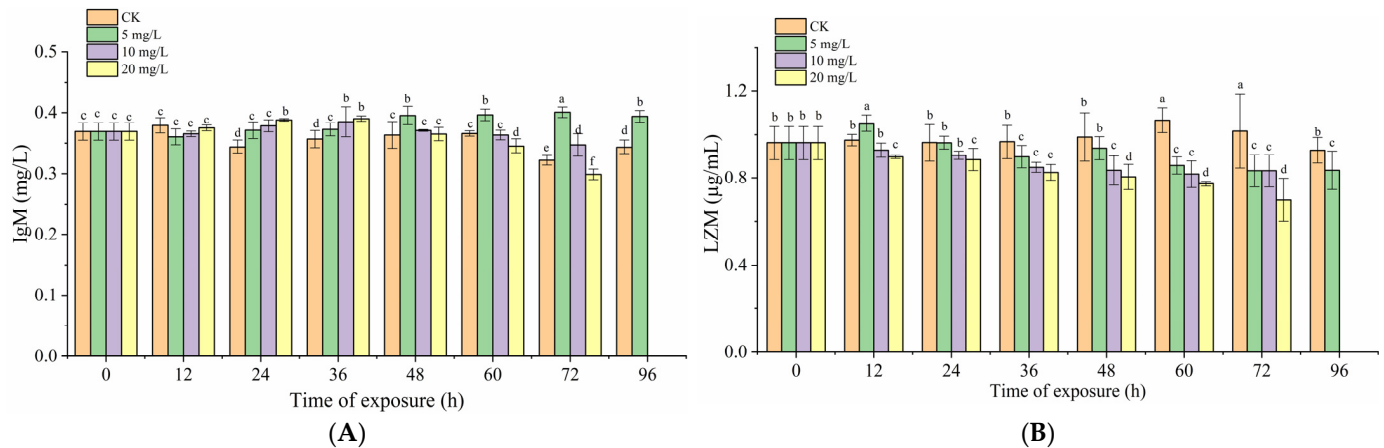


Figure 2. Changes in humoral immune parameter (A) IgM and (B) LZM levels in pearl gentian grouper exposed to various nitrite concentrations over 96 h. Data are means $\pm$ SD. Different lowercase letters indicate that there are significant differences among groups ($p < 0.05$).

3.4. Effects of Nitrite Exposure on Oxidative Stress in Grouper

Figure 3 shows the changes of oxidative stress in grouper exposed to nitrite. The level of GSH was increased from 12 to 24 h, and then it was decreased with increasing concentrations of nitrite. Following exposure to N-10 and N-20 after 48, 60, and 72 h, there were marked reductions in the activities of SOD and GSH compared with CK ($p < 0.05$). Meanwhile, there was a negative correlation between serum GSH levels and exposure concentrations at 48, 60, and 72 h. In contrast, MDA levels were elevated following exposure to N-20 for 48 and 72 h compared with CK ($p < 0.05$). Similar changes in the MDA activities were observed in the N-20 group at 60 h. Meanwhile, the correlation between serum MDA levels and exposure concentrations was positive at 12 and 24 h.

3.5. Effects of Nitrite Exposure on FAAs in Grouper

Table 3 shows the remarkable changes in FAAs according to the nitrite concentrations and exposure time. The contents of total FAAs were increased in the N-10 and N-20 groups at 72 h. In addition, the aspartic acid of umami amino acids was elevated considerably in the N-20 group after 72 h compared with CK ($p < 0.05$). There was a similar change in the alanine of the sweet amino acids after 72 h of exposure to N-10 and N-20 ($p < 0.05$). The valine acid of bitter amino acids was elevated considerably in the N-10 group after 60 and 72 h compared with CK ($p < 0.05$). The total sweet amino acids and fresh amino acids in the high concentration group showed an increasing trend.

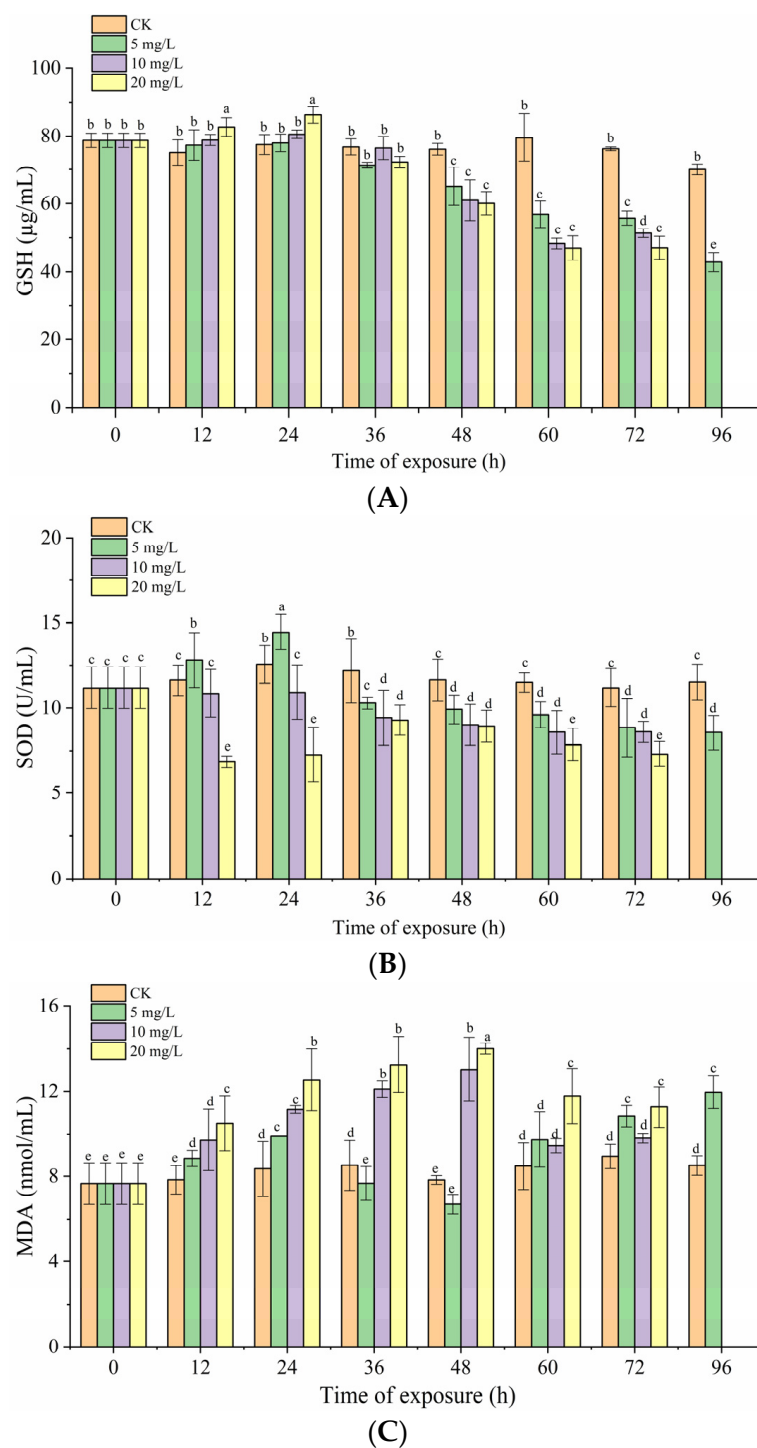


Figure 3. Changes in antioxidant capacity (A) GSH, (B) SOD, and (C) MDA levels in pearl gentian grouper exposed to various nitrite concentrations over 96 h. Data are means $\pm$ SD. Different lowercase letters indicate that there are significant differences among groups ($p < 0.05$).

Table 3. Changes in FAAs of pearl gentian grouper exposed to various concentrations of nitrite for 96 h.

Nitrite Concentration	Time of Exposure (h)	Free Amino Acids						
		Asp ^{&}	Thr [#]	Ser [#]	Glu ^{&}	Gly [#]	Ala [#]	Cys
0 mg/L	0 h	5.15 ± 0.04 ^a	26.53 ± 0.03 ^a	27.37 ± 0.03 ^a	60.79 ± 0.03 ^a	169.89 ± 0.16 ^a	100.23 ± 0.14 ^a	3.28 ± 0.03 ^a
	12 h	3.09 ± 0.07 ^c	27.53 ± 0.04 ^a	24.59 ± 0.00 ^b	47.63 ± 0.04 ^c	188.94 ± 0.33 ^a	84.43 ± 0.07 ^b	1.55 ± 0.06 ^c
	24 h	2.423 ± 0.04 ^d	21.35 ± 0.30 ^b	17.86 ± 0.43 ^c	46.01 ± 0.61 ^c	117.21 ± 0.35 ^b	51.46 ± 0.92 ^d	2.58 ± 0.05 ^b
	36 h	2.25 ± 0.01 ^d	20.77 ± 0.04 ^b	16.40 ± 0.02 ^c	26.73 ± 0.01 ^e	121.66 ± 0.12 ^b	49.73 ± 0.14 ^d	0.49 ± 0.01 ^d
	48 h	1.87 ± 0.01 ^e	13.50 ± 0.13 ^d	14.37 ± 0.10 ^d	32.59 ± 0.05 ^d	61.04 ± 0.03 ^c	47.10 ± 0.02 ^d	0.38 ± 0.06 ^d
	60 h	1.38 ± 0.06 ^e	17.76 ± 0.35 ^c	13.15 ± 0.02 ^d	31.79 ± 0.12 ^d	83.88 ± 0.05 ^c	40.56 ± 0.32 ^d	0.40 ± 0.00 ^d
	72 h	1.59 ± 0.01 ^e	16.14 ± 0.03 ^c	13.63 ± 0.01 ^d	26.94 ± 0.01 ^e	48.34 ± 0.01 ^d	47.45 ± 0.04 ^d	0.41 ± 0.04 ^d
	96 h	1.68 ± 0.07 ^e	9.62 ± 0.41 ^e	13.06 ± 0.20 ^d	23.86 ± 0.05 ^e	21.35 ± 0.18 ^d	44.54 ± 0.15 ^d	0.28 ± 0.02 ^d
5 mg/L	0 h	5.15 ± 0.04 ^a	26.53 ± 0.03 ^a	27.37 ± 0.03 ^a	60.79 ± 0.03 ^a	169.89 ± 0.16 ^a	100.23 ± 0.14 ^a	3.28 ± 0.03 ^a
	12 h	3.37 ± 0.05 ^c	20.46 ± 0.10 ^b	24.54 ± 0.11 ^b	43.69 ± 0.01 ^b	171.10 ± 0.24 ^a	72.16 ± 0.10 ^c	1.05 ± 0.03 ^c
	24 h	2.17 ± 0.01 ^d	16.35 ± 0.01 ^c	19.54 ± 0.03 ^c	38.88 ± 0.01 ^d	127.74 ± 0.06 ^b	62.38 ± 0.06 ^c	1.12 ± 0.01 ^c
	36 h	2.17 ± 0.03 ^d	19.70 ± 0.04 ^c	23.50 ± 0.03 ^b	42.59 ± 0.02 ^c	112.92 ± 0.22 ^b	80.43 ± 0.08 ^b	0.50 ± 0.06 ^d
	48 h	2.00 ± 0.02 ^d	14.13 ± 0.31 ^d	15.90 ± 0.13 ^c	38.41 ± 0.04 ^d	57.42 ± 0.00 ^c	53.05 ± 0.10 ^d	0.63 ± 0.00 ^d
	60 h	4.08 ± 0.06 ^b	20.20 ± 0.11 ^b	24.61 ± 0.11 ^b	50.84 ± 0.09 ^b	130.15 ± 0.03 ^b	82.42 ± 0.16 ^b	0.58 ± 0.01 ^d
	72 h	2.22 ± 0.05 ^d	7.16 ± 0.03 ^e	8.37 ± 0.00 ^e	22.33 ± 0.03 ^e	40.10 ± 0.03 ^d	43.42 ± 0.06 ^d	1.00 ± 0.05 ^d
	96 h	3.57 ± 0.12 ^c	12.59 ± 0.07 ^d	16.24 ± 0.11 ^c	34.97 ± 0.34 ^d	81.19 ± 0.24 ^c	79.85 ± 0.17 ^c	0.44 ± 0.03 ^d
10 mg/L	0 h	5.15 ± 0.04 ^a	26.53 ± 0.03 ^a	27.37 ± 0.03 ^a	60.79 ± 0.03 ^a	169.89 ± 0.16 ^a	100.23 ± 0.14 ^a	3.28 ± 0.03 ^a
	12 h	2.6 ± 0.25 ^d	17.34 ± 0.97 ^c	24.10 ± 0.53 ^b	44.20 ± 0.17 ^c	146.93 ± 0.42 ^b	78.90 ± 0.98 ^c	1.13 ± 0.43 ^c
	24 h	2.05 ± 0.02 ^d	14.87 ± 0.27 ^d	22.10 ± 0.15 ^b	32.84 ± 0.13 ^d	123.02 ± 0.51 ^b	65.50 ± 0.16 ^c	0.66 ± 0.02 ^d
	36 h	1.51 ± 0.02 ^e	13.50 ± 0.33 ^d	24.77 ± 0.15 ^b	39.35 ± 0.02 ^d	109.86 ± 0.09 ^b	71.23 ± 0.12 ^c	0.44 ± 0.11 ^d
	48 h	1.25 ± 0.04 ^e	7.172 ± 0.09 ^e	14.57 ± 0.02 ^d	27.71 ± 0.05 ^e	40.23 ± 0.11 ^d	48.28 ± 0.05 ^d	0.07 ± 0.05 ^d
	60 h	1.66 ± 0.00 ^e	12.66 ± 0.11 ^d	17.77 ± 0.08 ^c	32.08 ± 0.04 ^d	71.84 ± 0.06 ^c	58.13 ± 0.02 ^d	0.19 ± 0.02 ^d
	72 h	3.14 ± 0.13 ^c	19.44 ± 0.08 ^c	24.41 ± 0.10 ^b	49.57 ± 0.58 ^c	68.35 ± 0.11 ^c	92.57 ± 0.09 ^b	0.73 ± 0.04 ^d
	96 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20 mg/L	0 h	5.15 ± 0.04 ^a	26.53 ± 0.03 ^a	27.37 ± 0.03 ^a	60.79 ± 0.03 ^a	169.89 ± 0.16 ^a	100.23 ± 0.14 ^a	3.28 ± 0.03 ^a
	12 h	2.00 ± 0.08 ^d	17.29 ± 0.95 ^c	13.35 ± 0.33 ^d	31.95 ± 0.12 ^d	80.04 ± 0.06 ^c	42.45 ± 0.05 ^d	0.38 ± 0.02 ^d
	24 h	2.38 ± 0.00 ^d	15.03 ± 0.37 ^c	14.34 ± 0.14 ^d	34.72 ± 0.11 ^d	53.63 ± 0.04 ^c	51.04 ± 0.03 ^d	0.32 ± 0.01 ^d
	36 h	2.19 ± 0.01 ^d	13.45 ± 0.33 ^d	19.64 ± 0.02 ^c	34.39 ± 0.02 ^d	158.47 ± 0.23 ^a	66.48 ± 0.18 ^c	0.62 ± 0.00 ^d
	48 h	2.20 ± 0.01 ^d	7.11 ± 0.12 ^e	13.52 ± 0.20 ^d	28.41 ± 0.41 ^e	57.45 ± 0.03 ^c	42.94 ± 0.18 ^d	0.04 ± 0.01 ^d
	60 h	2.67 ± 0.09 ^d	12.62 ± 0.14 ^d	9.55 ± 0.03 ^e	29.44 ± 0.01 ^e	51.56 ± 0.05 ^c	46.50 ± 0.10 ^d	0.55 ± 0.01 ^d
	72 h	2.62 ± 0.09 ^d	19.36 ± 0.15 ^c	13.89 ± 0.10 ^d	34.12 ± 0.06 ^d	41.87 ± 0.15 ^d	65.07 ± 0.10 ^c	0.45 ± 0.02 ^d
	96 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nitrite Concentration	Time of Exposure (h)	Free Amino Acids						
		Val [*]	Met	Ile [*]	Leu [*]	Tyr [*]	Phe [*]	Lys
0 mg/L	0 h	13.52 ± 0.05 ^c	6.92 ± 0.06 ^c	10.68 ± 0.05 ^d	17.17 ± 0.05 ^c	7.56 ± 0.14 ^c	4.52 ± 0.11 ^e	69.36 ± 013 ^d
	12 h	12.96 ± 0.04 ^c	7.32 ± 0.03 ^c	10.55 ± 0.04 ^d	16.31 ± 0.07 ^c	7.65 ± 0.08 ^c	4.73 ± 0.04 ^e	89.34 ± 0.04 ^b
	24 h	12.65 ± 0.37 ^c	7.45 ± 0.58 ^c	15.57 ± 0.03 ^b	19.34 ± 0.20 ^c	9.74 ± 0.21 ^b	8.18 ± 0.01 ^c	69.84 ± 0.47 ^d
	36 h	13.69 ± 0.03 ^c	7.85 ± 0.04 ^c	11.83 ± 0.02 ^d	17.55 ± 0.00 ^c	7.08 ± 0.07 ^c	6.80 ± 0.14 ^d	68.40 ± 0.04 ^d
	48 h	13.83 ± 0.02 ^c	8.95 ± 0.03 ^b	12.73 ± 0.01 ^c	19.52 ± 0.01 ^c	9.34 ± 0.01 ^b	9.73 ± 0.02 ^c	46.34 ± 0.06 ^f
	60 h	12.36 ± 0.18 ^c	7.94 ± 0.02 ^c	10.78 ± 0.13 ^d	16.49 ± 0.12 ^c	6.74 ± 0.04 ^c	6.37 ± 0.01 ^d	49.71 ± 0.14 ^f
	72 h	14.07 ± 0.03 ^c	9.09 ± 0.05 ^b	12.28 ± 0.02 ^c	19.25 ± 0.04 ^c	9.22 ± 0.14 ^b	8.72 ± 0.01 ^c	47.58 ± 0.25 ^f
	96 h	14.72 ± 0.11 ^c	8.97 ± 0.13 ^b	13.24 ± 0.10 ^c	20.57 ± 0.17 ^b	9.19 ± 0.19 ^b	9.83 ± 0.01 ^c	50.39 ± 0.18 ^e
5 mg/L	0 h	13.52 ± 0.05 ^c	6.92 ± 0.06 ^c	10.68 ± 0.05 ^d	17.17 ± 0.05 ^c	7.56 ± 0.14 ^c	4.52 ± 0.11 ^e	69.36 ± 0.13 ^d
	12 h	15.38 ± 0.01 ^b	8.84 ± 0.02 ^b	11.87 ± 0.01 ^d	18.68 ± 0.01 ^c	7.56 ± 0.00 ^c	7.02 ± 0.06 ^e	73.37 ± 0.08 ^c
	24 h	16.93 ± 0.01 ^b	10.85 ± 0.03 ^a	13.96 ± 0.01 ^c	22.72 ± 0.02 ^b	10.36 ± 0.02 ^a	9.76 ± 0.03 ^c	96.74 ± 0.01 ^a
	36 h	18.88 ± 0.03 ^b	11.41 ± 0.02 ^a	16.57 ± 0.00 ^a	25.96 ± 0.04 ^a	11.45 ± 0.01 ^a	11.99 ± 0.03 ^b	74.74 ± 0.05 ^a
	48 h	15.18 ± 0.04 ^b	9.10 ± 0.05 ^b	13.51 ± 0.00 ^c	21.08 ± 0.02 ^b	8.67 ± 0.01 ^b	9.00 ± 0.06 ^c	70.16 ± 0.04 ^c
	60 h	17.25 ± 0.04 ^b	9.32 ± 0.01 ^b	13.58 ± 0.00 ^c	21.06 ± 0.04 ^b	7.86 ± 0.02 ^c	7.38 ± 0.08 ^d	83.65 ± 0.15 ^b
	72 h	8.75 ± 0.09 ^d	5.44 ± 0.13 ^d	6.98 ± 0.01 ^f	10.57 ± 0.02 ^d	4.76 ± 0.04 ^d	3.65 ± 0.01 ^a	87.13 ± 0.02 ^b
	96 h	18.85 ± 0.07 ^b	10.19 ± 0.10 ^a	15.27 ± 0.04 ^b	25.17 ± 0.06 ^a	10.49 ± 0.03 ^a	7.62 ± 0.04 ^d	35.78 ± 0.02 ^g
10 mg/L	0 h	13.52 ± 0.05 ^c	6.92 ± 0.06 ^c	10.68 ± 0.05 ^d	17.17 ± 0.05 ^c	7.56 ± 0.14 ^c	4.52 ± 0.11 ^e	69.36 ± 0.13 ^d
	12 h	15.18 ± 0.94 ^b	8.86 ± 0.47 ^b	12.43 ± 0.64 ^c	18.63 ± 0.10 ^c	7.59 ± 0.02 ^c	5.95 ± 0.07 ^{Be}	69.11 ± 0.19 ^d
	24 h	16.40 ± 0.04 ^b	9.70 ± 0.02 ^b	14.38 ± 0.03 ^b	21.83 ± 0.09 ^b	9.76 ± 0.10 ^b	10.33 ± 0.10 ^{Ac}	63.51 ± 0.16 ^d
	36 h	17.66 ± 0.07 ^b	10.75 ± 0.14 ^a	15.63 ± 0.05 ^b	23.93 ± 0.02 ^b	11.12 ± 0.01 ^a	11.76 ± 0.02 ^{Bb}	64.56 ± 0.07 ^d
	48 h	13.19 ± 0.11 ^c	8.50 ± 0.16 ^b	12.08 ± 0.04 ^c	18.53 ± 0.05 ^c	9.14 ± 0.01 ^b	9.62 ± 0.02 ^{Bd}	35.79 ± 0.06 ^g
	60 h	15.07 ± 0.04 ^b	9.36 ± 0.01 ^b	13.36 ± 0.02 ^c	20.67 ± 0.00 ^b	9.73 ± 0.01 ^b	10.28 ± 0.02 ^{Ac}	48.98 ± 0.01 ^f
	72 h	22.98 ± 0.04 ^a	11.90 ± 0.07 ^a	17.32 ± 0.00 ^a	27.89 ± 0.01 ^a	11.78 ± 0.06 ^a	12.17 ± 0.07 ^{Aa}	97.18 ± 0.05 ^a
	96 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20 mg/L	0 h	13.52 ± 0.05 ^c	6.92 ± 0.06 ^c	10.68 ± 0.05 ^d	17.18 ± 0.05 ^c	7.56 ± 0.14 ^c	4.52 ± 0.11 ^e	69.36 ± 0.13 ^d
	12 h	10.59 ± 0.01 ^c	7.16±0.03 ^c	8.62 ± 0.01 ^e	12.95 ± 0.02 ^d	5.97 ± 0.01 ^d	5.84 ± 0.04 ^e	69.46 ± 0.00 ^d
	24 h	13.13 ± 0.04 ^c	7.03 ± 0.05 ^c	10.87 ± 0.02 ^d	16.82 ± 0.01 ^c	6.78 ± 0.01 ^c	6.50 ± 0.02 ^d	74.10 ± 0.06 ^c
	36 h	13.87 ± 0.02 ^c	7.54 ± 0.02 ^c	10.74 ± 0.05 ^d	16.42 ± 0.01 ^a	6.72 ± 0.01 ^c	4.99 ± 0.01 ^e	88.48 ± 0.06 ^b
	48 h	12.79 ± 0.06 ^c	6.37 ± 0.12 ^c	9.99 ± 0.00 ^d	15.08 ± 0.06 ^c	6.05 ± 0.01 ^c	6.86 ± 0.02 ^d	59.47 ± 0.09 ^e
	60 h	8.90 ± 0.01 ^d	5.17 ± 0.05 ^d	6.83 ± 0.01 ^f	9.81 ± 0.01 ^d	4.38 ± 0.05 ^d	3.35 ± 0.04 ^f	76.82 ± 0.07 ^c
	72 h	14.29 ± 0.03 ^c	8.68 ± 0.04 ^b	11.99 ± 0.04 ^c	18.62 ± 0.06 ^c	9.13 ± 0.00 ^b	6.95 ± 0.09 ^d	74.07 ± 0.15 ^c
	96 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 3. Cont.

Nitrite Concentration	Time of Exposure (h)	Free Amino Acids						
		Nh3	His *	Arg	Total	Umami	Sweet	Bitter
0 mg/L	0 h	19.16 ± 0.07 ^d	7.79 ± 0.04 ^c	11.34 ± 0.39 ^e	561.26	65.94	324.02	61.24
	12 h	19.50 ± 0.04 ^d	7.70 ± 0.00 ^c	15.10 ± 0.05 ^c	568.92	50.72	325.49	59.90
	24 h	15.59 ± 0.14 ^d	7.60 ± 0.08 ^c	12.79 ± 0.10 ^d	437.64	48.43	207.88	73.08
	36 h	15.94 ± 0.02 ^d	7.39 ± 0.01 ^c	12.91 ± 0.03 ^d	407.47	28.98	208.56	64.34
	48 h	14.91 ± 0.08 ^e	7.54 ± 0.09 ^c	10.80 ± 0.02 ^e	324.54	34.46	136.01	72.69
	60 h	15.49 ± 0.23 ^d	7.19 ± 0.07 ^c	11.86 ± 0.03 ^e	333.85	33.17	155.35	59.93
	72 h	17.69 ± 0.07 ^d	7.63 ± 0.11 ^c	11.77 ± 0.02 ^e	311.80	28.53	125.56	71.17
	96 h	18.76 ± 0.04 ^d	7.20 ± 0.03 ^c	11.71 ± 0.11 ^e	278.97	25.54	88.57	74.75
5 mg/L	0 h	19.16 ± 0.07 ^d	7.79 ± 0.04 ^c	11.34 ± 0.39 ^e	561.26	65.94	324.02	61.24
	12 h	18.51 ± 0.06 ^d	9.19 ± 0.07 ^b	13.4 ± 0.04 ^d	520.19	47.06	288.26	69.70
	24 h	17.71 ± 0.05 ^d	8.89 ± 0.03 ^b	18.35 ± 0.03 ^b	494.34	41.05	226.01	82.56
	36 h	17.66 ± 0.09 ^d	10.47 ± 0.08 ^a	19.27 ± 0.05 ^b	499.99	44.76	236.55	95.11
	48 h	16.11 ± 0.01 ^d	7.78 ± 0.04 ^c	15.66 ± 0.12 ^c	367.79	40.41	140.50	75.22
	60 h	23.83 ± 0.47 ^c	9.57 ± 0.05 ^b	17.35 ± 0.07 ^c	523.73	54.92	257.38	76.70
	72 h	21.48 ± 0.03 ^c	4.74 ± 0.02 ^d	12.42 ± 0.01 ^e	290.52	24.55	99.05	39.45
	96 h	29.34 ± 0.11 ^b	8.52 ± 0.06 ^b	9.70 ± 0.17 ^f	399.60	38.54	189.87	85.83
10 mg/L	0 h	19.16 ± 0.07 ^d	7.79 ± 0.04 ^c	11.34 ± 0.39 ^e	561.26	65.94	324.02	61.24
	12 h	17.75 ± 0.11 ^d	8.34 ± 0.23 ^b	12.01 ± 0.03 ^e	491.05	46.80	267.27	68.12
	24 h	17.28 ± 0.02 ^d	9.09 ± 0.00 ^b	12.28 ± 0.02 ^e	445.57	34.89	225.49	81.76
	36 h	19.79 ± 0.30 ^d	9.78 ± 0.02 ^b	13.59 ± 0.03 ^d	459.10	40.86	219.36	89.80
	48 h	18.01 ± 0.33 ^d	7.00 ± 0.02 ^c	8.72 ± 0.01 ^f	279.86	28.96	110.25	69.56
	60 h	18.60 ± 0.08 ^d	8.26 ± 0.02 ^b	12.15 ± 0.04 ^e	360.71	33.74	160.40	77.29
	72 h	24.35 ± 0.22 ^c	11.51 ± 0.02 ^a	21.50 ± 0.05 ^a	516.63	52.71	204.77	103.49
	96 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20 mg/L	0 h	19.16 ± 0.07 ^d	7.79 ± 0.04 ^c	11.34 ± 0.39 ^e	561.27	65.94	324.02	61.25
	12 h	15.47 ± 0.12 ^d	6.30 ± 0.02 ^c	15.09 ± 0.04 ^c	344.91	33.95	153.13	50.27
	24 h	17.16 ± 0.03 ^d	7.08 ± 0.09 ^c	15.03 ± 0.07 ^c	345.96	37.10	134.04	61.18
	36 h	22.54 ± 0.04 ^c	7.72 ± 0.01 ^c	14.59 ± 0.01 ^d	488.85	36.58	258.04	60.46
	48 h	16.45 ± 0.22 ^d	6.74 ± 0.00 ^c	11.72 ± 0.04 ^e	303.19	30.61	121.02	57.51
	60 h	26.57 ± 0.34 ^b	4.59 ± 0.04 ^d	14.41 ± 0.00 ^d	313.72	32.11	120.23	37.86
	72 h	34.37 ± 0.45 ^a	7.77 ± 0.04 ^c	16.78 ± 0.07 ^c	380.03	36.74	140.19	68.75
	96 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Note: Different lowercase letters indicate that there are significant differences among groups ($p < 0.05$). * Denotes bitter amino acids, & denotes umami amino acids, and # denotes sweet amino acids.

3.6. Effects of Nitrite Exposure on Light Microscopy of Gill Tissues

A histological evaluation of the H&E-stained gill tissues was performed following 96 h of exposure to various concentrations of nitrite. Figure 4 shows that there was a normal gill structure in the control (Figure 4A). Gill lamellae were organized in parallel on both sides of the gill filaments, and the epithelial cells were flat and normally arranged. The mitochondrial-rich cells were distributed on secondary gill lamellae. However, gill filaments started to curl, shrink, and fold with the extension of exposure time. The gill lamellae of the N-5 (Figure 4C), N-10, (Figure 4D), and N-20 (Figure 4E) groups were curled 48 h after nitrite exposure; the gill lamellae of the N-10 (Figure 4D) group became longer; and the gill epithelial cells of the N-20 (Figure 4E) group were swollen. After 72 h of exposure to nitrite, the epithelial cells of the gill lamella of the N-5 (Figure 4G) group began to swell, and the gill lamella became longer. The epithelial cells in the N-10 (Figure 4H) group were further swollen, and the gill lamellae were further elongated. Meanwhile, the distance between gill lamellae widened compared with CK (Figure 4F). A widening of gill spacing and hypertrophy of epithelial cells in the gill lamellae were observed in the N-20 (Figure 4I) group. Meanwhile, the mitochondria-rich cells were enlarged, and gill lamellae were more curled. Moreover, the epithelial cell width and lamellae length of grouper gill were measured (Figure 5A,B). The width of gill epithelial cells of grouper increases with time. The length of the gills became shorter with the prolongation of exposure time.

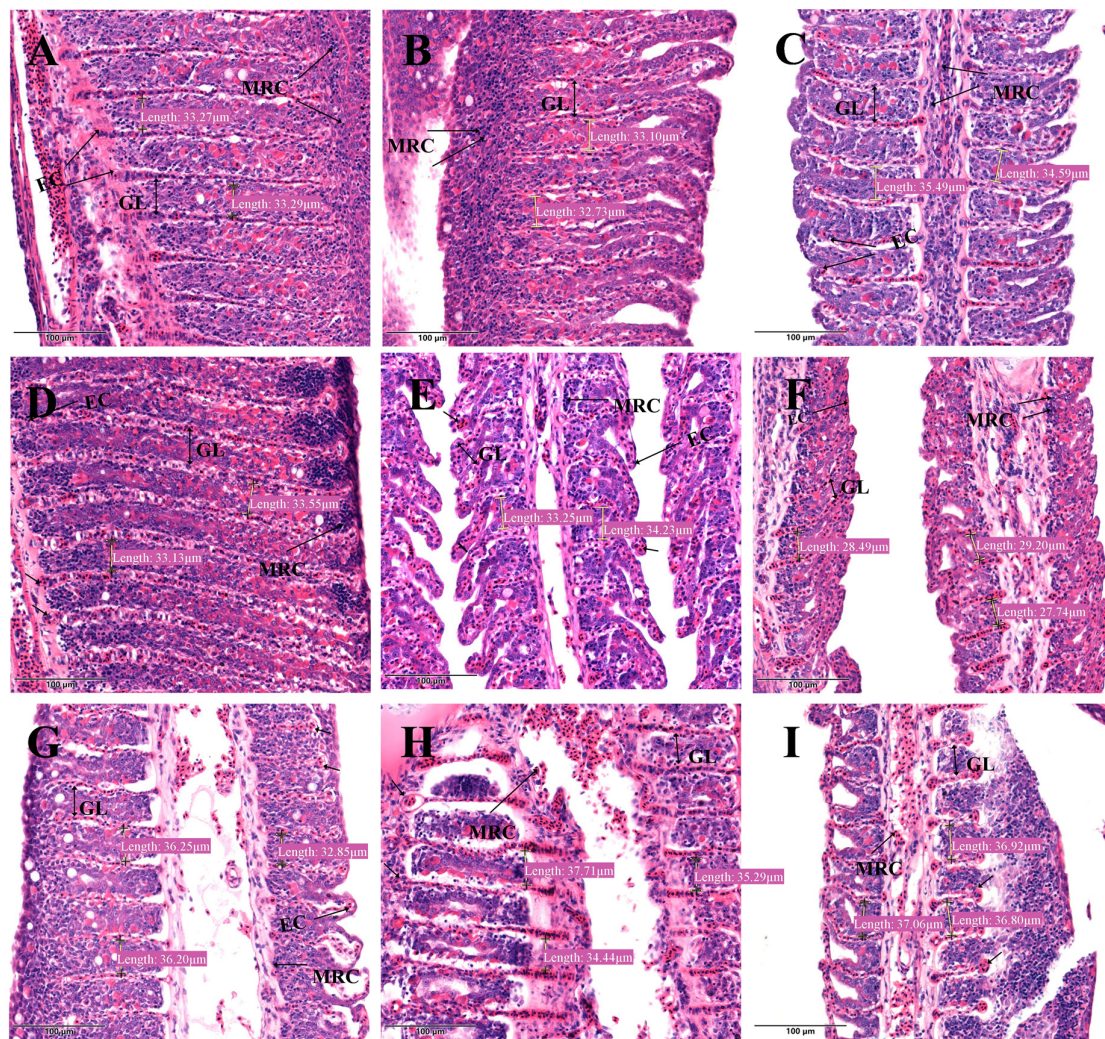


Figure 4. Representative H&E-stained sections showed changes in gill structure in pearl gentian grouper following 0, 48, and 72 h of exposure to various concentrations of nitrite: (A) 0 mg/L-0 h, (B) 0 mg/L-48 h, (C) 5 mg/L-48 h, (D) 10 mg/L-48 h, (E) 20 mg/L-48 h, (F) 0 mg/L-72 h, (G) 5 mg/L-72 h, (H) 10 mg/L-72 h, and (I) 20 mg/L-72 h. Bars = 100 μm. Gill lamella (GL), epithelial cell (EC); mitochondrial-rich cell (MRC), and epithelial cell hyperplasia (↑).

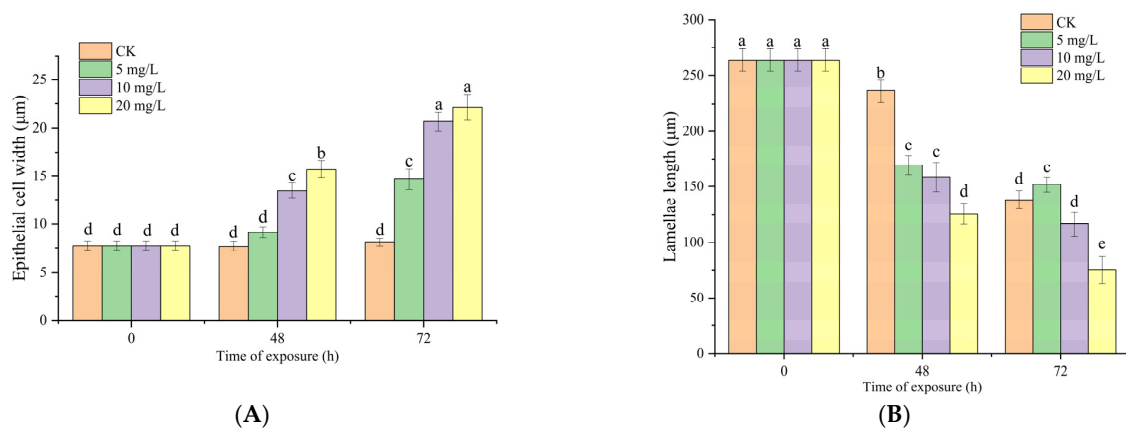


Figure 5. Quantification of fish gill structure. Epithelial cell width of gill (A) and lamellae length of gill (B) in grouper. Different lowercase letters indicate that there are significant differences among groups ($p < 0.05$).

3.7. Effects of Nitrite Exposure on SEM of Gill Tissues

Figure 6 shows that normal secondary lamellae were present in the control (Figure 6A) gills, which were equally spaced in parallel. There was no damage shedding of each cell. The gill structure was not changed significantly over time, while the small gill segments appeared to be shrunk and deformed. After 48 h exposure to nitrite, the gill tissues of the N-5 (Figure 6C), N-10 (Figure 6D), and N-20 (Figure 6E) groups were slightly disordered and shrunk. The interlamellar spacing between the gill lamellae of the N-10 and N-20 groups became smaller, and irregular folds appeared. The arrangement of gill lamellae in the N-0 (Figure 6B) group is still relatively regular. When exposed for 72 h, the surface of the gill sheets of the N-0 (Figure 6F) group was no longer smooth, and some cell atrophy appeared. The gill lamellae were folded and bent more severely in the N-20 (Figure 6I) group as compared to those in the N-10 (Figure 6H) group. Meanwhile, an increased number of mucous cells, damaged epithelial cells, and disorganized filaments and lamellae were observed in the N-10 (Figure 6H) and N-20 (Figure 6I) groups. Moreover, the interlamellar distance and lamellae surface area of grouper gill lamellae were measured. The interlamellar distance and lamellae surface area in all nitrite-treated samples showed a decreasing trend with the extension of exposure time compared with the CK (Figure 7A,B).

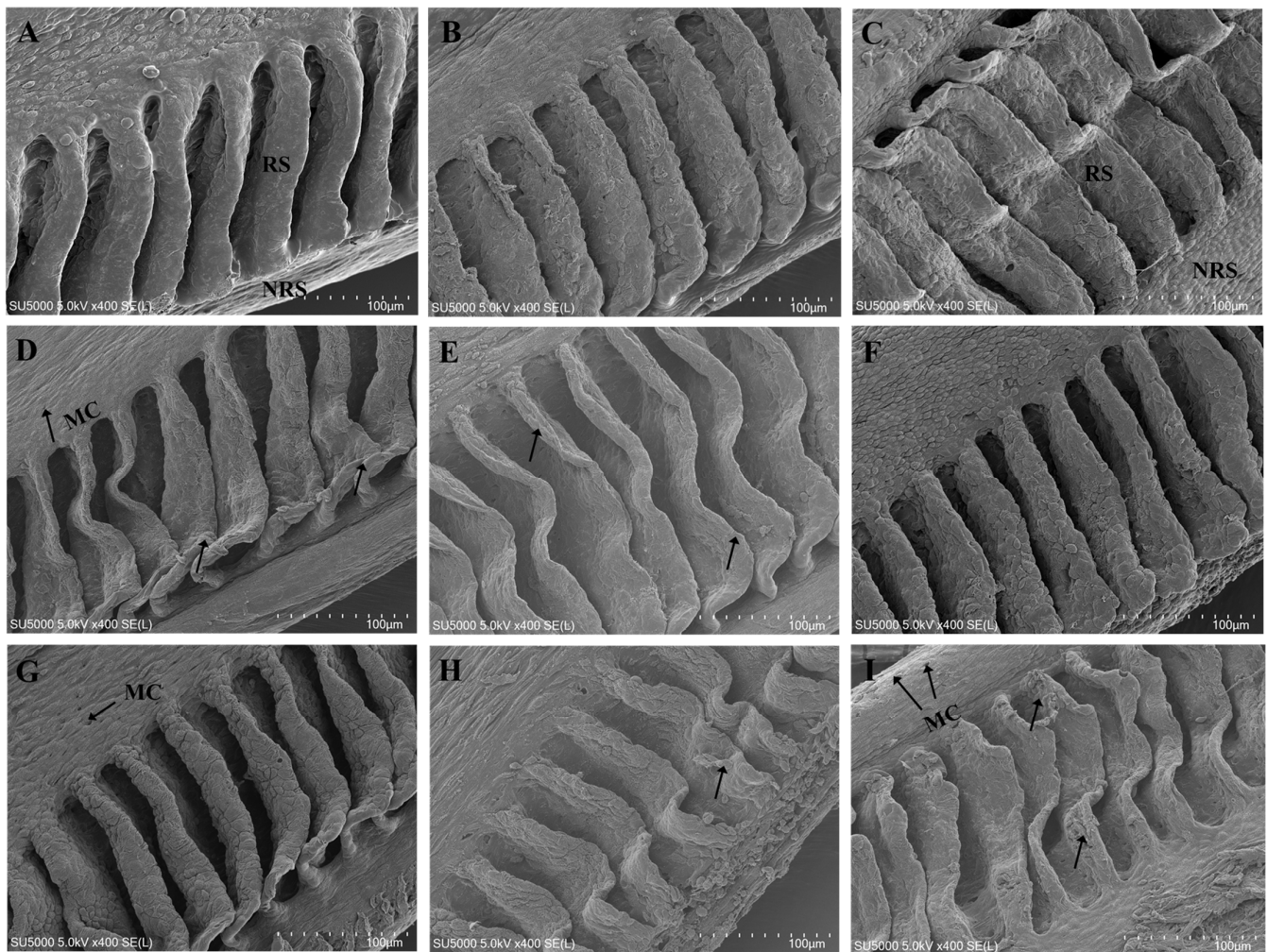


Figure 6. Representative SEM micrographs showed changes in the gill structure of pearl gentian grouper following 0, 48, and 72 h of exposure to various concentrations of nitrite: (A) 0 mg/L-0 h, (B) 0 mg/L-48 h, (C) 5 mg/L-48 h, (D) 10 mg/L-48 h, (E) 20 mg/L-48 h, (F) 0 mg/L-72 h, (G) 5 mg/L-72 h, (H) 10 mg/L-72 h, and (I) 20 mg/L-72 h. Bars = 100 μ m. Non-respiratory surface (NRS), respiratory surface (RS), mucous cell (MC), and gill lamella disorder ($\uparrow$).

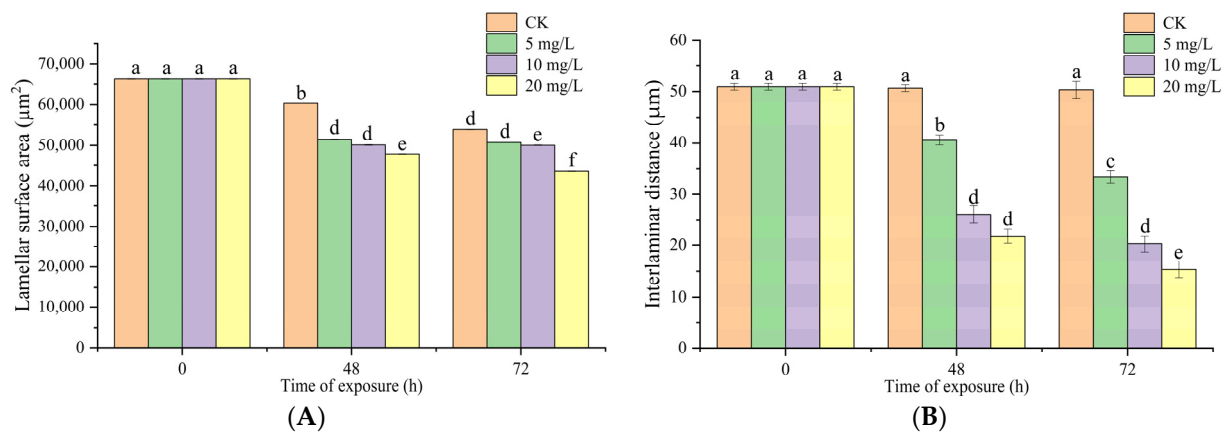


Figure 7. Quantification of fish gill structure. Lamellar surface area of gill (A) and interlamellar distance of gill (B) in grouper. Different lowercase letters indicate that there are significant differences among groups ($p < 0.05$).

4. Discussion

Nitrite can damage the aquatic environment. To clarify the plausible mechanisms of nitrite-stress-induced toxicity to pearl gentian grouper, the present study was conducted to establish a model of nitrite stress for 96 h. The effects of nitrite on gill tissues, serum biochemical function, meat flavor, and immune response of pearl gentian grouper were analyzed. Significant increases in the serum AST and ALT activities of fish were observed in this study when exposed to the N-20 group for 48 and 72 h. These findings were consistent with previous reports [21,32]. The increase in serum aminotransferase activity observed in fish possibly resulted from a disturbance in nitrogen metabolism [10]. In this case, the body needed to regulate glutamate production and protein synthesis by increasing the transaminase activity.

AKP is a class of membrane-bound glycoproteins that can be directly involved in the metabolic transfer of phosphate groups in organisms. AKP activity reflects the metabolic function and immunity of the body, so it is commonly used to assess fish health indicators [33]. In the present work, a marked increase in levels of serum AKP in the N-10 and N-20 groups was found, which could be attributed to high-nitrite-concentration-induced liver damage. These findings were consistent with the conclusion reached by Jia that nitrite exposure causes liver tissue damage [21].

COR, as an important stress hormone, is involved in the physiological processes of growth, energy metabolism, and immune response of the body [11]. The plasma COR levels are increased when the organism is stimulated. Gao et al. [34] have shown that serum COR contents of *Takifugu rubripes* are significantly elevated after 96 h of exposure to nitrite. The effects of stress-induced elevations of plasma COR on non-specific defense mechanisms in fish have been demonstrated [35]. In the present study, we found a marked increase in serum COR concentrations with nitrite exposure, indicating a nitrite-induced activation of the hypothalamic–pituitary–adrenal axis [11]. One thing worth noting in this article is that COR levels were significantly lower in the control group at 96 h compared to 0 h. This was probably due to the reduction in the number of fish in the tank due to sampling and was related to the replacement of seawater. The water-environment conditions of the control group were more obvious.

The non-specific immune system of fish is the first line of defense against pathogens and is used to maintain the homeostasis of the organism. The LZM, an immune factor, reflects cellular immune function and the organism's ability to fight infection [36]. Studies have shown that nitrite exposure leads to reduced LZM activities in turbot [21]. Similarly, LZM levels were significantly decreased following exposure for 48, 60, and 72 h in the N-20 group. IgM is a primary constituent of the humoral immune system in fish [37]. Several studies have shown that environmental pollutants, such as nitrite, heavy metals,

carbon dioxide, and ammonia, can inhibit IgM [38,39]. Similarly, our data revealed that the serum IgM levels of fish were sharply decreased after exposed to N-20 for 60 and 72 h. The findings indicated that nitrite provoked immunotoxicity and the suppression of humoral immunity in grouper.

Oxidative stress is mainly caused by ROS and reactive nitrogen. SOD represents the first line of defense of the body against oxidative stress in the antioxidant response of fish. The superoxide anion O_2^- is disproportionated by SOD to H_2O_2 [40]. SOD and GSH activities in serum were remarkably declined in the N-10 and N-20 groups after 48 and 72 h of exposure compared with CK, thus indicating that the enzymes were severely impaired in the high-concentration group, resulting in a reduced ability of the organism to scavenge superoxide anions and causing oxidative damage to cells. Gao [34] has also suggested that higher nitrite exposure sufficiently and effectively prevents the physiological antioxidant system from eliminating excess ROS, and severe oxidative damage may occur. MDA is the end product of lipid peroxidation, and it is often used to assess nitrite-induced oxidative injury in the liver of aquatic species [41]. Findings indicated that exposure to higher concentrations of nitrite led to a marked elevation of serum MDA levels.

Gills serve as the respiratory organs of aqueous organisms and are used for acid–base balance, filtration, and excretion of nitrogen compounds [42]. Gills are in contact directly with the water body. Therefore, they are vulnerable to damage by toxic substances, including nitrite [43]. Frances et al. [44] observed that silver perch exposed to nitrite over 25 days show significant changes in gill histological morphology, such as epithelial hypertrophy and capillary dilation. Benli et al. [45] found that Nile tilapia show chlorosis and capillary congestion after exposure to ammonia. Furthermore, Shimura et al. [46] reported that the gills of medaka fish exposed to nitrate exhibit epithelial necrosis, a reduced number of mucous cells, and disorganized gill tissues. The research showed that changes in gill structure, including the curling of gill filaments, hypertrophy of epithelial cells, and an increase in mucous cells, occurred when the nitrite concentrations were increased. SEM can detect large surface areas of gills and can process a large number of gill samples. In the present study, mucous cells were increased after nitrite exposure. These cells favor protecting the gill surface from mechanical damage [47]. The secondary lamella tissue was disturbed as the concentration of nitrite was increased.

FAAs, which have a sweet, sour, or bitter character, are considered to be an important taste property in aquatic products. A total of 17 FAAs were detected in the grouper. The flavored amino acids in fish are mainly composed of umami amino acids of glutamate (Glu) and aspartic acid (Asp); sweet amino acids of threonine (Thr), glycine (Gly), serine (Ser), and alanine (Ala); and bitter amino acids of valine (Val) and leucine (Leu) [48]. The contents of total FAAs were increased in the N-10 and N-20 groups after 72 h of exposure. This may be caused by an increase in proteolytic metabolism [49]. Reddy et al. [50] obtained the same result, as they observed that the levels of FAAs in the fish are significantly increased when freshwater fish are exposed to higher concentrations of zinc sulfate.

From the above experimental results, we concluded the possible mechanisms of oxidative stress, blood biochemistry, and immunity of pearl gentian grouper by nitrite exposure, as shown in Figure 8.

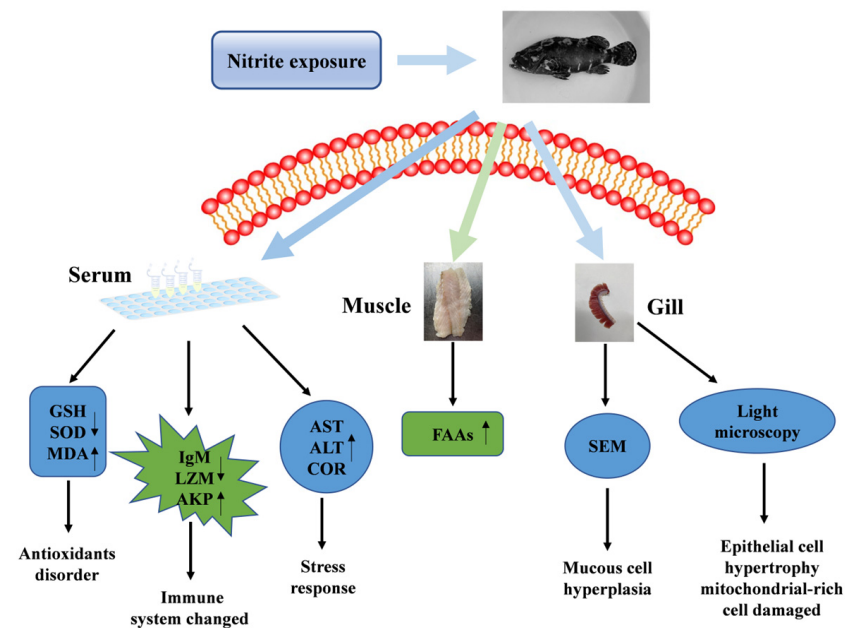


Figure 8. Possible mechanisms of nitrite-induced toxicity of the pearl gentian grouper. ↑ indicates elevated levels of indicators; ↓ indicates decreased levels of indicators.

5. Conclusions

Conclusively, the present findings indicated that nitrite exposure increased AST, ALT, COR, AKP, and MDA activities; decreased IgM, LZM, GSH, and SOD levels; and enhanced FAAs levels. Additionally, nitrite exposure led to the proliferation and hypertrophy of epithelial cells in gill lamellae and an increase in the mucous cells. Collectively, exposure to higher concentrations of nitrite had a toxic effect on the physiological changes in pearl gentian grouper. However, further long-term studies would be necessary to better understand the impacts of nitrite on fish.

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