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Effects of ammonia nitrogen stress on the blood cell immunity and liver antioxidant function of *Sepia pharaonis*

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ABSTRACT

The objective of this study was to investigate toxicity effects of ammonia nitrogen on the cuttlefish *Sepia pharaonis*. The effects of ammonia nitrogen stress (1, 3, 6, and 12 mg/L) on the blood cell immunity and liver antioxidant function were studied. The results showed that the number of blood cells, respiratory burst activities of blood cells, the phagocytosis rate of blood cells, Cu/Zn–superoxide dismutase (Cu/Zn–SOD) and catalase (CAT) activities and relative gene expression, and MDA content of the cuttlefish *S. pharaonis* were significantly affected by the ammonia nitrogen ($P < 0.05$). The number of apoptotic blood cells, respiratory burst activities of blood cells and contents of MDA were increase, phagocytosis of blood cells, SOD and CAT activity were decrease with long-term exposure to low concentration (1 mg/L) or high concentration (>3 mg/L) of ammonia nitrogen stress, and action degree is positively related to the stress of ammonia nitrogen concentration and time length treated. In summary, the ammonia nitrogen stress could result in declining phagocytic percentage, decreasing the activity of phagocytic, SOD and CAT, increasing the contents of MDA. It resulting in an imbalance of non-specific immune system and damaging antioxidant defense system, reducing the ability of eliminating free radicals, damaging cell membrane structure, then its functions of the cells and tissues of the body, which was a contributory cause of ammonia poisoning.

1. Introduction

Ammonia nitrogen is a common pollution stress factor in aquaculture water. As the final product of protein catabolism, ammonia nitrogen accounts for more than half of the nitrogen-containing waste released by most fish (Ruyet et al., 1995). The decomposition of animal excreta, residual bait, and plankton debris in the aquaculture system is likely to cause a sharp increase in ammonia nitrogen content in the water (Zhao et al., 1997; Dutra et al., 2016). When the ammonia concentration in aquaculture water is too high, ammonia can enter into the tissue cells of cultured animals through body surface infiltration, gill epithelium absorption, and other channels, increasing the ammonia nitrogen content in their body fluids (Eddy et al., 2005). When the stimulation time of ammonia nitrogen is beyond the tolerance limit and exceeds the regulation threshold of the organism, the antioxidant system and immune system functions of the organism are inhibited or impaired (Randall and Tsui, 2002; Romano and Zeng, 2007; Yue et al., 2010). This creates favorable conditions for pathogens to invade the organism (Wilson et al., 2007; Paust et al., 2011; Sinha et al., 2012), thus increasing the infection rate of animal diseases severe liver and organ failure, growth retardation, death, and other phenomena (Foss et al., 2004).

Sepia pharaonis, a globally important economic cephalopod, is mainly distributed in the Indian Ocean-Western Pacific Ocean. Because of overfishing and the deterioration of the marine ecological environment, its resources are declining daily. Its yield has been unable to keep pace with people's increasing demands (Peng et al., 2017). Researchers have found that tabby squid is characterized by large size (up to over 5 kg), fast growth (up to 0.5 kg within 4 months of cultivation), strong disease resistance, and high market demand, making it a cephalopod with great development prospects for cultivation (Jiang et al., 2018). However, during the breeding of *S. pharaonis*, it was found that it was unusually sensitive to ammonia pollution, and it's (body weight: 8 g) semi-lethal concentrations of ammonia nitrogen at 24 h and 96 h were 27.72 mg/L and 15.33 mg/L, respectively, which were significantly lower than those of other aquaculture species, deeming this species to be more sensitive to ammonia pollution stress than most fish, shrimp, and crabs (Peng et al., 2017). When the level of ammonia nitrogen in the water is a threshold levels, it will cause cuttlefish ink-jet and even a large

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number of deaths, which has become one of the important bottlenecks affecting the development of *S. pharaonis* aquaculture.

Therefore, it is of great significance to investigate the effects of ammonia nitrogen on antioxidant enzymes activities, hematology and immune response and to explore the mechanism of ammonia nitrogen poisoningcin cuttlefish, which could be used in the establishment of methods for the alleviation of ammonia nitrogen toxicity and in the cultivation of *S. pharaonis* (Gestal and Castellanos, 2015). The blood cells of tabby squid are an important part of cellular immunity, and the liver is the most important detoxifying organ (Storelli et al., 2006). Therefore, this experiment specifically used *S. pharaonis* as the research object: (1) to explore the effects of ammonia stress on the number of blood cells, phagocytotic rate of blood cells, respiratory burst activity of blood cells, and antioxidant enzyme activity in the liver of *S. pharaonis*; (2) to evaluate the effects of ammonia stress on its cellular immune function and antioxidant defense system; and (3) to provide a theoretical basis for understanding the mechanism of the cephalopod's toxicity and immune response to ammonia nitrogen.

2. Materials and methods

2.1. Animals and feeding conditions

The experiment was carried out at the Xiang Shan Lai Seedling Farm in Zhejiang Province from May to July 2020. The *S. pharaonis* used in the experiment was obtained from Xiang Shan to develop their seedling farm. Six hundred sixty cuttlefish of *S. pharaonis* of neat size and good vitality and health (average weight of 13.80 ± 0.11 g) were temporarily raised in 200 L indoor plastic buckets a week before the experiment. The water used during the maintenance period was sand-filtered seawater (salinity: 29.0 ± 1.0; temperature: 24 ± 0.5 °C; dissolved oxygen level: 7.53 ± 0.63 mg /L; pH: 8.02 ± 0.13; ammonia nitrogen content: 0.02 ± 0.01 mg /L; nitrous nitrogen content: 0.02 ± 0.01 mg /L). During the maintenance period, chilled shrimps were fed at 15% of their body weight three times per day (8:30, 12:30, and 17:30). The water was changed once a day (9,30) and was changed to 80% of the water. Physiocochemical parameters of seawater was measured once a day (16,00). Salinity, temperature, dissolved oxygen level, and pH were determined using a YSI Pro DSS (YSI; www.ysi.com). Ammonia nitrogen and nitrate nitrogen contents were determined using the HACH kit (HACH, DR-2000, Loveland, Colorado, USA).

2.2. Experimental procedures

The preliminary experiments results showed that the higher concentration that the cuttlefish of *S. pharaonis*(13.80 g) can tolerate without mortality after 72 h ($LC_{0,\ 72\ h}$) was 12.50 mg/L. According to the preliminary experimental results, five gradients were designed for this experiment. The ammonia concentration was 0 (control group), 1 mg/L, 3 mg/L, 6 mg/L, and 12 mg/L, respectively. The total of 540 cuttlefish were randomly allocated to 15 individual tanks (200 L, 1.2 m diameter × 1.3 m height) at a density of 36 cuttlefish per tank. The 15 individual test tanks were divided into five groups (5 ammonia concentrations × 3 tanks). The five different ammonia concentrations were adjusted with 10 mg/L ammonium acetate solution. The cuttlefish were reared under a natural photoperiod in aerated and UV-irradiated seawater. The cuttlefish juveniles were not fed diets during the experimental period. Every 12 h, 90% of the seawater in each tank was refreshed (at 8:00 and 18:00) with seawater at the same temperature, salinity, pH, and ammonia nitrogen concentration. The physiocochemical parameters of seawater was measured at the experimental period (Table 1).

Table 1
Seawater quality parameters during the experimental period.

	Ammonia treatment(mg/L)				
	0 (Control)	1	3	6	12
Ammonia-N (mg/L)	0.02 ± 0.01	1.06 ± 0.02	3.02 ± 0.02	6.14 ± 0.05	12.04 ± 0.08
Salinity	29.0 ± 1.0	29.0 ± 1.0	29.0 ± 1.0	29.0 ± 1.0	29.0 ± 1.0
Temperature (°C)	24.0 ± 0.5	24.0 ± 0.5	24.0 ± 0.5	24.0 ± 0.5	24.0 ± 0.5
pH	8.02 ± 0.12	8.03 ± 0.14	7.98 ± 0.15	7.96 ± 0.17	8.00 ± 0.19
DO	7.43 ± 0.61	7.51 ± 0.53	7.47 ± 0.58	7.52 ± 0.52	7.48 ± 0.63
Nitrite-N (mg/L)	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.02	0.034 ± 0.02

2.3. Sample collection

The sampling times were 0 h, 6 h, 12 h, 24 h, 48 h, and 72 h. Five cuttlefish were randomly selected from each parallel group. After anesthesia with 0.15 mol/L $MgCl_2$, blood was quickly extracted from the heart of the tabby cuttlefish with a sterile 1-mL syringe after dissection. Then, the blood was mixed with anticoagulation agents (0.45 mol/L NaCl, 0.60 mol/L $C_6H_{12}O_6$, 0.03 mol/L $Na_3C_6H_5O_7 \bullet 2\ H_2O$, 0.026 mol/L $C_6H_8O_7$, and 0.01 mol/L ethylenediamine tetraacetic acid (EDTA)-2Na; pH 7.45), mixed evenly (1:1, *v*/v) to make a blood cell suspension sample, and stored at 4 °C. At the same time, the liver samples were divided into two parts, one part for the gene expression analysis and one part for the enzyme activity determination.

2.4. Sample analysis

2.4.1. Determination of blood cell number

Blood cell suspension (0.1 mL) was dropped onto the blood count board and counted under an optical microscope (OlympusBX-50), counting and calculating the number of blood cells per unit volume.

2.4.2. Determination of the phagocytotic rate of blood cells

Vibrio harveilli was inoculated on 2116 E slant solid medium and cultured for 18–24 h (27 °C). The *V. harveilli* on the solid slant were washed with sterile saline, centrifuged, suspended, and prepared into a bacterial suspension at a concentration of approximately 10^7 cells/mL.

Blood cell samples (200 mL) were centrifuged for 5 min (4 °C; 3000 rpm), the supernatant was discarded, and then the precipitate was washed with sodium dimethyl arsenate buffer. Afterwards, the precipitate was resuspended to form a blood cell suspension of 10^6 cells/mL. After adding 0.1 mL of the bacterial suspension and 0.1 mL of the blood cell suspension in a sterile plastic concave hole plate, the mixture was placed under wet incubation for 30 min (37 °C) in the box, and then 50 μL of the mixture was placed on the slide. After smearing, the samples were often warm dried for 2 min, followed by the use of methanol for 5 min, Jim for dyeing for 15–20 min, decoloring for 5–10 min, dry, and the samples were finally sealed. Finally, under an Olympus optical microscope (10 × 100), the number of phagocytic blood cells was observed and counted, 200 blood cells were randomly counted, and *the phagocytotic rate of blood cells* (PR) was calculated using the following formula: PR = (the number of phagocytic blood cells /200 blood cells) × 100.

2.4.3. Determination of respiratory burst activities of blood cells

A 96-well plate was used to determine the respiratory burst activity. Before the test, the wells were pre-plated with L-polylysine solution at a concentration of 0.2%, and then dried for later use. A 100-blood cell suspension was added to each test well, and the supernatant was then centrifuged for 15 min (300 *g*). Then, 100 μL of 3-glucan (Sigma) at a concentration of 4 mg/mL (dissolved in phosphate-buffered saline [PBS]) was added and incubated for 30 min at 27 °C. After centrifugation for 15 min (300 *g*), the supernatant was discarded, and 100 μL PBS was added to rinse the precipitate once more. Next, 100 μL 0.3% of 4-nitro blue tetrazolium chloride (NBT) solution was added and

incubated at room temperature (20 °C) for 30 min. After centrifugation for 10 min (800 *g*), the supernatant was discarded, and the precipitate was fixed with 200 μL anhydric methanol, rinsed with 200 μL 70% methanol three times, and dried. After adding 120 μL KOH at a concentration of 2 mol/L and 120 μL of dimethyl sulfoxide into the wells, the precipitate was mixed with light shock for 3 min. Finally, the absorbance value was measured at 630 nm using a microplate reader. The blood cell respiratory burst activity of *S. pharaonis* was expressed as OD_{630nm} (O_2^- yield).

2.4.4. Preparation and analysis of the crude enzyme extract activity

After the sample was washed with 0.86% pre-cooled physiological salt, appropriate tissue was weighed, 0.75% pre-cooled physiological saline was added according to a 1:9 (g/mL) mass-volume ratio, and homogenized in an ice bath. After centrifugation for 15 min (4 °C; 12,000 rpm), the supernatant was immediately removed for the determination of protein and enzyme activity.

Enzyme activities, namely, those of Cu/Zn–superoxide dismutase (Cu/Zn–SOD), catalase (CAT), and malondialdehyde (MDA), were measured using the test methods of protein kits, namely, CAT kit, Cu/Zn–SOD kit, and MDA kit of the Nanjing Jian Cheng Institute of Biological Engineering. The Cu/Zn–SOD activity was determined using the xanthine oxidase method. Catalase activity was determined by the ammonium molybdate method. Malondialdehyde content was determined by the thiobarbituric acid method.

2.4.5. Relative quantity analysis of genes

The sample was placed in a 1.5-mL Eppendorf tube treated with diethyl pyrophosphate amide (DEPC) water containing 0.4 mL RNA extraction reagent. The samples were stored in a refrigerator at −80 °C for testing.

2.4.5.1. RNA extraction. The Liver tissues (50–100 mg) were taken, fully ground in liquid nitrogen, and 1 mL of Trizol added for extraction. Total RNA was extracted according to Trizol instructions. And quantity of RNA was determined by measuring its absorbance at 260 and 280 nm, and its integrity was tested by electrophoresis in 2% agarose gel.

2.4.5.2. First-strand synthesis of cDNA. Total RNA (0.2 mL) and 1 μL of 3′-adaptor (primer) were added to a 10-mL PCR tube and placed in a water bath (70 °C, 5 min). Then, the mixture was placed in an ice bath for 2 min, centrifuged and was added with the following reagents (Table 2). After the first (42 °C, 60 min) and second water bath (72 °C, 10 min) treatments, the mixture was placed on ice. Finally, the prepared cDNA template was embedded and stored at −20 °C.

2.4.5.3. Primer design. The fragment sequences of the genes to be detected in this study (Cu/Zn–SOD, CAT) were obtained from the transcriptological database of *S. pharaonis* constructed in our laboratory. These sequences were designed with fluorescent quantitative primers using PrimerPremier 5.0 software and synthesized by the Shanghai Sangon Bioengineering Technology Services Co., LTD. The primer sequences are shown in Table 3.

2.4.5.4. Quantitative reverse transcription PCR was used to detect the relative expression levels of genes. SYBR Green quantitative fluorescent PCR kit (Roche, Switzerland) and Light Cycler® 96 real-time quantitative fluorescent PCR instrument (Roche, Switzerland) were used for quantitative fluorescent PCR detection. The reaction conditions and reaction system were according to the instructions of the fluorescence quantitative PCR kit, using the $2^{-\Delta\Delta Ct}$ method to calculate relative gene expression.

Table 2
cDNA synthesis system and dosage.

Reagents	Consumption(μL)
5× First-Strand Buffer	4
10 mmol/L dNTP	2
Rnase inhibitor	1
Reverse Transcriptase	2

Table 3
RT-qPCR primer sequences of all genes used in the present experiments.

Gene	Forward (5′-3′)	Reverse (5′-3′)
Zn/Cu SOD	GAGACTTTCGTTAGGACGGATA	AGCCATTCCCCTTATTTCAC
CAT	TTCGTTTCTCTACCGTTGGTG	AAGTCCCAGTTACCGTCTTCC
β-Actin	GACTCCTACGTAGGAGACGA	CGTTGAAGGTCTCGAACATGA

2.5. Data processing and analysis

All experimental data were expressed as mean ± standard deviation (Mean ± SD), and SPSS17.0 software (SPSS, Chicago, IL, USA) was used for correlation analysis, one-way analysis of variance (ANOVA), and Duncan's multiple comparison analysis ($P < 0.05$).

3. Results

3.1. Effects of ammonia stress on blood cell count, respiratory burst, and blood cell phagocytosis of S. pharaonis

3.1.1. Changes in the number of blood cells of S. pharaonis under ammonia stress

Ammonia stress had a significant effect on *the number of blood cells* in the blood lymphocytes of *S. pharaonis* ($P < 0.05$). Within 72 h of the test, each treatment group showed a relatively obvious trend of decline, and the rate of decline in the later period of the test (after 48 h) tended to be flat. The number of decreased total blood cells was significantly negatively correlated with ammonia concentration ($P < 0.05$). After 72 h, the total number of blood cells in the 1, 3, 6, and 12 mg/L ammonia nitrogen treatment groups decreased by 13.09%, 28.89%, 56.59%, and 58.13%, respectively, compared with that of the control group (Fig. 1).

3.1.2. Changes in respiratory burst activities of blood cells of S. pharaonis under ammonia nitrogen stress

Ammonia stress had a significant effect on the blood cells respiratory burst activities of *S. pharaonis* ($P < 0.05$), which was significantly higher than that of the control group. The respiratory burst activities of blood cells in the ammonia nitrogen treatment group showed a rapid rise from

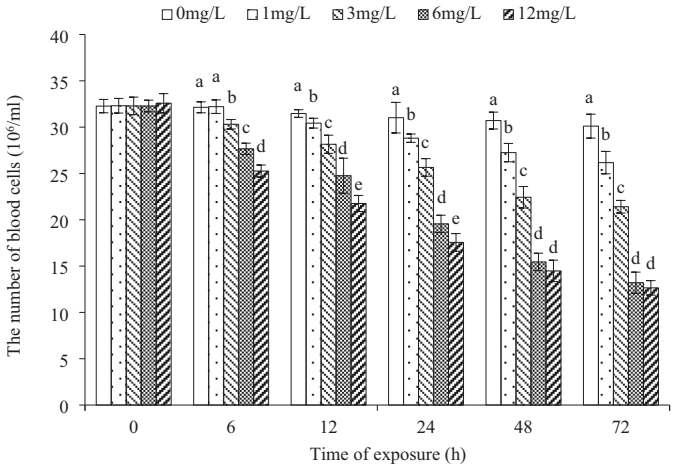

Fig. 1. Time course change of the number of blood cells of *S. pharaonis* exposed to different concentrations of ammonia nitrogen. Different letters donate significant difference between groups ($P < 0.05$) at the same time. The same as below.

0 to 24 h. And respiratory burst activities of blood cells were positively related to the treating time and concentration of ammonia nitrogen. After 72 h, the respiratory burst activities of blood cells in the treatment groups with 1, 3, 6, and 12 mg/L ammonia nitrogen was increased by 30.30%, 50.39%, 57.32%, and 64.04%, respectively, as compared with the that of the control group (Fig. 2).

3.1.3. Changes in the phagocytosis rate of blood cells of S. pharaonis under ammonia nitrogen stress

The effects of ammonia nitrogen stress on the phagocytotic rate of *S. pharaonis* blood cells significantly ($P < 0.05$). The effects of ammonia nitrogen stress on the phagocytotic rate of *S. pharaonis* blood cell significantly ($P < 0.05$). With the prolonging of stress, the phagocytic rate of blood cells tendency were activated first and then inhibited. The highest phagocytosis rate of blood cells of 1 mg/L, 3 mg/L, 6 mg/L and 12 mg/L treatment groups occurred at 12 h, 12 h, 12 h and 6 h, respectively. The phagocytosis rate of blood cells of 3 mg/L, 6 mg/L and 12 mg/L treatment groups were significantly lower than control group after 48 h, And phagocytosis rate of blood cells were positively related to the treating time and concentration of ammonia nitrogen. After 72 h, the phagocytosis rates in the 1, 3, 6, and 12 mg/L treatment groups were reduced by 6.22%, 25.76%, 46.59%, and 54.63%, respectively, compared with that of the control group (Fig. 3).

3.2. Effects of ammonia stress on the antioxidant enzyme activities and relative gene expression in the liver of S. pharaonis

The Cu/Zn–SOD activity in *S. pharaonis* liver was significantly affected by ammonia stress ($P < 0.05$). The Cu/Zn–SOD activity showed the effect of small amplitude promotion and then inhibition, and the Cu/Zn–SOD activity in the treatment group after a 72-h exposure was significantly lower than that of the control group, with time and dose effects. The Cu/Zn–SOD activity was not significantly different from that of the control group ($P > 0.05$) after 6 h and 24 h of 1 mg/L ammonia nitrogen exposure. The activity was significantly higher than that of the control group ($P < 0.05$) after 12 h and 48 h, while the activity at 72 h was significantly lower than that of the control group ($P < 0.05$). The activity of Cu/Zn–SOD first increased, then fluctuated, and finally decreased during the test period. The activity of Cu/Zn–SOD was significantly higher than that of the control group ($P < 0.05$) at 6–24 h after 3 mg/L ammonia nitrogen exposure; the difference between the control group and the Cu/Zn–SOD was not significant after 48 h ($P > 0.05$), while the activity of Cu/Zn–SOD was significantly lower than that of the control group ($P < 0.05$) after 72 h, and the Cu/Zn–SOD activity showed an inverted u-shaped trend within the test period. At a 6-mg/L ammonia nitrogen exposure at the 12 h and 24 h, the Cu/Zn–SOD activity was significantly higher than that of the control group ($P < 0.05$), reached the highest level after 24 h and 48 h after the sharp drop, and was significantly lower than that of the control group ($P < 0.05$) and the Cu/Zn–SOD activity in the test cycle showed a Λ-type performance trend. After 6 h of the 12 mg/L ammonia nitrogen treatment, the Cu/Zn–SOD activity was significantly higher than that of a control group ($P < 0.05$), peaked after 12 h, and dropped significantly, being significantly lower than that of a control group ($P < 0.05$). The Cu/Zn–SOD activity in the test cycle showed a Λ-type performance trend (Fig. 4).

Ammonia stress had a significant effect on the expression of the Cu/Zn–SOD gene in the liver of *S. pharaonis* ($P < 0.05$). The Cu/Zn–SOD gene expression scale firstly showed a trend of first promotion, and then inhibition. The expression level of the Cu/Zn–SOD gene was higher than that of the control group under both 1 mg/L and 3 mg/L ammonia nitrogen exposure during this test period, and the expression level of Cu/Zn–SOD gene increased at first, fluctuated within a certain value range, and then decreased. After 6 h of exposure to 6 and 12 mg/L ammonia nitrogen, the amount of Cu/Zn–SOD gene expression was significantly higher than that of the control group ($P < 0.05$). The maximum dropped dramatically after 12 h, and after 48 h, was significantly lower than that of the control group ($P < 0.05$). The test cycle in the amount of Cu/Zn–SOD gene expression in the expression type Λ trend (Fig. 5).

Ammonia stress had a significant effect on CAT activity in the liver of *S. pharaonis* ($P < 0.05$). Catalase activity showed a small effect of first

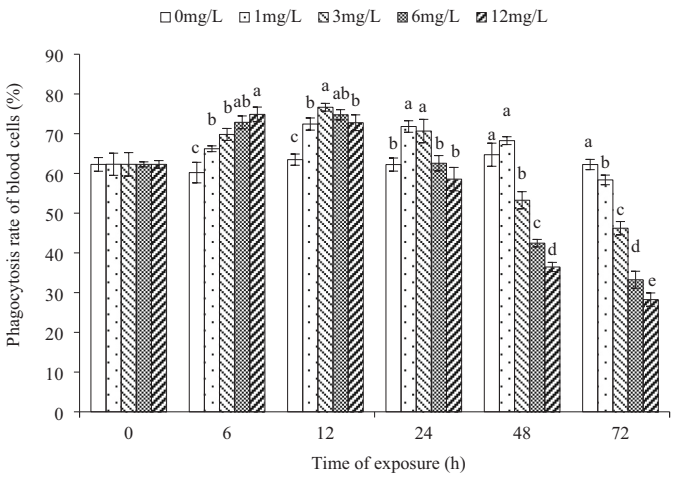

Fig. 3. Time course change of the phagocytosis rate of blood cells of *S. pharaonis* exposed to different concentrations of ammonia nitrogen.

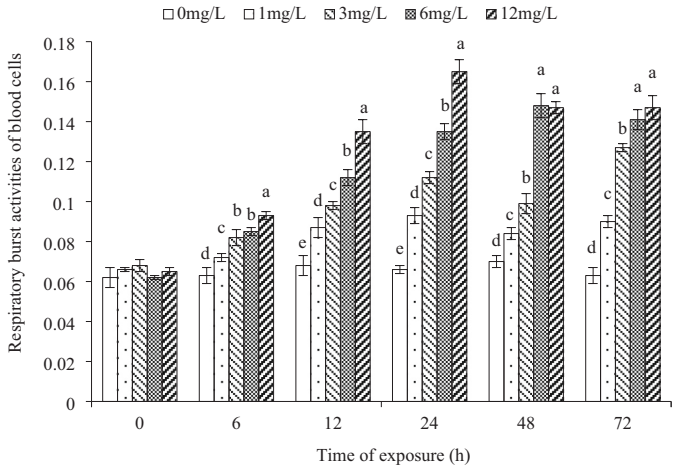

Fig. 2. Time course change of the respiratory burst activities of blood cells of *S. pharaonis* exposed to different concentrations of ammonia nitrogen.

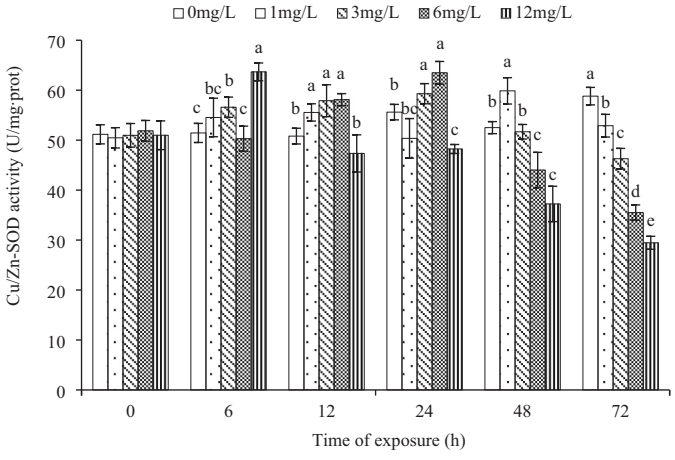

Fig. 4. Changes of Cu/Zn-SOD activity with time of exposure in liver tissue of *S. pharaonis*.

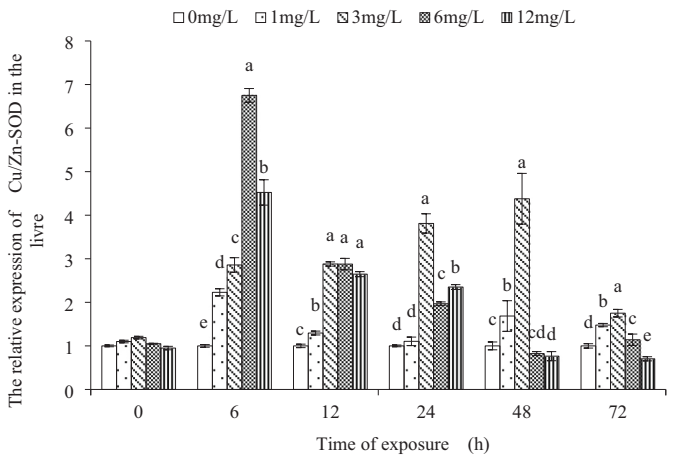

Fig. 5. Relative expression levels of Cu/Zn-SOD in liver of *S. pharaonis* after exposure to different concentrations of ammonia nitrogen.

promotion, and then inhibition, and after 72 h exposure, CAT activity was significantly lower than that of the control group, with time and dose effects. Catalase activity in the 1 mg/L ammonia nitrogen treatment group was similar to that of Cu/Zn–SOD during the whole test period, which fluctuated after the increase, and finally showed a downward trend. The activity of Cu/Zn–SOD at 6 h, 12 h, and 48 h after 3 mg/L ammonia nitrogen exposure was significantly higher than that of the control group ($P < 0.05$). The difference between the control group was not significant after 24 h ($P > 0.05$), and the activity at 72 h was significantly lower than that of the control group ($P < 0.05$), which fluctuated and finally decreased after the increase in the test cycle. At 6 and 12 mg/L ammonia nitrogen exposed for 6–12 h, CAT activity was significantly higher than that of the control group ($P < 0.05$), reaching the highest level after 12 h and 24 h after the sharp drop, which was significantly lower than that of the control group ($P < 0.05$). Catalase activity in the test cycle showed a Λ-type trend (Fig. 6).

Ammonia stress had a significant effect on CAT gene expression in the liver of *S. pharaonis* ($P < 0.05$). The CAT gene expression scale showed a trend of first promotion, and then inhibition. After 1 mg/L ammonia exposure for 12 h, the CAT gene expression level was higher than that of the control group ($P < 0.05$). The CAT gene expression levels first increased, fluctuated within a certain value range, and then decreased. At 3 mg/L ammonia exposure, the expression level of the 6–48 h CAT gene was higher than that of the control group ($P < 0.05$), and after 72 h, the expression level of the CAT gene was lower than that of the control group. The expression level of the CAT gene first increased, fluctuated within a certain value range, and then decreased. At 6 and 12 mg/L after exposure to 6 h ammonia, the amount of CAT gene expression was significantly higher than that of the control group ($P < 0.05$) and the maximum, dropping dramatically after 24 h and 48 h, and later was significantly lower than that of the control group ($P < 0.05$). The amount of CAT gene expression in the test cycle showed a Λ-type trend (Fig. 7).

Ammonia stress had a significant effect on MDA content in the liver of *S. pharaonis* ($P < 0.05$). Malondialdehyde content in the liver gradually increases with the increase in exposure concentration, and the extension of exposure time and has time and dose effects. After exposure to 1 mg/L ammonia for 48 h, the MDA content in the liver was significantly higher than that of the control group ($P < 0.05$). The MDA content in the liver was significantly higher than that of the control group after exposure to 3 mg/L, 6 mg/L, and 12 mg/L ammonia for 6 h ($P < 0.05$). Moreover, there was a significant difference in the MDA content between the treatment groups at different concentrations 24 h after treatment ($P < 0.05$) (Fig. 8).

4. Discussion

4.1. Effects of ammonia stress on the blood cell immune function of Sepia officinalis

The immune system of cephalopods is a non-specific immune system that cannot use immune proteins and antibodies mediated to resist the invasion of pathogens like most arthropods (Malham et al., 1998; Castellanos and Gestal, 2013). The immune system of squid mainly includes phagocytosis of blood cells and serum factors mediated by a variety of non-immunoglobulins (Cheng and Bivalves, 1987). Among them, blood cells play a very important role in the defense system of cephalopods, which can remove pathogens and repair wounds by phagocytosis and cysts (Foss et al., 2009.). demonstrated that both the blood cells and the attached oval cells in the gill heart of *S. officinalis* could remove these foreign bodies by phagocytosis or cysts by infusion or injection of isoprogenies and bacteria (Honti et al., 2014). Changes in the blood cell morphology, structure, number, phagocytosis rate, and respiratory burst of cephalopods are considered important parameters for studying and evaluating the immune defense capability of cephalopods (Zhang et al., 2013).

Many studies have confirmed that in fish, crustaceans, and molluscs, a decrease in the number of blood cells results in a decrease in the body resistance to pathogens (Moullac and Haffner, 2000; Gagnaire et al., 2006; Xu et al., 2008). In this study, it was found that the number of

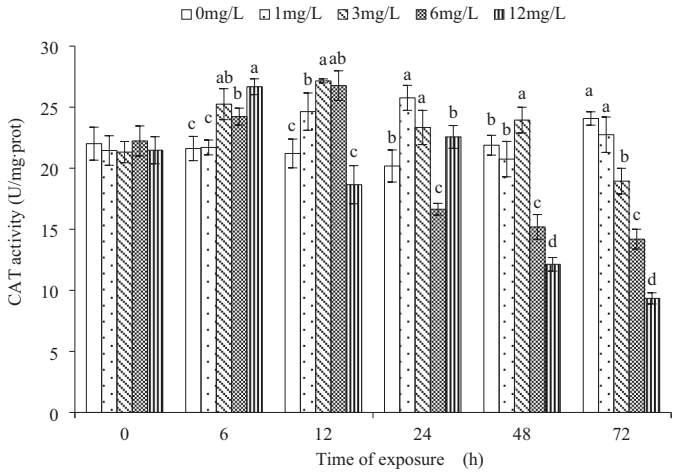

Fig. 6. Changes of CAT activity with time of exposure in liver tissue of *S. pharaonis.*

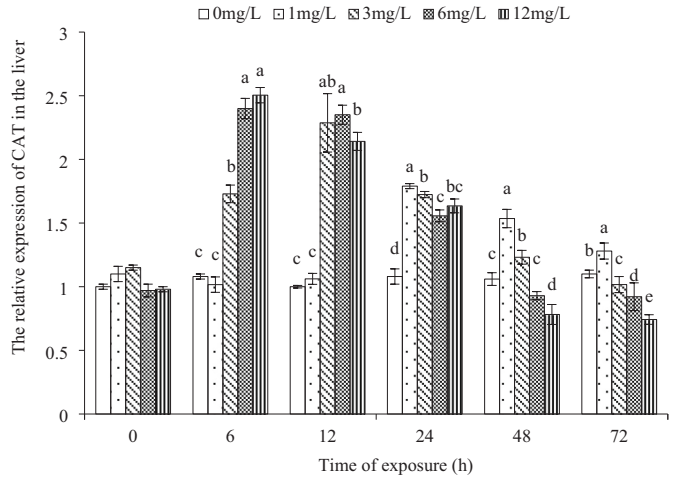

Fig. 7. Relative expression levels of CAT in liver of *S. pharaonis* after exposure to different concentrations of ammonia nitrogen.

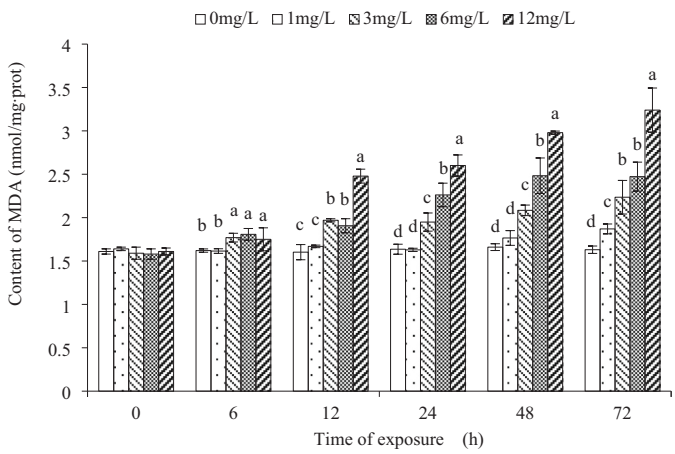

Fig. 8. The content of MDA with time of exposure in liver tissue of *S. pharaonis*.

blood cells in the blood lymphocytes of *S. pharaonis* decreased significantly under ammonia stress. The decrease in blood cells may be caused by the migration and infection of some blood cells on the one hand and the rupture and death of blood cells on the other hand. This also indicates that the number of blood cells decreases during the immune response due to phagocytosis, the cysts to remove pathogens, and lysis. The decrease in blood cells also suggests that they are involved in cellular immune defense as the main body of immune defense. However, the significant decrease in blood cells indicates that the stress of a high concentration of ammonia nitrogen will lead to the death of blood cells and in the decline of immune resistance of *S. pharaonis*. In summary, when *S. pharaonis* was exposed to the initial stress of a low concentration of ammonia nitrogen, the decrease in blood cell number indicated that cellular immunity played an extremely important role in the direct phagocytosis and killing of exogenous microorganisms. When exposed to higher levels of ammonia nitrogen over a long time, the number of blood cells decreased significantly, indicating damage.

Studies have shown that most blood cells of molluscs have a classic respiratory burst phenomenon. Under the stimulation of pathogenic microorganisms, phagocytes produce a large amount of ROS after experiencing respiratory bursts, killing the things that are engulfed inside the cell (Cheng et al., 2005; Deng et al., 2012). The results of this study showed that, under the stress of low ammonia nitrogen, respiratory burst activities of blood cells of *S. pharaonis* showed a significant increase, followed by a flat, and then a rising trend. This indicates that at the beginning, under ammonia nitrogen stress and strong oxidative stress, respiratory burst activities of blood cells increases, and a large amount of ROS is rapidly produced. The body will be damaged to different degrees, and respiratory burst activities of blood cells increases. Reactive oxygen species are more or less related to the depth of cell apoptosis, and excessive ROS production impairs the mitochondrial membrane permeability and respiratory chain (Halliwell, 1999; Suzuki et al., 2002; Cheng et al., 2005).

The phagocytosis of blood cells is the primary method of immune defense against pathogen invasion in molluscs, and the phagocytosis rate of blood cells is one of the most important indicators of immune defense ((Schram et al., 2014); Mouriès et al., 2002). The results of this study showed that the phagocytosis rate of the blood cells increased slightly at first, and then decreased significantly under stress. Finally, the phagocytosis rate was significantly negatively correlated with the concentration of ammonia nitrogen. Moreover, the phagocytosis rate of the blood cells of *S. pharaonis* under ammonia nitrogen stress presented an obvious dose response effect. A similar phenomenon has also been reported in other species. When the heavy metal Pb^{2+} contaminated scallops (*Chlamys farrys*), the phagocytosis ability of blood cells decreased (Wang et al., 2011). The phagocytosis rate of blood cells in mussels under Cu^{2+} stress also increased, and then decreased rapidly (Pipe et al., 1999). Little and Kraaijeveld (2004) proposed that the regulation of the immune system by the body requires a lot of energy, and when the body is in a bad environment for a long time, it will be in an unfavorable state due to energy consumption. Therefore, under the stress of a certain concentration of ammonia nitrogen, when the concentration of ammonia nitrogen is lower than a certain value in a certain time, the increase in phagocytosis can be stimulated; otherwise, phagocytosis can be inhibited.

In summary, ammonia stress increases the respiratory burst activities of blood cells, the phagocytosis rate of blood cells, and blood cell apoptosis. However, with an increase in stress concentration and stress time, resulting in nonspecific immune system damage.

4.2. *Effects of ammonia stress on the antioxidant defense system of S. pharaonis liver*

As is well known, under adverse environmental stress, oxidative stress in cultured animals will occur, and a large amount of ROS will be produced. As a cytotoxic molecule, ROS have high activity and strong oxidative reaction ability and can attack nucleic acids, proteins, lipids, and other cellular biological macromolecules through oxidative action (Ortuño et al., 2002; Lushchak et al., 2005; Kim et al., 2015). Under normal physiological conditions, the enzyme system and antioxidants in the body can maintain the balance between ROS production and clearance. However, under adverse environmental stress, the antioxidant system will interfere, and the balance of ROS production and clearance will be disrupted, resulting in an increase in ROS content in cells (Hegazi et al., 2010;(Zhang et al., 2012)). In this study, the results show that the 3 mg/L, 6 mg/L, and 12 mg/L after exposure to the 72 h ammonia nitrogen tabby squid Cu/Zn–SOD and CAT enzyme activity was significantly lower than that of the control group, with the Cu/Zn–SOD and CAT gene expression being restrained and significantly lower than those of control group, which fully proved the high concentration and prolonged stress conditions inhibits the tabby squid antioxidant defense system from obstructing the ROS generation and clearing the balance of excessive intracellular ROS, then eventually leads to tissue and organ damage, affecting their normal function, which was a contributory cause of ammonia poisoning.

Copper/zinc–superoxide dismutase and CAT are important antioxidant enzymes in organisms with special physiological activities. Among them, Cu/Zn–SOD is the primary substance for ROS elimination in organisms, which can resist and block the damage caused by ROS in cells and can timely repair the damaged cells. Additionally, CAT is the peroxidase body mark of the enzyme, which accounts for 40% of the total body peroxidase enzyme (GuI et al., 2015; Lushchak et al., 2005; Zhang et al., 2011). As important enzyme scavengers, Cu/Zn–SOD and CAT are coordinated and can effectively remove metabolic processes to produce ROS. The biological active oxygen in the body is maintained at a low level, preventing damages caused by increased active oxygen content from the process of membrane lipid peroxidation and other damages. This study shows that ammonia nitrogen can affect the antioxidant capacity of tabby squid and cause oxidative stress. In the low concentration of 0–48 h trial with a high concentration of 0–6 h trial Cu/Zn–SOD and CAT activity is higher than that of the control group, and with the stress time extended significantly lower than the control group, has obvious time effect. This indicated that both low concentration and short-time exposure to ammonia nitrogen showed a certain “toxic excitant effect.” However, with an increased duration of exposure to ammonia nitrogen stress, this effect disappeared, and the activities of Cu/Zn–SOD and CAT decreased significantly. Similar phenomena were observed in hostile environments with species such as *Radix*, *Pseudobagrus fulvidraco*, and European bass (*Dicentrarchus labrax*) (Wei and Liu, 2015; Sinha et al., 2015). At the same time, in this study, with an increased duration of exposure to ammonia nitrogen stress, the mRNA expression of liver antioxidant enzymes was also inhibited. It was further confirmed that both long-term exposure to low concentrations

and short-term exposure to high concentrations of ammonia nitrogen would damage the antioxidant enzyme system in the liver of *S. pharaonis*.

Malondialdehyde is one of the most important biochemical indicators of oxygen damage and is one of the metabolites of lipid peroxidation. It is widely used as an important evaluation index of oxidative damage to the cell membrane during oxidative stress (Lepage et al., 1991). The MDA content indicates the degree of lipid peroxidation of the cell membrane and indirectly reflects the degree of cell membrane damage. Li et al. (2016) reported that the increase in nitrite nitrogen exposure, body wall, and MDA content increased with an increase in the concentration of nitrite nitrogen stress and increased exposure time and content. This shows that a long-term exposure to low and high concentrations of ammonia nitrogen, trepang organization continued accumulation of free radicals, which can cause membrane lipid peroxidation, leading to a loss of the cell membrane system and interfering with the normal metabolism of cells and material exchange, which could lead to cell death. In this experiment, the MDA content in the liver of *S. pharaonis* showed a gradual increase with the increase in ammonia nitrogen exposure concentration and the extension of exposure time, having both time and dose effects. This indicates that the concentration of ammonia nitrogen stress directly affects the content of MDA in the liver of *S. pharaonis*, and under a long-term exposure to ammonia nitrogen stress conditions, the aggravation of lipid peroxidation in the liver of *S. pharaonis* causes damage to the cell membrane, leading to cell activation disorder, and then a series of pathological changes.

5. Conclusion

All the results indicated that ammonia nitrogen stress could result in declining phagocytic percentage, decreasing the activity of phagocytic, SOD and CAT, increasing the contents of MDA. There action degree is positively related to the stress of ammonia nitrogen concentration and time length treated. It resulting in an imbalance of non-specific immune system and damaging antioxidant defense system, reducing the ability of eliminating free radicals, and causing oxidative damage to the cell membrane, then its functions of the cells and tissues of the body, which was a contributory cause of *S. pharaonis* ammonia poisoning. Findings from this study could be used in the establishment of methods for the alleviation of ammonia nitrogen toxicity and in the cultivation of aquatic animals.

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Author contributions

CY, PR, and JX conceived and designed the experiment. HZ, PR and JM ran the experiments. The paper was written by CY and revised by PR. JX provided funding and helped discussing the results.

Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled “Effects of ammonia nitrogen stress on the blood cell immunity and liver antioxidant function of *Sepia pharaonis*”.

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