Comparative Biochemistry and Physiology, Part C 156 (2012) 75–79

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

# *In vitro* toxicity of nitrite on haemocytes of the tiger shrimp, *Penaeus monodon*, using flow cytometric analysis

Jian-An Xian [a,b], An-Li Wang [a,*], Xuan-Ming Hao [b], Yu-Tao Miao [a], Bin Li [a], Chao-Xia Ye [a], Shao-An Liao [a]

[a] Key Laboratory of Ecology and Environment Science in Guangdong Higher Education, Guangdong Provincial Key Laboratory for Healthy and Safe Aquaculture, College of Life Science, South China Normal University, Guangzhou 510631, People's Republic of China
[b] School of Physical Education & Sports Science, South China Normal University, Guangzhou 510631, People's Republic of China

ARTICLE INFO

*Article history:*
Received 31 January 2012
Received in revised form 17 April 2012
Accepted 17 April 2012
Available online 27 April 2012

*Keywords:*
Nitrite
Haemocyte
*Penaeus monodon*
Apoptosis
Flow cytometry

ABSTRACT

This study investigated the *in vitro* effects of nitrite on reactive oxygen species (ROS) production, NO production, esterase activity and cell apoptosis of *Penaeus monodon* haemocytes. Haemocytes were *in vitro* exposed to different dose of nitrite (0, 0.1, 0.5, 1, 5 and 10 μM). Cellular responses of nitrite-treated haemocytes were determined by flow cytometry. The results revealed that haemocytes treated by nitrite *in vitro* showed conspicuous time- and dose-dependent decreases in ROS and NO production as well as esterase activity. Additionally, 0.1 and 0.5 μM nitrite did not affect the apoptotic cell ratio during the 3 h experimental time, while significant increases in apoptotic cells were observed after haemocyte exposure to nitrite at 1 μM for 3 h, and at 5 or 10 μM for 1 h. These results indicated that nitrite suppresses cellular functions, including production of ROS and NO, and activity of esterase. Cell apoptosis of haemocytes would be induced by extracellular nitrite as doses exceed 1 μM.



## 1. Introduction

Nitrite, an intermediate product either during bacterial denitrification of nitrate or bacterial nitrification of ammonia, is the most common pollutant in intensified aquaculture or in recirculated water. For shrimps and prawns, elevated environmental nitrite has been reported to retard growth and larval development, to induce methaemocyanin formation, to cause hypoxia in tissue, to impair the respiratory metabolism, to affect nitrogen excretion, to depress immune ability, to increase susceptibility to bacterial infection and cause death in extreme cases (Chen and Chen, 1992; Chen and Cheng, 1995a, 1995b; Jensen, 2003; Lin and Chen, 2003; Tseng and Chen, 2004; Chand and Sahoo, 2006; Mallasen and Valenti, 2006).

Haemocytes play vital role in the immune function of shrimp, including phagocytosis, encapsulation, cytotoxicity, storage and release of the prophenoloxidase system (Johansson et al., 2000). Loss and damage of circulating haemocytes would depress the immune ability, increase the susceptibility against pathogens, and even endanger the survival of shrimp (Lorenzon et al., 1999; Yeh et al., 2004). However, less effort has been directed at determining toxic effects of nitrite on haemocytes at a cellular level. Our *in vivo* previous study has showed the oxidative toxicity mechanism of aquatic nitrite on shrimp haemocytes (Xian et al., 2011). Nevertheless, the *in vitro* toxic influence of nitrite is still unclear, which may help us to further understand the toxicity mechanism of nitrite on shrimp haemocytes.

Reactive oxygen species (ROS), including superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl ($HO^-$), singlet oxygen ($^1O_2$) and peroxyl ($ROO^-$), play an important role in phagocyte-mediated killing of pathogens (Raman et al., 2008). Under normal conditions, the production and destruction of ROS are well regulated, whereas under environmental stress, the balance between the production of ROS and the antioxidant defense mechanisms is disturbed (Xian et al., 2010). It had been reported that stress of various environment factors, such as nitrite (Xian et al., 2011), heavy metals (Xian et al., 2010) and pH (Wang et al., 2009), would induce ROS production in shrimp haemocytes. Overproduction of ROS may lead to oxidative damage to tissue macromolecules including DNA, proteins and lipids (Wang et al., 2009), and subsequently induce cell apoptosis (Orrenius, 2007). Recent studies found that reactive nitrogen species (RNS) such as nitric oxide (NO) also acts as a cytotoxic molecule contributed to microorganisms killing in invertebrates (Raman et al., 2008). Similarly, NO production could also be affected by environmental stress (Canesi et al., 2008). Esterase is a type of lysosomal enzyme involved in the intracellular degradation of pathogenic or foreign elements. Numerous studies showed that esterase activity would be modified by various stimulants, such as naphthalene (benzo[a]pyrène, phenanthrene, anthracene and fluoranthene), pesticides (paraoxon and chlorohalonil) (Gagnaire et al., 2006), Ni, Cd and Cu (Mazón et al., 1998; Zvereva et al., 2003). These studies might suggest that non-specific esterase activity was a sensitive indicator for ecotoxicological research. Effect of nitrite on activity of esterase was analyzed in the present study.

* Corresponding author. Tel./fax: +86 2085210141.
*E-mail address:* wanganl@scnu.edu.cn (A.-L. Wang).

1532-0456/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2012.04.001

Flow cytometry which analyzes the individual cell in a short time could provide an objective, reproducible and sensitive characterization of a large number of haemocytes. This technique has been used in *in vitro* studies of toxic effects of aquatic contamination on several species of bivalve (Brousseau et al., 2000; Gagnaire et al., 2004). In the present study, ROS production, NO production, esterase activity and apoptotic cell ratio of nitrite-treated haemocytes were measured by flow cytometry, in order to investigate the *in vitro* cytotoxicity of nitrite on shrimp haemocytes.

## 2. Materials and methods

### 2.1. Animals

*Penaeus monodon* ($9.30 \pm 1.09$ g) were obtained from a commercial shrimp farm in Zhuhai, Guangdong Province, China. They were maintained in the laboratory with diluted seawater at 20‰, pH 7.9–8.0 and controlled temperature ($24 \pm 2$ °C), with continuous water circulation. Prior to experimental use, animals were acclimated to the laboratory conditions for one week, and fed twice daily with commercial shrimp feed. Only apparently healthy shrimp in the intermoult stage were used.

### 2.2. Preparation of haemocyte suspension

A 200 μL haemolymph sample was extracted from each shrimp by a 25 gauge needle and 2.5 mL syringe containing an equal volume of ice-cold anticoagulant solution (AS, glucose 20.5 g $L^{-1}$, sodium citrate 8 g $L^{-1}$, sodium chloride 4.2 g $L^{-1}$, pH 7.5). Haemolymph from ten animals was pooled to reduce interindividual variation, and then the pooled haemolymph was diluted with AS to obtain a final concentration of $1 \times 10^6$ cells $mL^{-1}$.

### 2.3. Exposure protocol

The xenobiotic used in this study was $NaNO_2$. The compound was prepared as a 100 mM solution in AS, and then be diluted in AS to obtain $NO_2^-$-N working solutions at doses of 10, 50, 100, 500 and 1000 μM, respectively. A volume of 30 μL working solution was added to 2970 μL of haemocyte suspension to obtain different doses (0.1, 0.5, 1, 5 and 10 μM) of $NO_2^-$-N, respectively. The cells were incubated at room temperature, and subsamples were collected to determine the cellular responses after 0, 1, 2 and 3 h exposure.

### 2.4. Flow cytometry

Flow cytometry was performed with a FACSCalibur equipped with a single argon ion laser with filtered emission at 488 nm. Photomultiplier bandpass filters for fluorescence were 530 nm (green fluorescence, FL1 detector) and 585 nm (yellow/orange fluorescence, FL2 detector). For each subsample, 10,000 events were counted.

### 2.5. Reactive oxygen species (ROS) production

To monitor the level of ROS, the cell-permeant probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma) was used as described previously (Xian et al., 2010). A volume of 200 μL haemocyte suspension was incubated with 10 μM DCFH-DA for 30 min at room temperature in the dark. Then the fluorescence of cells was recorded on FL1 detector of flow cytometer. ROS production was expressed as mean fluorescence of DCF.

### 2.6. NO production

The fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA, Molecular Probes) was used to detect intracellular NO production. This probe is cell-permeant and passively diffuses across cellular membranes. Once inside the cells, it is deacetylated by intracellular esterases to become DAF-FM. After reacting with NO, DAF-FM is further converted to DAF-FM triazole ($\lambda_{excitation} = 495$, $\lambda_{emission} = 515$) which exhibits about a 160-fold greater fluorescence quantum efficiency. A volume of 200 μL haemocyte suspension was incubated with 10 μM DAF-FM DA for 60 min at room temperature in the dark. Then the fluorescence of cells was recorded on FL1 detector of flow cytometer. Results were given as mean of DAF-FM fluorescence, in arbitrary FL1 units.

### 2.7. Apoptotic cell ratio

The apoptotic haemocytes were examined using Annexin V-FITC/PI apoptosis detection kit (Invitrogen) following the manufacturer's instructions as described previously (Xian et al., 2010). Briefly, haemocytes were washed with AS and resuspended at about $3 \times 10^6$ cells $mL^{-1}$ in 1× Annexin V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). One hundred microliters of haemocyte sample was stained with 5 μL of Annexin V-FITC and 10 μL of 50 μg $mL^{-1}$ PI working solution for 15 min in the dark. Then 400 μL 1× Annexin V binding buffer was added to each tube, and the cells were immediately analyzed by flow cytometry. Results were expressed as Annexin V-FITC/PI dot plot. Cells stained negative with both probes represent live cells (quadrant a). Cells stained positive with Annexin V-FITC and negative with PI are early apoptotic cells (quadrant b). Cells stained positive with both Annexin V-FITC and PI are in the end stage of apoptosis, undergoing necrosis, or already dead (quadrant c). Apoptotic cell ratio was expressed as the percentage of cells in quadrants b and c (Fig. 4).

### 2.8. Non-specific esterase activity

Esterase activity was measured using the non-specific liposoluble substrate fluorescein diacetate (FDA, Molecular Probes). A volume of 200 μL haemocyte suspension was incubated with 5 μM FDA for 30 min at room temperature in the dark. Then the fluorescence of cells was recorded on FL1 detector of flow cytometer. Esterase activity was expressed as mean fluorescence of FDA.

### 2.9. Statistical analyses

Cell Quest© software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) was used to create logical markers and mean fluorescence intensity analyses of fluorescence data. All data are presented as means ± standard deviation for three independent experiments. A multiple comparison (Tukey) test was conducted to compare the significant differences among treatments using SPSS 13.0 program (SPSS Inc., Chicago, IL, USA). A $P$ value $< 0.05$ was considered significant.

## 3. Results

### 3.1. ROS production

Effect of nitrite exposure on ROS production is presented in Fig. 1. No significant change was shown in the control treatment ($P > 0.05$). After exposure to nitrite at doses from 0.1 to 10 μM, time-dependent significant decreases were shown in ROS production of haemocytes ($P < 0.05$).

### 3.2. NO production

As shown in Fig. 2, NO content in haemocytes of control treatment increases slightly, and significant increase was observed at 3 h of nitrite incubation ($P < 0.05$). On the contrary, production of NO was