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Research Article

Effect of Exogenous Nitric Oxide on *Enterocytozoon hepatopenaei* Copy Numbers and Immunity of *Exopalaemon carinicauda*

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To explore the effect of nitric oxide (NO) on *Enterocytozoon hepatopenaei* (EHP) invading the shrimp, sodium nitroprusside was used as an exogenous NO donor to investigate the effects of different concentrations (0 μg/L (control group), 0.3 μg/L, 0.6 μg/L, and 0.9 μg/L) of sodium nitroprusside on the EHP copy number and the structure in the hepatopancreas of *Exopalaemon carinicauda* infected by EHP. The results showed that the EHP copy number in the sodium nitroprusside group was significantly lower than that in the control group, and the hepatopancreas in the sodium nitroprusside injection group had less structure damage than the control group. Additionally, superoxide dismutase (SOD) activity in the control group and 0.3 μg/L groups increased and then decreased, while alkaline phosphatase (AKP) activity decreased and then increased, with the same trend, and, the inducible nitric oxide synthase (iNOS) activity in the haemolymph of the 0.3 μg/L group was significantly higher than that of the control group at 3 days ($P < 0.05$). The NO content the 0.3 μg/L group was also higher from 1 to 4 days than that in the control group and significantly higher than that in the control group at 3 days ($P < 0.05$). The injection of NO could reduce the EHP copy number in infected *E. carinicauda* and slow down the pathological changes in the hepatopancreas structure, which might be related to the increase in NO content.

1. Introduction

Enterocytozoon hepatopenaei (EHP) is an intracellular parasitic fungus that causes hepatopancreatic microsporidiosis (HPM) in shrimp [1, 2]. HPM can infect a variety of marine and freshwater shrimp including *Litopenaeus vannamei*, *Penaeus monodon*, and *Macrobrachium rosenbergii*. Infected shrimp grow slowly and can lead to death in severe cases. The disease is prevalent in shrimp-farming countries, such as Thailand, India, Vietnam, Malaysia, Indonesia, and China, and is hazardous to global shrimp farming [3, 4]. Horizontal transmission of EHP occurs through EHP-contaminated feed, bait, diseased shrimp residues, and aquaculture water containing EHP spores, and EHP can also be transmitted vertically from the parent host to the offspring host [5]. EHP spores are easily inactivated outside the host. According to one study, EHP spores that have been frozen at -20°C for more than 2 hours or immersed in 15 mg/L KMnO4, 40 mg/L 65% active chlorine, or 20% ethanol for 15 minutes will inhibit their polar filament release and fail to form budding bodies, thereby losing their ability to proliferate. Salachan et al. discovered that live EHP

spores being in water for 10 days without oxygenation or treated with 20 ppm calcium hypochlorite (concentration 90%) for 24 hours would lose their activity [7]. All the above studies investigated the inactivation of EHP *in vitro*, but because of its thick cell wall consisting of titin and protein and intracellular parasitism [8], no effective drugs have been reported to inhibit EHP infestation in living organisms.

Nitric oxide (NO) is a kind of small molecule substance with wide functions and unique properties in organisms, which is synthesized by nitric oxide synthase (NOS) [9]. NO plays a role in the regulation of nerve and muscle activity, feeding, defense, and environmental stress [10], and different concentrations of NO can play different roles [11]. It has been found that NO has the effect of removing *Vibrio harveyi* infected by *Litopenaeus vannamei* [12]. It is found in *Procambarus clarkii* of various molluscs and crustaceans that NO produced by microbial infection can enhance the ability of blood cells to gather bacteria, thereby enhancing the bactericidal capacity of blood cells [13]. Sodium nitroprusside (SNP, the molecular formula is $Na_2 [Fe(CN)_5NO] \cdot 2H_2O$) is the most widely used NO source in biological research, which can hydrolyze to release NO [13]. It has been found that the immune defense ability of *Eriocheir sinensis* injected with SNP has been improved, but whether exogenous NO provided by SNP is effective in slowing down EHP infection has not been studied [13].

Exopalaemon carinicauda, belonging to Arthropoda, Crustacea, Decapoda, Nematoda, and Palaemonidae, is an important economic shrimp in China [14, 15]. It is a potentially ideal model organism for the study of marine shrimp, as its growth physiology, reproductive development, and genetic breeding have been widely studied [16–22]. At present, the infection of EHP is also found in the process of cultivation of *E. carinicauda*, but there are still few drug studies on the treatment of EHP. Therefore, this study takes *E. carinicauda* as the research object, selects SNP as the NO donor, and explores the effect of different concentrations of SNP on the prevention of EHP infection, in order to reduce the loss of EHP in the cultivation industry of *E. carinicauda*.

2. Materials and Methods

2.1. Preparation of Crude Extracts of EHP Spores. According to Yang's methods [23], the hepatopancreas of EHP-infected *E. carinicauda* was homogenized by adding twice the volume of phosphate-buffered saline (PBS) to the tissue and then filtered through a filter membrane (effective pore size 10 μm) to remove residual cells or other impurities. The precipitate was resuspended with a small amount of PBS and repeated 3 times; the collected precipitate was centrifuged at 1000 r/min for 2 min to remove the supernatant; the precipitate was centrifuged at 9000 r/min for 5 min to remove the supernatant and repeated 3 times; the precipitate was resuspended with a small amount of PBS to obtain the crude extract of EHP spores and set aside at 4°C.

According to the method of Jiang et al. [24], the crude extract of EHP spores was dyed with 2% Phloxine B and then dropped onto a hemocytometer covered with a coverslip.

The number of spores in the crude extract of EHP spores was calculated to be 7.5×10^7 pieces/mL.

2.2. Injection of Exogenous Nitric Oxide and the EHP Artificial Infection Test. Healthy shrimps were taken from the aquaculture pond in Lianyungang, China, with a body length of 3.5 ± 0.3 cm and a weight of 1.5 ± 0.3 g. After being transported back to the laboratory, 9 randomly selected shrimp were determined to be free of EHP infection by qPCR detection and were then temporarily raised at 28°C, pH 8.1 ± 0.2, and salinity 25‰ for three days. At the beginning of the study, the toxicity of nitroprusside to *Exopalaemon carinicauda* was studied by the acute toxicological test. By calculating the mortality rates of 24, 48, and 72 hours (Table 1), the safe concentrations at each time point were 34.326 mg/L, 24.278 mg/L, and 18.709 mg/L, respectively, as calculated by Jia's method [25].

The study set up four groups with 30 shrimps in each group, i. e. control group, group A, group B, and group C, repeating three times for each group. The concentration of SNP (purchased from Aladdin) injected into the pericardial cavity of individuals in each group was 0 (control group), 0.3 (group A), 0.6 (group B), and 0.9 μg/L (group C). Finally, each shrimp was injected with 10 μl SNP solution, and the final NO concentrations of each group were 0 (control group), 1.0069 (A group), 2.0138 (B group), and 3.0206 (C group) nM, respectively. After injection, the shrimp were placed in water with a concentration of 321 EHP spores/mL for infection. During the period, we would feed the shrimp with formulated feed (HaidaHailong, China) at 6 a.m. and 8 p.m. The shrimps were anesthetized by freezing. The hepatopancreas of 9 shrimps were taken at 0, 24, 48, 72, and 120 h after infection to measure EHP cope numbers and superoxide dismutase (SOD) and alkaline phosphatase (AKP) activities, the hepatopancreas sections were manufactured, and haemolymph was taken to measure nitric oxide synthase (iNOS) activity and nitric oxide content.

2.3. EHP qPCR Standard Curve Plotting and Hepatopancreas EHP Copy Number Detection. Total DNA from the hepatopancreas was extracted using Ezup Column Animal Genome DNA Extraction Kit (see the instructions for specific steps) (Sangon Biotech, Shanghai, China).

The EHP qPCR standard curve was performed using DNA from EHP *E. carinicauda* as the template, according to the method of Santhoshkumar et al. [26]. The primer pair was EHP-F (5′GCCTGAGAGATGGCTCCCACGT3′)/EHP-R(5′GCGTACTATCCCCAGAGCCCGA3′), and the amplification product was 510 bp in length. After PCR amplification, 1% agarose gel was used for detection. The PCR reaction system was a $2 \times$ Taq Mix II (Dye plus) (Vazyme, Nanjing, China) reaction system, and the PCR reaction program was set at 95°C for 3 min, 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and 72°C for 5 min, for a total of 30 cycles. According to the method of Jiang et al. [27] EHP standard DNA was cloned. Then, standard DNA was extracted and diluted to 20 ng/μl to obtain a copy number of 3.62×10^{10} copies/μL. The standards were

Table 1: Experimental results of acute toxicity of sodium nitro-prusside on *Exopalaemon carinicauda*.

Concentration (mg/L)	Mortality rate		
	24 h (%)	48 h (%)	72 h (%)
5	5.56	13.89	22.22
10	13.89	16.67	27.78
15	19.44	27.78	38.89
20	19.44	41.67	52.78
25	27.78	44.44	66.67
0	0.00	0.00	2.78

concentrated in a gradient of 3.63×10^9, 3.63×10^8, 3.63×10^7, 3.63×10^6, and 3.63×10^5 copies/μL.

According to the method of Liu et al., the copy number of hepatopancreas EHP was detected [28]. Sample DNA was diluted to 20 ng/μL with the primer pair SSUR-F(5'GTAGCGGAACGGATAGGG3')/SUR-R(5'CCAGCATTGTCGGCATAG3'), and the amplification product length was 185 bp, after which a copy number of EHP in the hepatopancreas was calculated. The qPCR reaction system was made according to the instructions of the Cham Q Universe SYBR qPCR Master Mix kit with 10 μL of reagents. The program running qPCR is as following: first 95 °C for 30s, and then 40 cycles in this order (60 °C for 1 min, 95 °C for 10 s, and 60 °C for 30 s).

2.4. Preparation of Hepatopancreatic Tissue and Masson Staining. Hepatopancreatic tissues were fixed with 4% paraformaldehyde for 24 h and then used for paraffin sectioning and Masson staining. Neutral gum was used to seal the sections, and then, the sections were observed.

2.5. Detection of Nonspecific Immunological Indicators. The haemolymph of *E. carinicauda* was extracted and assayed for NO content by the one-step method and iNOS activity by the colorimetric method; the hepatopancreas was crushed to measure SOD activity by the WST-1 method and AKP activity by the microenzymatic method with reference to the kit (Nanjing Jiancheng Institute of Biological Engineering, Nanjing, China).

2.6. Data Analysis. Relevant data were analyzed using SPSS 25 (IBM Corporation), multiple comparisons were performed using Duncan's one-way ANOVA, and significant differences were $P < 0.05$ [29]. Graphs were drawn using Origin 2018 (OriginLab Inc) software, and the data were analyzed by the mean ± standard deviation (mean ± SD).

3. Results and Analysis

3.1. Effect of Exogenous Nitric Oxide on EHP Copy Numbers in the Hepatopancreas of E. carinicauda. The EHP copy numbers in the hepatopancreas of individual *E. carinicauda* were measured at each time point using qPCR, and the results are shown in Figure 1. The EHP copy numbers in the hepatopancreas of each individual group showed an overall increasing trend with time, and there was no significant difference in the EHP copy numbers of each group in the first 3 days. On the fourth day, the EHP copy number of the control and C groups increased significantly ($P < 0.05$) and the relative copy number of EHP in the hepatopancreas of the group injected with exogenous NO was markedly lower than that of the control group. Specifically, the EHP copy numbers of groups A, B, and C were 0.25 times, 0.31 times, and 1.03 times higher than those of the control group. On the 5th day, the EHP copy numbers of the control group were markedly higher than those of other groups ($P < 0.05$), while those of group B were markedly lower than those of group C ($P < 0.05$). There was no significant difference between groups A and B. The EHP copy numbers of groups A, B, and C were 0.25, 0.22, and 0.3 times of those of the control group.

3.2. Effect of Exogenous Nitric Oxide on the Hepatopancreas of E. carinicauda. The results of individual hepatopancreas sections at each time point are shown in Figure 2: one day after EHP infection, some of the groups showed deformation of the lumen cross-section and vacuolation of the hepatic tubules and a few EHP spores appeared in the interstitial space of the hepatic tubules in the control group (Figure 2-A1) and group B (Figure 2-C1); at 3 days of infection, basophilic inclusion was observed only in the control group (Figure 2-A2), although the interstitial enlargement and vacuolation of hepatic tubules increased and spores aggregated in all groups. At day 5 of infection, the hepatopancreas of each group had shrimp liver enterocyte spores and EHP spores, but the control group (Figure 2-A3) had the atrophied and deformed hepatopancreas, and the degree of hepatopancreas vacuolization was more severe in group A (Figure 2-B3), group B (Figure 2-C3), and group C (Figure 2-D3).

3.3. Effect of Exogenous Nitric Oxide on the Immunity of E. carinicauda. Combining the changes in the hepatopancreas EHP copy number and tissue structure revealed that exogenous nitric oxide could slow down the EHP infestation of *E. carinicauda*. iNOS activity and NO content in haemolymph and the SOD activity and AKP activity in hepatopancreatic tissues were examined on behalf of each group. The results were compared with those of the control group as follows.

The effect of exogenous NO on the iNOS activity of *E. carinicauda* is shown in Figure 3. The trend of change in haemolymph iNOS activity in group A increased and then decreased. iNOS activity in group A was significantly lower than that in the control group on the first day ($P < 0.05$) but was significantly higher and peaked in group A on the third day.

The effect of exogenous NO on the NO content of *E. carinicauda* is shown in Figure 4. The trend of NO content in haemolymph increased and then decreased, with the NO content in group A being significantly higher than that in the control group at 1 and 4 days ($P < 0.05$).

The effect of exogenous NO on superoxide dismutase of *E. carinicauda* is shown in Figure 5; the trend of SOD activity in both groups increased and then decreased. The difference in SOD activity between the two groups was not significant ($P > 0.05$). The effect of exogenous nitric oxide on alkaline
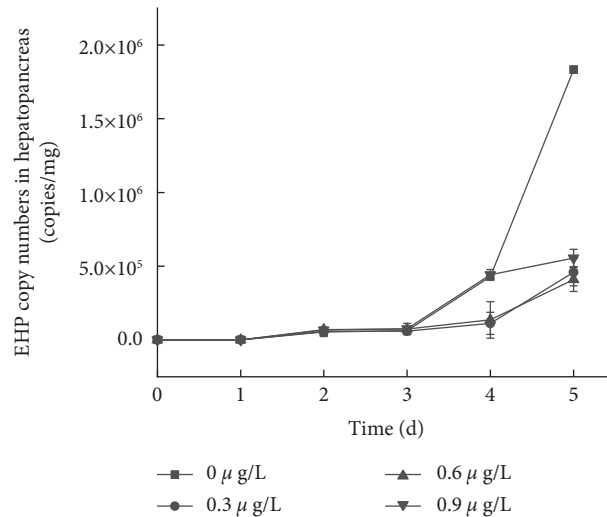
FIGURE 1: EHP copies of the EHP-infected hepatopancreas of *Exopalaemon carinicauda* after exogenous NO injection.
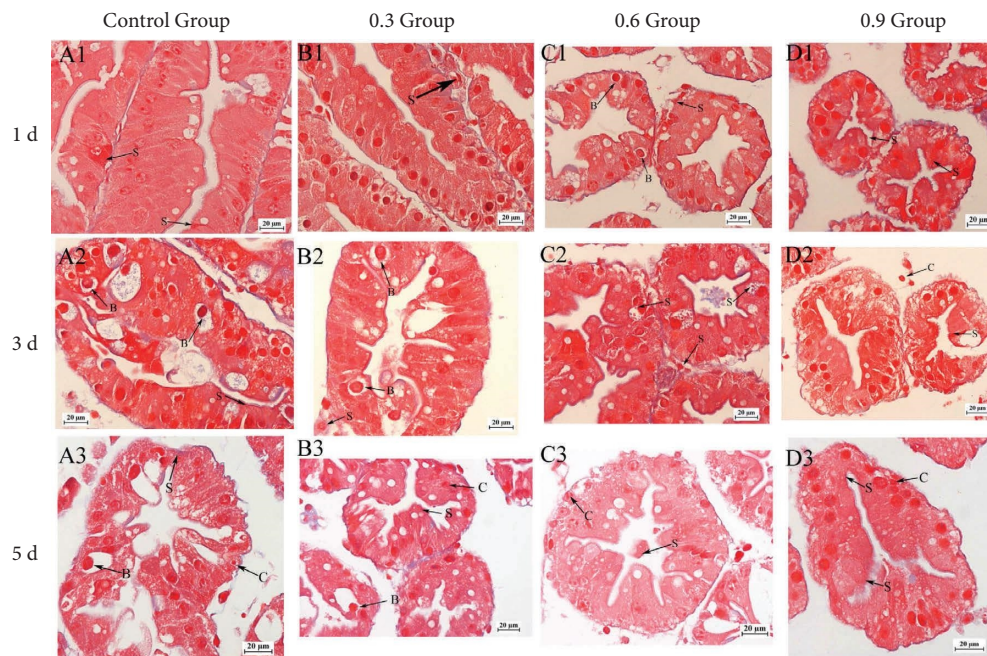

FIGURE 2: The structure of the hepatopancreas of *Exopalaemon carinicauda* infected with EHP by exogenous NO injection. The control group at 1 d, 3 d, and 5 d corresponded to A1, A2, and A3, respectively; group A at 1 d, 3 d, and 5 d corresponded to B1, B2, and B3, respectively; group B at 1 d, 3 d, and 5 d corresponded to C1, C2, and C3, respectively; group C at 1 d, 3 d, and 5 d corresponded to D1, D2, and D3, respectively. B, basophilic inclusion; S, spore; C, cyst.

phosphatase activity in *Exopalaemon carinicauda* is shown in Figure 6, while the trend of AKP activity in both groups at 4 d was significantly higher than the other times and AKP activity in group A was significantly higher than that in the control group ($P < 0.05$).

4. Discussion

As a highly reactive free radical, nitric oxide (NO) and its oxidized derivatives contribute to the antimicrobial function of free radicals. For examples, in mammals and invertebrates, it is involved in immunological responses such as cell signaling and cytotoxic molecules, as well as in reaction to parasite infections [11, 30]. In the present study, we found that exogenous NO also had the same effect of slowing down EHP infestation, such as the EHP copy number being significantly lower in the 0.3 μg/L, 0.6 μg/L, and 0.9 μg/L groups than that in the control group at day 5 ($P < 0.05$). Moreover, the degree of hepatopancreatic lesions was also much less in infected individuals than that in the control. In addition, this study also found that the degree of hepatopancreas injury of *Exopalaemon carinicauda* at each
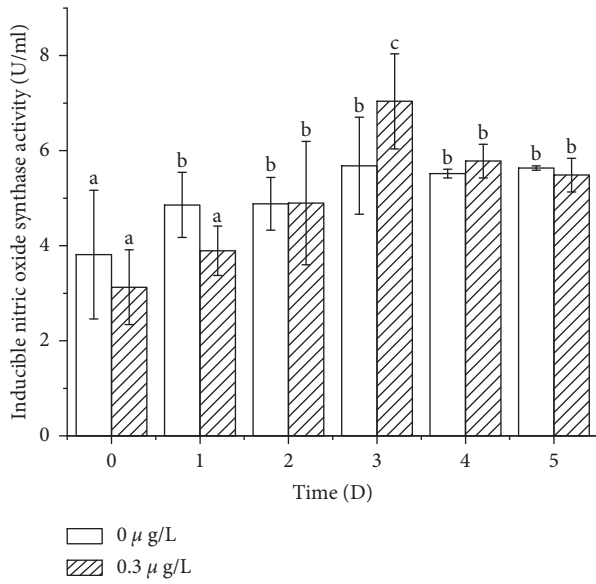
FIGURE 3: Effect of exogenous NO injection on the activities of nitric oxide synthase in *Exopalaemon carinicauda*.
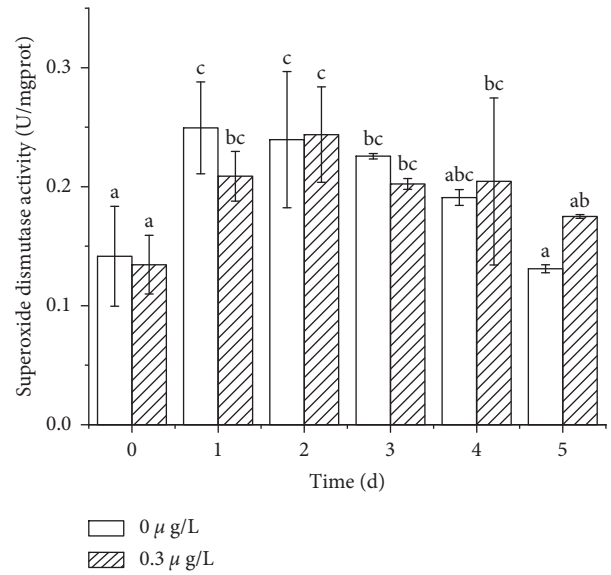

FIGURE 5: Effect of exogenous NO injection on the activities of superoxide dismutase in *Exopalaemon carinicauda*.
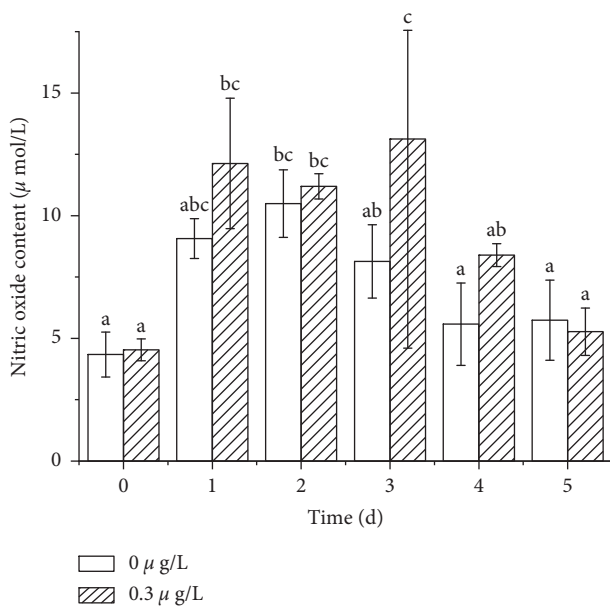

FIGURE 4: Effect of exogenous NO injection on nitric oxide content in *Exopalaemon carinicauda*.
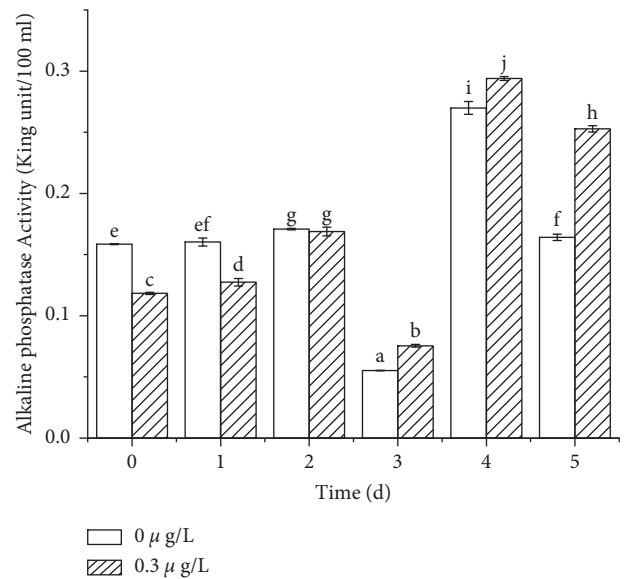

FIGURE 6: Effect of exogenous NO injection on the activities of alkaline phosphatase in *Exopalaemon carinicauda*.

sampling time point was consistent with an increase in the EHP copy number, and the same result was obtained when EHP infected *Litopenaeus vannamei* [28], indicating that the higher the EHP copy numbers, the greater the damage to tissues and organs. This study showed that NO can inhibit the increase in the EHP copy number and reduce symptoms to a certain extent.

It has been shown that NO and exogenous NO induced by organisms are toxic to parasites such as filarial worms, nematodes, and trematodes [30], while injection of sodium nitroprusside (SNP) increased the NO content in the intestine of *Antheraea pernyi* compared to the control group and

inhibited *Nosema pernyi* from attacking the intestine of *A. pernyi* [31]. In the present study, NO levels were higher in *E. carinicauda* than in the control group from days 1–4 after the injection of 0.3 μg/L. The EHP copy number in group A was also lower than that in the control group at this time, while in group A, NO levels decreased and the EHP copy number increased at day 5. Therefore, we believe that NO produced by SNP is one of the reasons for slowing down EHP infection.

The hepatopancreas participates in organism metabolism and innate immune response, and its structural integrity will affect its function [32]. In this study, the hepatopancreas was more intact and structurally clear in the 0.3 μg/L group than that in the 0.6 μg/L and 0.9 μg/L groups,

suggesting that SNP injected at 0.3 μg/L was instead more effective in slowing down EHP invasion. The 0.6 μg/L and 0.9 μg/L groups were able to release more NO, but excess NO promotes inflammation and leads to cellular and tissue dysfunction [33]. This study found that the damage to the hepatopancreas structure in these two groups is more severely damaged than that in the 0.3 μg/L group. At the same time, although SNP injection increased NO levels in the haemolymph of E. carinicauda, it inhibited inducible nitric oxide synthase (iNOS) activity in haemolymph. Specifically, the iNOS activity in the 0.3 μg/L group was lower than that in the control group at day 1, while in the 0.3 μg/L group, the NO content in haemolymph decreased at 3 d and the iNOS activity gradually increased. Because iNOS can catalyze the production of NO [13], when E. carinicauda can use exogenous NO to participate in the phagocytosis of the organism and defend against EHP infestation, iNOS activity in the 0.3 μg/L group was lower than that in the control group, but as the NO content of the organism decreased, iNOS activity gradually increased in the 0.3 μg/L group.

NO can slow down EHP infestation through phagocytosis, so we also investigated the phagocytosis-related superoxide dismutase (SOD) and alkaline phosphatase (AKP) activities in E. carinicauda. SOD is the first line of defense against excess reactive oxygen molecules in organisms and specifically dismutates O^{2-} into hydrogen peroxide (H_2O_2), while AKP is the material basis for phagocytic bactericidal activity [34, 35]. It was found that the effect of SNP injection on SOD in shrimp was insignificant and that there was a promotive effect on AKP. The AKP activity in group A was higher than that in the control group at 4 and 5 days, indicating that 0.3 μg/L SNP had a promotive effect on AKP in the hepatopancreas of E. carinicauda. The increase in NO content in L. vannamei will also increase the AKP activity level [36], whereas in Chinese mitten crab, injected with a total of 5.04 ml of 1000 μmol/L SNP (about 5×10^7 times that of the 0.3 μg/L group), AKP in the serum was inhibited after 48 h, which may be related to tissue dysfunction in the organism [13].

Data Availability

The results of the experimental data are presented in the form of pictures without additional supplements and presentations.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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