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Hematological constituents and ultrastructural changes in dark-banded rockfish, *Sebastes inermis*, under nitrite stress

In-Seok Park†, In Bon Goo, Young Ju Kim, Jae Wook Choi and Ji Su Oh

Division of Marine Environment and Bioscience, Korea Maritime University, 727 Teajong-Ro, Yeongdo-Gu, Busan 606-791, Korea

The acute toxicity and sublethal effects of nitrite on the dark-banded rockfish, *Sebastes inermis* (mean body weight: 83.3 ± 7.2 g), were studied under static conditions for a period of 96 h. The acute toxicity of nitrite was at the 50% lethal concentration (LC_{50}) of 700 mg/L. The sublethal effects on selected hematological parameters of the dark-banded rockfish, such as its osmolality, hematocrit, cortisol, alanine aminotransferase (ALT), and aspartate aminotransferase (AST), were measured after 0, 6, 12, 24, 48, 72, and 96 h of exposure to 0, 50, 100, 200, 400, or 700 mg/L nitrite. Sublethal nitrite caused a progressive reduction in the hematocrit of the fish, depending on the nitrite concentration and the exposure period. Exposure to 100–700 mg/L nitrite for 96 h caused a reduction in the hematocrit and an increase in cortisol, ALT, and AST compared with the control levels. Abnormal ultrastructural changes in the gills and liver tissues were observed in fish exposed to 700 mg/L nitrite for up to 96 h compared with the control tissues. Ultrastructural changes included atrophic gill mitochondria and hepatocytes that developed smooth endoplasmic reticulum and atrophic mitochondria. Although no rockfish mortality occurred at 500 mg/L nitrite, all the hematological parameters examined responded adversely to a nitrite dose of 200 mg/L for 96 h. These results show that although the acute toxic concentration of nitrite for the dark-banded rockfish is > 700 mg/L, sublethal concentrations of nitrite also negatively affect its hematological parameters.

Key words : Dark-banded rockfish, Hematological constituent, Nitrite, *Sebastes inermis*, Ultrastructural change

The intensification of aquaculture and higher stocking densities of the cultured species produce proportional increases in nitrogenous wastes. Together with ammonia, nitrite is the most common pollutant in culture systems. Nitrite is a toxic metabolite in water and is an intermediate in the bacterial oxidation of ammonia. Without the proper management of nitrification with biofilters, nitrite can easily accumulate in culture systems, and surplus nitrite exerts considerable stress on the fish, resulting in growth suppression, tissue damage, and mortality (Lewis and Morris, 1986). Nitrite absorbed through the gill and intestinal epithelium accumulates in the plasma, gill, liver, brain, spleen, muscle, and other tissues, like the bioaccumulation of a pollutant, with consequent effects on the fish tissues. The physiological responses of fish have been well documented in previous studies (Jensen, 2003). Although many studies of the uptake and toxicity of nitrite have been reported for both freshwater (Hilmy *et al*., 1987; Knudsen and Jensen, 1997; Huertas *et al*., 2002) and marine fish (Almendras, 1987; Grosell and

†Corresponding author: In-Seok Park
TEL : +82-51-410-4321, FAX : +82-51-405-4322
E-mail : ispark@hhu.ac.kr

Jensen, 1999), few data are available on nitrite toxicity at acute and sublethal concentrations on the dark-banded rockfish, *Sebastes inermis*.

The dark-banded rockfish (order Scorpaeniformes, family Scorpaenidae) is an ovoviviparous species (Choi *et al*., 2002), and is found from Hokkaido in Japan through the East Sea to Jeju Island in Korea, generally along rocky seashores (Choi *et al*., 2002). It is commercially important in Korea as one of the major food fish, and is considered to be a good candidate for mariculture because of its market potential, its suitability for production in a sea farming business, and its high tolerance of varying environmental conditions (Choi *et al*., 2005; Park *et al*., 2007).

Therefore, we studied the effects of acute nitrite toxicity at the median lethal concentration on the histopathology of gill and liver tissues, and the hematological responses at sublethal nitrite concentrations.

Materials and Methods

Young dark-banded rockfish, *Sebastes inermis*, were obtained from the Gyeongsangnam-Do Fisheries Resources and Research Institute (Tongyeong, Korea) and moved to the laboratory. The 700 fish were allocated to five 1100 L circular tanks in a recirculating culture system at the Fishery Genetics and Breeding Science Laboratory, Korea Maritime University (Busan, Korea), and cultured for one month in sand-filtered and aerated seawater (salinity, 34 ppt; temperature, 20.5 ± 0.5 °C; pH 7.9 ± 0.7; dissolved oxygen, 8.8 ± 0.5 mg/L; and ammonia, 0.01 mg/L). The fish were fed to satiation twice daily with a commercial diet composed of 45% protein, 8.5% lipid, 2% fiber, and 9% ash.

The fish were starved for 1 day before the 96 h acute toxicity test. Nitrite (sodium nitrite; Sigma, St Louis, MO, USA) was added to the tank to the appropriate nitrite concentrations, and a nitrite-free treatment was used as the control. Three replicate samples were examined for each test concentration and the control. Based on the calculated 96 h 50% lethal concentration (LC_{50} = 700 mg/L, Park *et al*., 2007), the effects of nitrite on selected hematological parameters of fish exposed to sublethal concentrations (700 mg/L) were examined: osmolality, hematocrit, cortisol, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). The test concentrations were 0 (control), 50, 100, 200, 400, and 700 mg/L nitrite. Ten individuals were placed in each test tank. Three replicates were used for each test concentration and the control. After 0, 6, 12, 24, 48, 72, or 96 h of exposure to nitrite, blood samples were taken from one fish in each replicate tank. These samples were collected from the caudal vein using a rinsed disposable syringe and mixed with 1.5 mL of anticoagulant (20 IU/mL heparin sodium; Choongwae Pharma, Seoul, Korea). The blood plasma was collected by centrifugation at 5,600g for 5 min and stored in a deep freeze (CLN-500 UW Nihon Freezer, Nihon Co., Tokyo, Japan) at −70 °C until analysis.

The hematocrit was measured after hematocrit centrifugation (IEC/MB Micro Hematocrit Centrifuge, DAMON/IEC Division, MA, USA) for 10 min. The cortisol content was measured according to method of Donaldson (1981), with the induction of an antigen−antibody reaction (Coat-A-Count TKCO Cortisol RIA Kit, DPC, USA), then measured with a radioimmunoassay

using the 1470 Wallac Wizard Automatic Gamma Counter (Cobra, Packard Co., USA). Osmolarity was measured based on the freezing point of the tissue Na content, with a Micro Osmometer (Fiske 210, Fiske, USA). AST and ALT were analyzed with the DTSC II Chemistry System (VITROS DT60 II and VITROS DTE II Johnson and Johnson Clinical Diagnostics Inc., NY, USA).

Ultrastructural changes in the gill and liver tissues of fish exposed to 700 mg/L nitrite and of the nitrite-free control were observed. For electron microscopy, the tissues were prefixed for 2 h at 4 °C in 2.5% glutaraldehyde solution buffered with 0.1 M phosphate buffer solution (phosphate-buffered saline [PBS], pH 7.2). After the samples were washed with PBS for 10 min, they were postfixed in 1% osmium tetroxide for 2 h at 4 °C. The samples were rewashed with PBS, then serially dehydrated in a series of ethanol from 50% to 100%, and embedded in Epon 812. Sections (80 nm thick) were cut with an ultramicrotome (LKB, Nova, Bromma, Sweden) and then stained with toluidine blue to determine the regions of interest. The sections were doubly stained with uranyl acetate and lead citrate solutions, and examined with a transmission electron microscope (60–80 kV; JEM 1200 E-X II, JEOL, Tokyo, Japan).

Statistical analyses of the results obtained after 0, 6, 12, 24, 48, 72, and 96 h of exposure were performed with SAS for Windows, V. 6.12 (SAS Institute, Cary, NC, USA), with a significance level of $P < 0.05$. Duncan's multiple-range test was used to test for significant differences between the exposure periods.

Results

Dose-dependent mortality occurred in the dark-banded rockfish, *Sebastes inermis*, exposed to 500 mg/L nitrite. Almost all fish survived exposure for 96 h to 0 or 500 mg/L nitrite, whereas only 75% and 25% of the fish survived exposure for 96 h to 600 and 800 mg/L nitrite, respectively.

The hematocrit decreased progressively with increases in both the nitrite concentration and the exposure period (Fig. 1). A greater reduction in the hematocrit was observed at all nitrite concentrations within the first 24 h compared with that in the subsequent part of the exposure period. The maximum reduction in the hematocrit was recorded in fish exposed to 700 mg/L nitrite for 96 h.

Dose- and time-dependent increases in osmolality were observed after 72 h of exposure to 700 mg/L nitrite

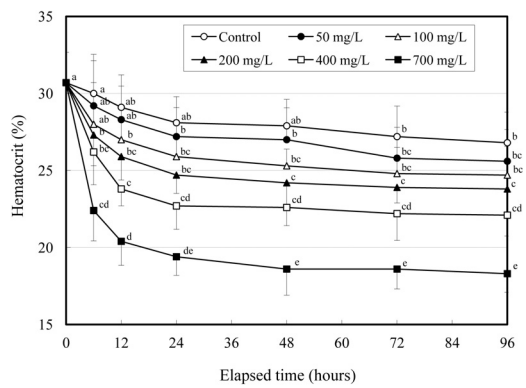

Fig. 1. Changes in hematocrit (means ± SE, $n = 3$) in fingerlings of the dark-banded rockfish, *Sebastes inermis*, during exposure for 96 h to sublethal nitrite concentrations. Means with the same letters at each nitrite concentration are not significantly different ($P < 0.05$).

Table 1. Changes in osmolality (mOsm/kg) in fingerling of the dark-banded rockfish, *Sebastes inermis*, during exposure for 96 h to sublethal nitrite concentrations*

Elapsed time (h)	Control	Nitrite concentration (mg/L)				
		50	100	200	400	700
0	394.2±18.24	394.2±18.24	394.2±18.24	394.2±18.24	394.2±18.24^{b}	394.2±18.24^{b}
6	392.8±29.83	392.8±26.73	395.7±30.13	394.0±17.78	397.2±21.13^{b}	396.3±19.81^{b}
12	394.9±31.79	393.4±27.72	395.5±23.43	397.3±29.12	395.9±19.81^{b}	397.7±26.61^{b}
24	393.0±24.67	396.1±26.41	394.9±31.42	398.0±19.99	401.3±39.08^{b}	402.0±29.72^{b}
48	391.8±25.41	395.9±19.40	399.6±21.37	402.8±30.03	407.2±17.41^{b}	409.8±26.72^{b}
72	392.4±28.60	395.8±30.47	401.0±29.33	397.8±30.42	412.9±31.79^{b}	418.7±29.14^{a}
96	390.6±22.41	398.2±27.72	405.1±30.06	409.8±29.17	420.1±40.10^{a}	425.5±35.79^{a}

* Means ± SE (n = 3) with the same letters at each nitrite concentration are not significantly different (P < 0.05).

(Table 1), with significant increases in fish exposed to 400 or 700 mg/L nitrite for 72–96 h (P < 0.05). The cortisol content increased continuously with increasing nitrite concentrations and exposure periods (Fig. 2). Compared with the control values, a significant increase in cortisol was observed after exposure for 12 h to 50–700 mg/L nitrite and after exposure for 48 h to all nitrite concentrations (P < 0.05). The maximum increase in cortisol was observed in fish exposed to 700 mg/L nitrite for 96 h.

As shown in Fig. 3, dose-dependent increases in ALT were observed with increasing exposure times at all nitrite concentrations, with significant increases in fish exposed to 50–700 mg/L nitrite for 96 h (P < 0.05). Similarly, dose-dependent increases in AST were observed with increasing exposure times (Fig. 4).

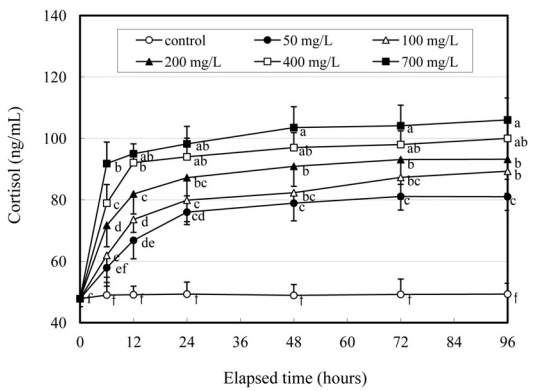

Fig. 2. Changes in cortisol (means ± SE, n = 3) in fingerlings of the dark-banded rockfish, *Sebastes inermis*, during exposure for 96 h to sublethal nitrite concentrations. Means with the same letters at each nitrite concentration are not significantly different (P < 0.05).

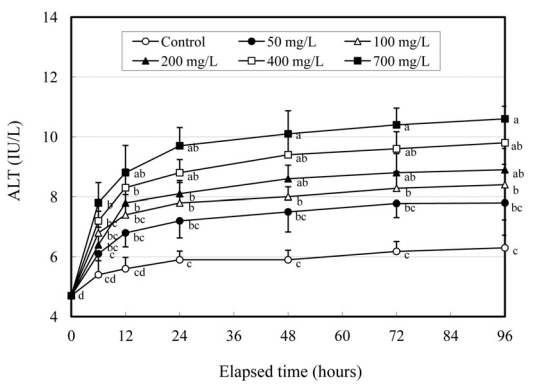

Fig. 3. Changes in alanine aminotransferase (ALT, means ± SE, n = 3) fingerlings of the dark-banded rockfish, *Sebastes inermis*, during exposure for 96 h to sublethal nitrite concentrations. Means with the same letters at each nitrite concentration are not significantly different (P < 0.05).

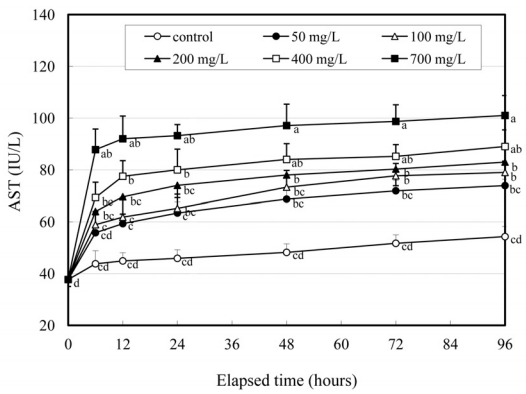

Fig. 4. Changes in aspartate aminotransferase (AST, means ± SE, n = 3) in fingerlings of the dark-banded rockfish, *Sebastes inermis*, during exposure for 96 h to sublethal nitrite concentrations. Means with the same letters at each nitrite concentration are not significantly different ($P < 0.05$).

Significant increases were observed in fish exposed to 50–700 mg/L nitrite for 96 h ($P < 0.05$). The maximum increase was recorded in fish exposed to 700 mg/L nitrite.

Electron microscopic analysis showed abnormal gross pathologies in the gill and liver tissues of fish exposed to 700 mg/L nitrite compared with the tissues of the control fish (Figs. 5 and 6). Transmission electron microscopic analysis of the gills after exposure to 700 mg/L nitrite for 96 h showed ultrastructural changes in the gill mitochondria, which were heterogeneously atrophic (Fig. 5). Severe cytological destruction of the hepatocytes was observed in fish exposed to 700 mg/L nitrite for 96 h (Fig. 6). The cells in the livers of these fish had developed smooth endoplasmic reticulum (SER) and their mitochondria were atrophic (Fig. 6). In contrast, those of the control fish were of uniform size and appearance.

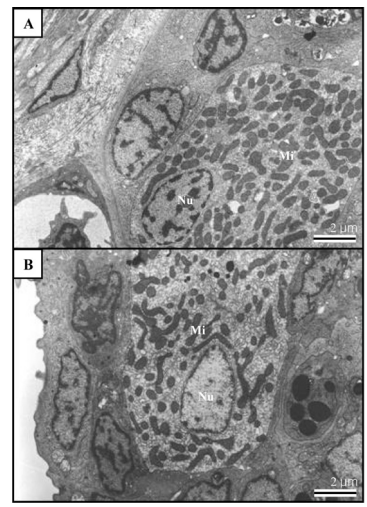

Fig. 5. Transmission electron micrographs of the chloride cell in gill tissue of the dark-banded rockfish, *Sebastes inermis*, during exposure for 96 h to 700 mg/L nitrite. (A) control, and (B) exposed fish. Nu: nucleus; Mi: mitochondria.

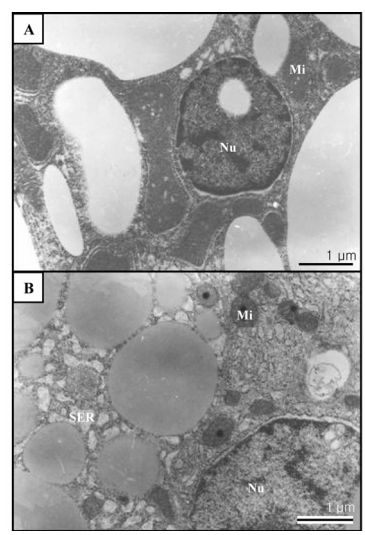

Fig. 6. Transmission electron micrograph of the hepatocyte in liver tissue of the dark-banded rockfish, *Sebastes inermis*, during exposed for 96 h to 700 mg/L nitrite. (A) control, and (B) exposed fish. Nu: nucleus; Mi: mitochondria; SER: smooth endoplasmic reticulum.

Discussion

The toxicity and effects of nitrite vary among fish species and also depend on the test conditions, including the fish size, water ion composition, and temperature (Perrone and Meade, 1977; Williams and Eddy, 1986; Alcaraz *et al*., 1997; Doblander and Lackner, 1997). Nitrite is actively taken up across the gills and/or intestinal epithelium in competition with chloride (Tomasso, 1994). Chloride and other anions in the water exert a protective effect against nitrite during activebranchial uptake, so small amounts of Cl^- are likely to afford some protection against high nitrite inputs (Eddy *et al*., 1983; Williams and Eddy, 1986). Consequently, the effects of nitrite on fish are more intense in Cl^--poor freshwater, so nitrite is more toxic in freshwater than in seawater (Grosell and Jensen, 1999; Jensen, 2003). Therefore, the observed 96 h LC_{50} (700 mg/L) for the dark-banded rockfish, *Sebastes inermis*, is very high compared with those reported for freshwater fish species: for example, 10.6 mg/L for the grass carp, *Ctenopharyngodon idella* 32 mg/L for the Nigerian catfish, *Clarias lazera* and 130 mg/L (72 h LC_{50}) for the Siberian sturgeon, *Acipenser baeri* (Hilmy *et al*., 1987; Alcaraz and Espina, 1997; Huertas *et al*., 2002; Park *et al*., 2007).

Plasma nitrite accumulation probably causes methemoglobinemia, which are detectable effects of nitrite intoxication (Lewis and Morris, 1986; Martinez and Souza, 2002; Jensen, 2003; Costa *et al*., 2004). Although methemoglobinemia is not directly related to high mortality in fish exposed to nitrite, the passage of nitrite into the blood stream may cause an increase in blood cell lysis (Jensen, 1990; Knudsen and Jensen, 1997; Costa *et al*., 2004), changes in the plasma electrolyte balance, and the efflux of K^+ from red blood cells, which is evident as an increase in the number of shrunken red blood cells (Jensen, 1992; Huertas *et al*., 2002; Martinez and Souza, 2002).

In this study, hematocrit were decreased dependant of dose and time, it supported preceding study. The dysfunctional erythrocytes may be removed from the blood circulation causing an oxygen shortage and thus a reduction in the hematocrit. A much greater reduction in the hematocrit occurred during the initial 24 h of the exposure period than in the subsequent part at all nitrite concentrations, suggesting an initial high rate of nitrite accumulation. Eddy *et al*. (1983) also reported a higher rate of nitrite uptake during the initial 24 h of nitrite exposure than later. A greater reduction in hematocrit at higher nitrite concentrations may indicate the possible exhaustion of the hemopoietic potential of the fish under hypoxic conditions (Reddy *et al*., 1992), perhaps also resulting in tissue hypoxia (Eddy and Williams, 1987; Jensen, 1990; Tomasso, 1994).

Tissue-level hypoxia and a change in the respiratory metabolism from aerobiosis to anaerobiosis require a greater energy supply to compensate for the increased respiratory metabolism. This may also indicate a dysfunction of the cell metabolism. In ourexperiment, a significant increase in cortisol in the fish during all nitrite treatments occurred after 12 h of exposure. The phenomenon of a dose-dependent reduction in glucose during exposure to nitrite has been reported in another study (Das *et al*., 2004; Park *et al*., 2007). Cortisol and glucose were found to increase in a stress-dependent

manner. Barton and Iwama (1991) reported that these increases are the result of metabolic responses and that glucose is produced as a result of gluconeogenesis in response to the action of the cortisol produced under stress. The steady reduction in the hematocrit and the dose-dependent increase in cortisol in the dark-banded rockfish exposed to 200–700 mg/L nitrite may indicate a failure of hemopoietic activity (Reddy *et al*., 1992; Das *et al*., 2004; Park *et al*., 2007).

The gill and liver tissues of the dark-banded rockfish exposed to 700 mg/L nitrite for 96 h showed severe cytological damage. Severe cytological destruction of the hepatocytes was observed in fish exposed to nitrite for 96 h. The hepatocyte mitochondria ofthe fish exposed to 700 mg/L nitrite were atrophic, in contrast to those of the control fish, which were uniform in size and appearance. Especially, smooth endoplasmic reticulum (SER) was increased in hepatocyte. This may indicate a detoxication of the cell metabolism. SER is organelle what activity to cellular detoxification (Rim *et al*., 1978). Fish gills are sensitive respiratory organs, and are the first point of contact between waterborne nitrite and the fish. In the present study, the exposure of the dark-banded rockfish to nitrite resulted in notable structural changes in the gill lamellae, including the accumulation of hemocytes in the hemocoelic space, swelling and fusion of the lamellae, and lifting of the lamellar epithelium, which have also been noted in the gill lamellae of other fish after exposure to insecticides (Park *et al*., 2007). As a matter of fact, most of the gill histopathological changes were largely nonspecific, as has been confirmed by the occurrence of similar changes under a wide range of toxicant-exposure conditions (Mallat, 1985). Other ultrastructural studies of fish gills and hepatocytes have shown similar results to ours. In contrast, the hepatocytes of fish exposed to cadmium (0.29 mg/L) show swelling and vesiculation of the mitochondria (Hugla and Thomé, 1999).

We studied the toxicity of nitrite for the dark-banded rock fish in terms of the physiological and ultrastructural changes it induces. Adverse effects of nitrite were observed on all the hematological parameters examined in fish exposed to 200 mg/L nitrite. We found that ultrastructural changes were useful parameters for the histopathological analysis of these fish. Further studies are required to classify the ultrastructural changes that occur under nitrite stress in more detail.

Acknowledgments

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