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Ammonia toxicity induces glutamine accumulation, oxidative stress and immunosuppression in juvenile yellow catfish *Pelteobagrus fulvidraco*

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ABSTRACT

A study was carried to test the response of yellow catfish for 28 days under two ammonia concentrations. Weight gain of fish exposure to high and low ammonia abruptly increased at day 3. There were no significant changes in fish physiological indexes and immune responses at different times during 28-day exposure to low ammonia. Fish physiological indexes and immune responses in the treatment of high ammonia were lower than those of fish in the treatment of low ammonia. When fish were exposed to high ammonia, the ammonia concentration in the brain increased by 19-fold on day 1. By comparison, liver ammonia concentration reached its highest level much earlier at hour 12. In spite of a significant increase in brain and liver glutamine concentration, there was no significant change in glutamate level throughout the 28-day period. The total superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GR) activities in the brain gradually decreased from hour 0 to day 28. Liver SOD, GPX and GR activities reached the highest levels at hour 12, and then gradually decreased. Thiobarbituric acid reactive substance brain and liver content gradually increased throughout the 28-day period. Lysozyme, acid phosphatase and alkaline phosphatase activities in the liver reached exceptionally low levels after day 14. This study indicated that glutamine accumulation in the brain was not the major cause of ammonia poisoning, the toxic reactive oxygen species is not fully counter acted by the antioxidant enzymes and immunosuppression is a process of gradual accumulation of immunosuppressive factors.

1. Introduction

Environmental ammonia is toxic to fish (Kim et al., 2015). Ammonia exposure typically results in reduction of fish growth (Sun et al., 2012), and causes gill hyperplasia (Benli et al., 2008), liver tissue degeneration, immune suppression (Cheng et al., 2015), hyper-excitability, coma, convulsions and fish mortality (Roumieh et al., 2013). A previous study surmised that the symptoms of ammonia poisoning in fish exposed to acute or chronic ammonia might be similar to the action of ammonia in mammals during hepatic encephalopathy (HE, Smart, 1978). In mammals, it is thought that brain glutamine accumulation can lead to astrocyte swelling under ammonia toxicity (Brusilow, 2002; Marquez et al., 2013). However, recent studies have confirmed that some fishes, such as mudskipper, can tolerate high levels of brain ammonia and glutamine (Chew et al., 2005; Ip et al., 2005). Thus, it is possible that the defense mechanism against ammonia toxicity in fishes is different from that in mammals, but there is a lack of knowledge regarding the effects of ammonia on fish.

Ammonia can lead to oxidative stress in cultured rat astrocytes *in vitro* (Murthy et al., 2001). The primary symptom involves the over-activation of N-methyl-d-aspartate (NMDA) type glutamate receptors (Hermenegildo et al., 2000), which leads to an overproduction of nitric oxide (NO), reactive oxygen species (ROS) and reactive nitrogen species (RNS, Murthy et al., 2001), resulting in per-oxidation which damages the normal function of cells and tissues, and finally reduces animal immunity and disease resistance (March, 1992). Sun et al. (2012) found that ammonia significantly restrained the antioxidant system in bighead carp. Chen et al. (2011) reported that the immune response of tilapia was restrained by ammonia exposure. To date, ammonia poisoning in fish has been shown follow multifactorial pathogenesis (Ip et al., 2005; Hegazi et al., 2010; Li et al., 2014). To the author's best knowledge, the link between glutamine accumulation, oxidative stress and immunosuppression remains undefined.

This study investigated the effects of ammonia toxicity on glutamine accumulation, antioxidant enzyme activities and immune responses in the brain and liver of yellow catfish *Pelteobagrus fulvidraco*. The aim of this study was to determine the link between glutamine accumulation, oxidative stress and immunosuppression, and the pathogenesis of ammonia poisoning in fish.

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2. Materials and methods

2.1. Animals, experimental conditions and sampling intervals

Juvenile yellow catfish (3.00 ± 0.08 g, mean ± S.D.) were captured from a fish farm in Jixing (Zhejiang, China), and transferred to Ningbo University. After the fish were acclimated with a commercial feed for 30 days, they were randomly stocked into six 500-L plastic tanks at a density of 40 fish per Tank. No attempt was made to separate the sexes.

Based on the report of the sublethal concentrations (5.71 mg L^{-1} TA-N) of ammonia in channel catfish (Colt and Tchobanoglous, 1978), the concentrations of total ammonia nitrogen (TA-N) were chosen. The experiment consisted of a low ammonia treatment [total ammonia nitrogen (TA-N) 0.01 mg L^{-1}, un-ionized ammonia (UIA-N) <0.001 mg L^{-1}] and a high ammonia treatment (TA-N 5.70 mg L^{-1}, UIA-N 0.12 mg L^{-1}). Fish were exposed to experimental water for 28 days. The desired ammonia concentrations were achieved by adding a solution of NH_4Cl (10 g L^{-1}) every 7 h. The TA-N levels were measured by nesslerization (Hegazi et al., 2010). Percentage of UIA-N was calculated using the equation of Johansson and Wedborg (1980). This equation gave the percentage of UIA-N as a function of pH and temperature. Corrections for pH measurements in low-ionic strength buffers (*i.e.* conversion to the Hansson scale) were performed according to Whitfield (1974). All tanks were supplied with dechlorinated tap water with a daily exchange rate of 1/3 tank volume. Water was continuously aerated using air stones. Water temperature and dissolved oxygen were measured daily with a HACH HQ30d oxygen meter (Hach Company, Loveland, USA). During the trial, water quality was maintained at 27–29 °C, dissolved oxygen 7.81 ± 0.13 mg L^{-1} and nitrite <0.5 mg L^{-1} (Griess reaction, Siikavuopio and Sæther, 2006). Photoperiod was maintained at 12 h light and 12 h dark.

Experimental fish were sampled on 0, 6, 12 and 24 h, and 3, 7, 14 and 28 days. For each exposure time, four fish from each tank were randomly collected, and then were anesthetized with tricaine methanesulfonate (MS-222) at 120 mg/L before weighing. Brain and liver were quickly removed and freeze-clamped with liquid nitrogen-precooled aluminum tongs. Samples were stored at −80 °C until analyses, which were performed within a month. After being executed, the head kidneys of four fish were removed for macrophage separation.

2.2. Ammonia, glutamine and glutamate content assays

Based on the report of Ip et al. (2005), the frozen brain and liver were weighed, ground to a powder in liquid nitrogen and homogenized three times in 6% trichloroacetic acid at 24,000g for 20 s each. The homogenate was centrifuged at 10,000g at 4 °C for 15 min. Ammonia analysis was determined following the methods of Bergmeyer and Beutler (1985). Freshly prepared NH_4Cl solution was used as the standard for comparison. Glutamine and glutamate were analyzed with a Shumadzu LC-6A amino acid analysis system (Kyoto, Japan), equipped with a Shim-pack ISC-07/S1504 Litype column, according to the methods of Ip et al. (2005). Results were expressed as μmol g^{-1} wet mass tissue.

2.3. Antioxidant enzyme activity and lipid peroxidation assays

The frozen brain and liver were weighed and homogenized in ice-cold phosphate buffer (50 mM, pH 7.4). The homogenate was centrifuged at 2000g in a cooling centrifuge at 4 °C for 15 min and the supernatant was saved. Total superoxide dismutase (SOD) activity was determined following the methods of Beauchamp and Fridovich (1971). One unit of SOD activity was calculated using the amount of superoxide dismutase required to inhibit the reduction of nitrobluete trazolium by 50%. Catalase (CAT) activity was determined by measuring the decrease in H_2O_2 concentration (Aebi, 1984). One unit of CAT activity was defined as the amount of CAT required to transform 1 μmol of H_2O_2 per min. Glutathione peroxidase (GPX) activity was measured following the methods of Flohé and Günzler (1984). One unit of GPX activity was defined as the amount of GPX required to oxidize 1 μmol of NADPH per min. Glutathione reductase (GR) activity was measured following the methods of Ching et al. (2009). Activities were expressed in nmol of NADPH oxidized per min per mg protein. The terminal product formed in the decomposition of polyunsaturated fatty acids mediated by free radicals was quantified as thiobarbituric acid reactive substances (TBARS) according to the methods of Buege and Aust (1978). All assays were determined with commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's instructions.

2.4. Lysozyme, alkaline phosphatase, acid phosphatase activity and respiratory burst assays

The enzyme activity levels were measured with commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Liver lysozyme (LYZ) activity was determined following the methods of Hultmark et al. (1980). The assay was based on the lysis of a lysozyme-sensitive Gram-positive bacterium *via* the lysozyme present in the homogenate. Liver alkaline phosphatase (AKP) was determined according to Engstad et al. (1992), and the acid phosphatase (ACP) according to Grove et al. (2006). The respiratory burst of macrophage cell was measured by the nitroblue tetrazolium reduction assay following the method of Secombes (1990).

2.5. Statistical analyses

Assayed parameter values were presented as means ± standard errors of the mean (S.E.M.). Data were used to compare the effects of the ammonia by a t-test. Data were used to compare the effects of the stress time by one-way ANOVA. If there was a significant *F*-test, subsequent comparisons of treatment means were performed using the Duncan's Multiple Range test. The significance level was set at $P < 0.05$. All analyses were performed using SPSS 18.0.0 (Chicago, USA) for Windows.

3. Results

As anticipated, no mortality were observed in fish exposed to high (5.70 mg L^{-1} TA-N) or low (0.01 mg L^{-1} TA-N) concentration of ammonia during the 28-day period ($P > 0.05$, Table 1). Fish body weight and weight gain abruptly increased at day 3, and then gradually increasing ($P < 0.05$), but no significant differences were found between high and low ammonia treatment groups ($P > 0.05$).

There were no significant changes in ammonia, glutamine and glutamate of brain and liver contents at different times during 28-day exposure to low concentration of ammonia ($P > 0.05$, Table 2). Fish exposed to high concentration of ammonia showed brain ammonia concentration increased significantly by 19-fold on day 1 (7.36 μmol g^{-1} tissue; $P < 0.05$), but was not significantly different from day 1 to day 28 ($P > 0.05$). By comparison, liver ammonia concentration reached its highest level (15.48 μmol g^{-1} tissue) much earlier than brain, increasing by 36-fold at hour 12, and then gradually decreasing ($P < 0.05$). At day 7, the fish brain glutamine concentration reached an exceptionally high level of 28.48 μmol g^{-1} tissue ($P < 0.05$). However, the brain glutamine concentration was lower than that of the liver at 12 h ($P < 0.05$). In spite of a significant increase in glutamine content in the brain and liver of fish ($P < 0.05$), there was no significant change in the brain glutamate level throughout the 28-day period ($P > 0.05$). During the 28-day period, brain ammonia and glutamine concentrations of fish in the treatment of high ammonia were higher than those of fish in the treatment of low ammonia ($P < 0.05$), there was no significant different in the brain glutamate level between high and low ammonia treatment groups ($P > 0.05$).

No significant differences were found in brain and liver antioxidant enzyme activities and lipid peroxidation levels at different times during