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Effect of ammonia-N on histology and expression of immunoglobulin M and component C3 in the spleen and head kidney of *Pelteobagrus vachellii*

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ABSTRACT

Ammonia-N is toxic to many aquatic animals and serves as a key stress factor in aquatic environments. The effects of ammonia-N stress on the immune response of darkbarbel catfish *Pelteobagrus vachellii* were investigated in this study. Changes in overall histology, and in the expression of complement C3 and immunoglobulin M (IgM) in spleen and head kidney, and lysozyme and C3 in serum, were measured in 1 and 5 mg/L ammonia-N. Hyperemia, melano-macrophage assembly and loose splenosis were evident in spleen tissue. Both lysozyme and component C3 were significantly reduced in serum ($P < 0.05$) under both stress treatments, although lysozyme was increased slightly at 24 h in the 1 mg/L treatment. C3 mRNA expression increased at 6 h in spleen and 6–12 h in head kidney then decreased rapidly ($P < 0.05$), although C3 mRNA recovered to control levels in spleen at 96 h after the 1 mg/L treatment ($P > 0.05$). IgM expression also increased significantly at 6–12 h in spleen and 6–24 h in head kidney after the 1 mg/L treatment ($P < 0.05$). A similar overall pattern were observed with 5 mg/L ammonia ($P < 0.05$); IgM mRNA expression was elevated at 6 h in spleen and 6–12 h in head kidney ($P < 0.05$) then decreased to levels below controls ($P < 0.05$). These results suggest exposure to 5 mg/L ammonia-N could damage the histological structure of spleen, diminish lysozyme and component C3 serum content, and suppress C3 and IgM expression in spleen and head kidney.

1. Introduction

Darkbarbel catfish (*Pelteobagrus vachellii*) is a relatively small but rapidly growing freshwater fish species in China, and growing demand has seen a considerable rise in market value (Qin et al., 2013), with production reaching 300,000 tons in 2013 (Qin et al., 2017a). However, close-breeding and intensive-density feeding, along with deteriorating aquatic environments has resulted in an increase in diseases affecting this important aquaculture species that include putrid skin disease and cephalosome disease. The incidence of cephalosome disease has increased by 30% and is accompanied with large economic losses.

Ammonia-N is a toxic substance that can affect fish growth and immunity, and is a key stress factor in the confines of aquaria and aquaculture systems (Kosenko et al., 1997). Ammonia is the main end product of protein catabolism in bony fish (teleosts), and this substance can accumulate to unsafe levels in densely populated aquatic environments (Hegazi et al., 2001). In addition, most biological membranes including gills are permeable to ammonia but relatively impermeable to ammonium ions (Nakada et al., 2007; Nawataet al., 2007). Consequently, chronic exposure to ammonia may reduce growth, cause gill hyperplasia and histopathological changes in gill epithelia, and induce hyperexcitability and ultimately death (Sun et al., 2012; Lease et al., 2003; Benli et al., 2009; Ip et al., 2001). Additionally, ammonia can affect the innate immune system of crustaceans; antibacterial and bacteriolytic activities, α2-macroglobulin were decreased in swimming crab (*Portunus trituberculatus*), and phagocytic and superoxide dismutase activities were decreased in white shrimp (*Litopenaeus vannamei*) (Yue et al., 2010; Liu and Chen, 2004). It believed that the immunosuppressive effects of ammonia contribute to disease outbreaks (Yue et al., 2010; Qin et al., 2017b). Knowledge of the relationship between ammonia stress and the immune response is therefore important for reducing fish diseases in the aquaculture industry, and is currently limited in bony fish.

The spleen functions as a major secondary immune organ that produces large quantities of IgM^+ and mature B cells (Zwollo et al., 2008; Bromage et al., 2004). Moreover, spleen is the main site where antigen processing and defense and against microbes takes place. Therefore, the spleen have an important effect on antigen presentation and initiation of the adaptive immune response (Li et al., 2017). The head kidney is the major site of antibody production, and plays a

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central role in organizing the stress response and coordinating various regulatory systems (Tort, 2011). Moreover, in fish, as the two important immune molecular, IgM was related to acquired immunity, being regarded as the main component of systemic immunity in teleosts fish and played a key role in acquired immunity (Zimmermana et al., 2011); while complement C3 was related to innate immunity, being regarded as the crossing of the three pathways of complement activation and served one of the bridges linking innate and acquired immunity (Hawlisch and Kohl, 2006). Therefore, in this study, the effect of ammonia exposure in *P. vachellii* were investigated.

2. Materials and methods

2.1. Animals and rearing conditions

Before carrying out the ammonia-N exposure treatments, adult *P. vachellii* (16.25 ± 1.36 g) were collected from the lab pond and acclimatized in the laboratory for 2 weeks with aerated water and natural light/dark cycles.

2.2. Experimental design

After acclimatization, fish were distributed between six 300 L plastics tanks supplied with aerated water (pH 7.4; NH_3 < 0.01 mg/L). Temperature and dissolved oxygen were measured daily and maintained at 20 ± 1 °C and 6.7 ± 1.6 mg/L, respectively. Two different ammonia-N concentrations of 1 and 5 mg/L were tested, and three replicates were performed for each treatment group of 40 randomly selected fish. During the experiment, ammonia-N concentrations were measured every 6 h, and the variation was at 1.20 ± 0.18 and 5.37 ± 0.23 for the 1 and 5 mg/L treatment groups, respectively. The ammonia-N concentration was adjusted with 10 g/L NH_4Cl.

2.3. Sample collection

Three fish were chosen at random from each tank and anaesthetized with neutralized MS222 at 0 (control), 6, 12, 24, 48 and 96 h after ammonia-N treatment. Spleen and head kidney tissue was frozen in Trizol reagent and stored at −80 °C for total RNA extraction. Blood was collected from the caudal vein using heparin sodium and incubated at 4 °C for 1 h, centrifuged at 1000*g* for 10 min, and used for serum lysozyme and component C3 determination.

2.4. Histology of spleen and kidney tissue

Small pieces of spleen was removed at 0 and 96 h, washed in phosphate buffer, fixed by immersion in Bouin's solution for 18 h, dehydrated in paraffin, and stained with hematoxylin and eosin (H & E).

2.5. Immune response assay

Serum component C3 was determined by enzymatic immunoassay (ELISA) using a commercial kit (CAT#:E032; Jiancheng Bioengineering Institute, Nanjing, China). Lysozyme was measured by nephelometric assay using a commercial kit (CAT#:A050; Jiancheng Bioengineering Institute). Both kits were used according to manufacturer's instructions.

2.6. Analysis of immune-related gene expression

The expression of IgM and C3 in head kidney and spleen tissue were separately detected by quantitative PCR analysis. Total RNA was extracted using RNAiso buffer (TaKaRa, Dalian, China) following the manufacturer's instructions, and 5 μg of purified RNA was used for reverse transcription. First strand cDNA was synthesized using the PrimeScript RT reagent kit and gDNA (TaKaRa, Dalian, China) following the manufacturer's instructions, and diluted 10-fold. Immune-related gene-specific primers were included in reactions (Table 1). SYBR Green RT-PCR assays were conducted to measure mRNA levels using the FastStart Essential DNA Green Master Kit (Roche, Basel, Switzerland) with the PCR temperature profile and reaction conditions specified by the manufacturer. Reactions were performed on a Light-Cycler Nano Real-Time PCR System (Roche). The comparative C_T method was used to analyze expression levels. ΔC_T was calculated as the difference in C_T between the target gene and internal control subtracted from the calibrator C_T to yield $\Delta\Delta C_T$. Expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 1
Primers used in gene expression analysis.

Gene	Genbank No.	Primer sequence (5 → 3)	Products length (bp)
IGM	JQ730737	TCCCCAAGGTTTACTTGCTCGCTCC CGATGGATCTGGATATGTGGCGCAC	257
C3	GU353333	GCACCACCCAGGCAACCATA CAGCACCAGTTCCCTTAGCAGTA	223
β-actin		CACTGTGCCCATCTACGAG CCATCTCCTGCTCGAAGTC	200

2.7. Statistical analysis

Data are presented as the mean ± standard deviation. Statistical comparisons were performed by one-way analysis of variance (ANOVA) using SPSS 19.0 software and the Duncan Multiple Range test was used to identify significant differences between treatments. The results were subject to analysis of *t*-test between 1 and 5 mg/L treatment groups within the same time points. Data were considered statistically significant at $P < 0.005$.

3. Results

3.1. Effects of ammonia-N on spleen

Fish exposed to different ammonia concentrations displayed histopathological changes in the spleen (Fig. 1). In spleen, hyperemia, melano-macrophage assembly and loose splenosis were observed with 1 mg/L ammonia, and hyperemia was even more obvious in the 5 mg/L group.

3.2. Immune response parameters

In the presence of 1 mg/L ammonia, serum lysozyme was generally decreased at 6, 12, 48 and 96 h, and lysozyme activity was significantly lower than controls at 0 h in 1 mg/L group ($P < 0.05$), as was the case with the 5 mg/L treatment group ($P < 0.05$; Fig. 2). In addition, a significant decrease appeared in the lysozyme activity in 5 mg/L group than that in 1 mg/L group at 24 h ($P < 0.05$).

Ammonia-N exposure had notable effect on complement C3 activity in *P. vachellii*, which also decreased with increasing exposure time in both treatment groups, compared with controls ($P < 0.05$; Fig. 3). The C3 activity of the 1 and 5 mg/L groups reached the lowest levels at 48 h and 96 h after treatment, respectively. ($P < 0.05$).

3.3. Expression profiles of immune-related genes

Table 2 shows the expression of C3 and IgM in the spleen. C3 expression increased by 58% and 53% at 6 h after treatment with 1 mg/L and 5 mg/L ammonia-N, respectively ($P < 0.05$), but expression was decreased compared with controls at 24 h. Similarly, in kidney, C3 mRNA levels were increased at 6–12 h with both treatments ($P < 0.05$), but expression was decreased at 48–96 h and 24–96 h in