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Nitrite-induced hepatotoxicity in Bluntsnout bream (*Megalobrama amblycephala*): The mechanistic insight from transcriptome to physiology analysis

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ABSTRACT

Previous studies have investigated the physiological responses to acute nitrite exposure in fish; however, little information is available for the underlying molecular mechanisms of nitrite toxicity in aquatic ecosystems. In an effort to understand the underlying mechanisms of nitrite tolerance and to illuminate global gene expression patterns modulated by nitrite toxicity, we sampled livers from juvenile *Megalobrama amblycephala* exposed in 0.1, 15 and 30 mg L^{-1} nitrite and performed short read (100 bp) next generation RNA sequencing (RNA-seq). The RNA-seq reads from all the exposures (≈24 million reads) were assembled into unigenes datasets according to an available reference transcriptome. Using reads from each nitrite concentration, we performed RNA-seq based gene expression analysis that identified a total of 357 differentially expressed genes. The differentially expressed genes were related to oxidative stress, apoptotic pathway, oxygen transport, immune responses and the metabolism of proteins and fats. Quantitative real-time polymerase chain reaction using six genes independently verified the RNA-seq results, the present study suggests several new candidate genes commonly regulated in liver of *M. amblycephala*. In addition to liver histology examinations, this study provides important mechanistic insights into nitrite-induced liver toxicity in a whole-animal physiology context, which will help in understanding the syndromes caused by nitrite poisoning.

1. Introduction

Nitrite is an intermediate product of bacterial nitrification of ammonia or denitrification of nitrate (Kroupova et al., 2005). Intensive aquacultural practices, where overcrowding and poor water re-circulation is commonplace, can act to increase water nitrite to concentrations that are toxic to aquatic animals (Jensen, 2003). The toxicity of nitrite has been reported at various levels of biological organization in fish. Nitrite exposure can adversely affect fish growth (Woo and Chiu, 1994), gill osmoregulatory function (Woo and Chiu, 1997), blood oxygen carrying capacity (Woo and Chiu, 1995; Jensen, 2003; Yidiz et al., 2006; Madison and Wang, 2006; Camargo and Alonso, 2006) and ionic homeostasis (Grosell and Jensen, 2000; Martinez and Souza, 2002; Jensen, 2003). Consider the

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fact that liver is the main organ for detoxification, little information has been observed for nitrite-induced alterations of gene expression of liver in farmed fish. Understanding the molecular mechanisms of nitrite toxicity will provide useful information for developing detoxification strategies and technologies.

Nitrite can occur in natural waterways and aquaculture systems due to high loads of organic material, fish densities and temperature (Hargreaves, 1998), making it important to study and understand nitrite tolerances and effects. Nitrites are actively transported into the bloodstream through the gills in some fish by means of chloride cells, previous study have explored the varying sensitivities of different species relative to Cl^- and nitrite uptake (Tomasso and Grosell, 2005). Acute toxicity of nitrite has been investigated in a number of fish species (Grosell and Jensen, 2000; Martinez and Souza, 2002; Huertas et al., 2002; Ferreira da Costa et al., 2004; Das et al., 2004); however, there are no reports for the effects of nitrite on juvenile blunt snout bream (*Megalobrama amblycephala*), which is farmed extensively as a principal species in Chinese freshwater polyculture systems. Aquaculture of this fish has expanded rapidly during the last decade because of fast growth rate, high feed efficiency ratio, tender flesh and high disease resistance (Zhou et al., 2008). Since prevalence of disease in cultured *M. amblycephala* is increasing, especially during summer months, high temperatures and elevated ammonia concentrations seem to trigger disease outbreaks that result in high mortality (He et al., 2006). Therefore, we predicted that *M. amblycephala* is relatively sensitive to nitrite exposure due to weak detoxification ability of liver tissue.

A previous study sequenced and characterized the juvenile *M. amblycephala* different tissues transcriptome using 454 pyrosequencing (Gao et al., 2012). These sequence data are a reference EST resource for large-scale gene expression analyses of *M. amblycephala*. Recently developed RNA sequencing technologies (RNA-seq) have clear advantages over existing approaches and could improve our understanding of the extent and complexity of the eukaryotic transcriptome including non-model organisms (Wang et al., 2010). This type of result could help to boost collaborative, comparative and integrative genomics studies. Therefore, to investigate the mechanisms underlying nitrite toxicity in *M. amblycephala* we measured several parameters: (1) the gene expression profiles of the liver; (2) differential expression of mRNA transcripts by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR); (3) hematological parameters; (4) histological alterations of the liver. This is the first study to document the capacity of nitrite exposure to induce transcriptome-wide gene expression changes in liver of *M. amblycephala* and link these changes to biological processes.

2. Materials and methods

2.1. Experimental animals

We obtained three hundred healthy *M. amblycephala* juvenile that were of a similar size (mean weight: 10.16 ± 1.24 g) from the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, China. The fish were transferred immediately to the aquatic laboratory and held in three 500-L fiberglass tanks ($N = 100$ fish/tank). During acclimation, each tank was supplied with pre-aerated municipal water at $20 \pm 1\,^{\circ}C$ (pH 7.45 ± 0.08; chloride 15–18 mg L^{-1}; dissolved oxygen 5.16–6.53 mg L^{-1}; total ammonia <0.5 mg L^{-1}; baseline nitrite <0.1 mg L^{-1}) with natural light and photoperiod. Fish were fed with a commercial pelleted diet twice daily at a ration of 3% total body weight. The water exchange rate was 33% per day and fecal matter was removed daily from the aerated tanks.

2.2. Nitrite exposure

After fasting for 24 h, fish were selected from the holding tank and transferred to nine plastic tanks (100 cm × 80 cm × 60 cm) of each test solution. Based on the LC50 estimate in our lab (unpublished), a 48-h nitrite exposure was used at three sodium nitrite levels: 0–0.1 (control), 15 and 30 mg L^{-1}, and three replicate tanks at each concentration were performed (20 individuals per tank). The ambient nitrite concentration of each exposure group was adjusted to the required value by adding stock solution of sodium nitrite. After 48-h exposure to nitrite, three fish were taken randomly from each tank were anesthetized with MS-222 (Sigma, St. Louis, MO, USA) at the concentration of 150 mg L^{-1}, and blood were taken from the caudal vein as a pooled sample. The total blood hemoglobin concentration (Hb) was measured by the cyanomethaemoglobin method. Methaemoglobin (MetHb) was measured in whole blood samples at 560, 576 and 630 nm (Benesch et al., 1973). Three fish from each tank were sacrificed with anesthesia overdose to obtain liver pooling samples that were stored at −80 °C until RNA extraction for RNA-seq analysis.

2.3. Library preparation and sequencing

Total RNAs from triplicates (each replicate consisted of liver pooled from three fishes) at the respective sampling. Total RNA was extracted from each tissue sample using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. To remove residual genomic DNA, the RNA samples were incubated with 10 units of DNA-free DNAse I (Ambion, USA) for 30 min at 37 °C. The quality and quantity of the purified RNA were determined by measuring the absorbances at 260 nm and 280 nm (A260/A280) using a Nanodrop ND-1000 spectrophotometer (LabTech, USA). RNA integrity was further verified by electrophoresis through a 1.5% (w/v) agarose gel. Poly(A) mRNA was isolated from each total RNA sample with oligo (dT) magnetic beads (Invitrogen). The purified mRNA was fragmented with the RNA fragmentation kit (Ambion) and used as template for first strand cDNA synthesis using random hexamer primers and reverse transcriptase (Invitrogen). The second strand cDNA was synthesized using RNase H (Invitrogen) and DNA polymerase I (New England Biolabs, USA). The Illumina Genomic DNA Sample Prep kit (Illumina, USA) was used to generate 100 bp + 100 bp paired-end cDNA libraries by following the manufacturer's protocol. The libraries were loaded onto flow cell channels for sequencing