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Antiviral activity of cathelicidin 5, a peptide from *Alligator sinensis*, against WSSV in caridean shrimp *Exopalaemon modestus*

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ABSTRACT

White spot disease caused by white spot syndrome virus (WSSV) is responsible for harming shrimp aquaculture industry and results in a pandemic throughout the world. Cathelicidin 5 treatment enhanced immune parameters including antioxidant enzyme activity and immune-related genes expression in shrimp *Exopalaemon modestus*. Shrimp treated with cathelicidin 5 and inoculated with white spot syndrome virus (WSSV) exhibited a significantly lower mortality rate and lower viral VP28 amplification and expression than control. This study addresses the role of cathelicidin 5 in immune stimulatory and antiviral activities that could protect *E. modestus* from WSSV infection.

1. Introduction

The caridean shrimp (*Exopalaemon modestus*), belongs to the Palaemonidae family, is mainly distributes in many fresh water lakes in China. It has great economic value in fishing and aquaculture for accounting for more than half of total shrimp products in some big lakes. However, this industry has suffered from various bacterial and viral pathogens. More than twenty viral pathogens were reported to infect shrimps, whereas white spot syndrome virus (WSSV), caused shrimp white spot disease (WSD), is the most serious virus [1,2]. Globally the losses of WSD infection approach $10 billion [3]. The World Animal Health Organization (OIE) has listed WSD as a notifiable crustacean disease, because WSD is considered as the most serious shrimp viral disease. WSSV is rod-shaped dsDNA virus with high lethal and stress dependence [4]. It can be vertically transmitted from infected broodstock to their offspring or horizontally from infected shrimp to healthy one via water [5,6]. The discovery of antibiotics is definitely a great achievement in bacteria disease, but it is not effective to cure virus disease of shrimp since no adaptive immunity exists in them [7,8].

Like other arthropod species, shrimp mainly rely on an innate immunity system to protect themselves [8]. The responses to infectious pathogens of the innate defense system of shrimp are grouped into humoral and cellular immunity. Cellular responses comprise several different mechanisms, such as apoptosis, encapsulation, phagocytosis and nodulation, whereas the humoral responses include the prophenoloxidase, antimicrobial peptides (AMPs) synthesis, and the clotting cascade [9,10].

Host defense peptides (HDPs, also referred to as antimicrobial peptides) play important role in killing or cleaning the infected pathogens directly as products of immune response [11]. HDPs in shrimp attract a lot of attention due to their function in killing bacteria or virus. They play critical roles in host immune response against pathogen invasions, such as bacteria, virus, fungi, and even parasites [12], and what's more, they can also promote and regulate the host immune responses [13,14].

Cathelicidin 5 is a member of HDPs that was identified from the Chinese alligator previously [15]. It played an important role in anti-infective responses. The results of previous study have indicated that cathelicidin 5 possess potent antimicrobial and immune-modulatory activities [15]. In peritonitis mice model, they exhibited effective protection for mice against bacterial infections through immune cells recruitment [15].

In this study, we identified the immune-protective effect of cathelicidin 5, a natural HDP from *Alligator sinensis*, on caridean shrimp with WSSV infection. Interestingly, we noticed that shrimp treated with cathelicidin 5 showed a more favorable survival rate. Therefore, it is

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possible that cathelicidin 5 may be exhibiting immunostimulant and antiviral activities. Accordingly, in the present study cathelicidin 5 was characterized in order to determine its immunostimulant and antiviral activities against WSSV. Cathelicidin 5 would be a potential precursor of novel peptide drugs for the treatment of shrimp virus disease.

2. Methods and materials

2.1. Shrimps and peptides

The healthy adult caridean shrimps (*E. modestus*), weighting 3.11 g ± 0.47 g, were purchased from a commercial farm in Suzhou, China. After collection, the shrimps were maintained in circulating tanks with aerated tap water at 23 ± 1 °C. The shrimps were maintained under conditions described upon for one week before experiment. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Soochow University (Suzhou, China). Tissues including hemolymph, hepatopancreas, and gill were subjected randomly to PCR assays to ensure that the shrimps were WSSV-free before experimental challenge.

Peptide (cathelicidin 5: TRRKFWKKVLNGALKIAPFLLG) was synthesized by the peptide synthesizer GL Biochem Ltd. (Shanghai, China). The crude peptides were purified by RP-HPLC, and the identity was analyzed by MALDI-TOF MS. Purity of the synthetic peptides was confirmed to be higher than 95%. Peptides were dissolved in PBS to 2 mg/mL as stock solution before experiments.

2.2. Virus and WSSV challenge

WSSV (GenBank accession No. AF332093.1) was purified and used in challenge experiments. WSSV infected shrimp, *Litopenaeus vannamei*, were kindly provided by Dr. Hongtuo Fu in Freshwater Fisheries Research Center, CAFS, Wuxi, China. The WSSV inoculum was prepared from WSSV infected shrimp according to Zhu et al. [16]. The viral infective value was 10^7 copies/mL (identified by qRT-PCR), and *E. modestus* were intramuscularly injected with 20 μL (10^0-10^7 copies/mL) filtered supernatant obtained from WSSV infected *Litopenaeus vannamei* in the viral challenge test. Subsequently, the infected shrimp were reared for additional 10 days. Based on the statistical data of the mortality experiment, we chose 10^4 copies/mL for the further tests.

Shrimps were treated in each of four groups: (1) control group (phosphate-buffered saline, 20 μL PBS injected); (2) WSSV challenged group were injected intramuscularly into healthy shrimp in the lateral area of the fourth abdominal segment using a syringe with a 29-gauge needle. (3) Pre-stimulation group. 2.5 μg cathelicidin 5 were injected into shrimps, and 24 h later, the prepared WSSV dilution was injected into shrimps. (4) Co-stimulation group. 2.5 μg cathelicidin 5 and WSSV dilution were simultaneously injected into shrimp. After WSSV challenge, survival rates were recorded daily for 6 days. Survival curves were plotted using KaplanMeier analysis and differences in survival rates were analyzed by the log-rank test. Differences with P values of < 0.05 were considered statistically significant.

2.3. Tissue samples collection

The gill, hemolymph, hepatopancreas, and muscle were collected form health or challenged shrimps. The samples were used immediately for immune parameters analysis or DNA/RNA isolation.

2.4. DNA extraction and viral load quantification

DNA was extracted using E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek, USA) following the manufacturer's instructions and protease K was used additionally at a final concentration of 2 μg/μL for sample digestion. Extracted DNA was subjected to electrophoresis in 1% agarose gel and quantified by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., USA).

Table 1
Summary of primers in this study.

Primer name	Sequence (5′–3′)
QVP28-F	AAACCTCCGCATTCCTGTGA
QVP28-R	TCCGCATCTTCTTCCTTCAT
JAK-F	TGCTGTTCCGACTGCGTTTC
JAK-R	GCGTGGAAGTCTGCTCGAAC
Relish-F	CTACATTCTGCCCTTGACTCTGG
Relish-R	GGCTGGCAAGTCGTTCTCG
proPO-F	TCCATTCCGTCCGTCTG
proPO-R	GGCTTCGCTCTGGTTAGG
Dorsal –F	GATGGAATGATAGAATGGGAAGC
Dorsal –R	CACTGGTACTCTTGTCTGGTGGTC
SOD-F	ATCACTCACGGACTGGTTCC
SOD-R	GAGAGAAACGCCCTTGTGAC
CAT-F	GCCCGTACAAGGAACTACCA
CAT-R	TGACGTTCTGCCTCATTCAG
Hsp70-F	CCTCCAGGACTTCTTCAACG
Hsp70-R	GGTCACGTCCAACAGCAAC
β-actin-F	GTGCCCATCTACGAGGGATA
β-actin-R	TAGGACTTCTCCAGCGAGGA

Viral load was quantified by WSSV envelope protein VP28 recombinant plasmid. The series of dilution of WSSV standard DNA was then prepared to get the WSSV copy number standard curve [17]. The six different concentrations were 10^9 copies/mL,10^8 copies/mL, 10^7 copies/mL,10^6 copies/mL,10^5 copies/mL, and 10^4 copies/mL. Diluted solutions of plasmid were used as standard samples to generate a standard curve in the quantitative real-time PCR, performed with primers QVP28F and QVP28R (Table 1) to determine the viral loads using a Mastercycler ep realplex (Eppendorf, Germany). Each assay was carried out in triplicate. Melting curves were produced to confirm that only one specific PCR product was amplified.

2.5. Total RNA isolation and synthesis of cDNA

The total RNA from tissues was isolated following the manufacturer's instruction of RNA extraction kit (Omega, Netherlands), treated with DNase I and column purified, and stored in a −80 °C freezer until analysis. The concentration of total RNA was quantified using a NanoDrop 2000 spectrophotometer (Hach, America), with OD260/OD280 between 1.80 and 2.00. Reverse transcription polymerase chain reaction (RT-PCR) was performed using cDNA synthesis kit (Takara, Japan) using total RNA (500 ng/μL).

2.6. Real-time quantitative PCR analysis

RT-qPCR was performed using a CFX96 Real Time PCR system (Bio-Rad, Foster City, CA, USA) along with gene-specific oligonucleotides designed from immune genes. qRT-PCR was performed using a SYBR Green (Applied Biosystems, USA) and normalized to β-actin gene of *E. modestus*. Primers of Janus Kinase (JAK), relish, prophenoloxidase (proPO), Dorsal, Catalase (CAT), heat shock protein 70 (Hsp 70), and superoxide dismutase (SOD) were shown in Table 1. The reactions were done in a 96-well plate in a 20 μL reaction volume, including 10 μL of SYBR mixture, 2 μL of cDNA, 0.5 mM of each genes primer, and 7 μL of nuclease-free water. The reaction steps involved an initial denaturation at 95 °C for 30 s, 40 cycles (95 °C for 15 s, 58 °C for 20 s to anneal), and 72 °C for 30 s. A dissolution curve temperature from 60.0 to 95.0 °C was created, and increased by 0.5 °C per 0.05 s.

2.7. Immune parameters analysis/antioxidant enzyme activity assays

Shrimp were randomly divided into five groups (20 per group): 0 μg/g (shrimps injected with equal volume of PBS buffer), 1 μg/g, 10 μg/g, 20 μg/g, and 40 μg/g cathelicidin 5. Shrimp were maintained

as described upon. Cathelicidin 5 was dissolved in PBS buffer and sterilized by filtration. Peptide was intramuscularly injected using a 1 mL plastic syringe. Hemolymph was collected at 0 h, 24 h, and 48 h after injection using a 1 mL plastic syringe. The hemolymph was plated at room temperature until clotted, and then stored at 4 °C overnight. The serum was extracted by centrifuge 5000 rpm at 4 °C for 10 min, and then was used for enzyme activity assay. The activities of lysozyme (LZM), acid phosphatase (ACP), alkaline phosphatase (AKP), and peroxidase (POD) were measured using corresponding detection kits (Jiancheng, Nanjing, China) according to the manufacturer's guidelines.

2.8. Mammalian cytotoxicity determination (in vitro toxicity)

HeLa Cells, Vero cells, HEK293T cells, and RAW264.7 cells were seeded in 96-well plates (5×10^4 cells per well) containing 100 μL medium and incubated at 37 °C overnight until cells reached approximately 90% confluence each well. Then the cell supernatant was replaced by the medium containing cathelicidin 5. A geometric series with a common ratio of 10 was designed covering cathelicidin 5 concentration respectively from 0.2, 2, 20, and 200 μg by using basal medium. The cells treated with PBS were used as the control. Cytotoxicity was determined following the protocol of alamerBlue® cell viability assay (Invitrogen, USA) after cathelicidin 5 treatment for 48 h. Briefly, 10 μL alamarBlue® reagent is added directly to complete media of each well, the plates are incubated at 37 °C to allow cells to convert resazurin to resorufin for 4 h, and the fluorescence signal is measured using an excitation between 530 and 560 nm and an emission at 590 nm by Synergy H4 Hybrid Reader (BioTek, USA).

2.9. Shrimp toxicity determination (in vivo toxicity)

E. modestus were divided into five groups (20 shrimps in each) and the experiment was performed in three replicates in order to ensure the reproducibility of the results. Shrimps were injected with series concentrations of cathelicidin 5, 1 μg/g, 10 μg/g, 20 μg/g, 40 μg/g, and 80 μg/g, and mortality was recorded daily. Control groups were injected with 20 μL of 1x PBS. The shrimps were maintained in circulating tanks Values ± standard deviation for 3 independent experiments are shown.

2.10. Histological assay

Twenty-four hours post WSSV infection, shrimps from 4 groups were sacrificed and the hepatopancreas tissues were fixed in 10% formalin and paraffin embedded. Hematoxylin and eosin (H&E) staining was conducted for histopathology.

2.11. Data analysis and statistics

Statistical significances were calculated with an unpaired two tailed Student's t-test and Log-rank (Mantel-Cox) Test (for survival data only) using GraphPad Prism 8 software (San Diego, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity of cathelicidin 5 in vitro and in vivo

In crustacean, no permanent cell line has been reported. Nonetheless, several mammalian cell lines (HeLa cells, Vero cells, HEK293T cells and RAW264.7 cells) were used to identify the cathelicidin 5 toxic. As presented in Fig. 1, cathelicidin 5 was demonstrated no toxic to cells cultured with 48 h at dose increased according to alamarBlue® cell viability test.

Before the animal experiment, we examined the toxicity of the peptides to shrimps. *E. modestus* was used to evaluate the toxicity of the cathelicidin 5 *in vivo*. The use of shrimp as an invertebrate animal model to test *in vivo* toxicity is crucial because the toxicity *in vivo* can be significantly different, in some cases. For example, the toxicity of the cathelicidin 5 increased *in vivo* while the *in vitro* toxicity decreased in comparison to the mammalian cell lines. Compared with the control group, we observed more than half moribund shrimps at higher concentrations of cathelicidin 5 (40 μg/g) at about 72 h after injection (Table 2). Therefore, although the lower concentration of cathelicidin 5 (1 μg/g, 10 μg/g, and 20 μg/g) treated groups showed lesser toxicity than did the high concentration of cathelicidin 5 (40 μg/g) treated group on shrimp, but at high concentrations (80 μg/g), the cathelicidin 5 had a toxic effect on the shrimp. Further, the degree of lethality was found to be directly proportional to the concentration of the cathelicidin 5 used. Our results highlight the use of an animal model for toxicity evaluation to better select a host defense peptide for further investigative steps or drug development.

3.2. Antiviral activity of cathelicidin 5 in vivo

As shown in Fig. 2A, the final cumulative mortalities of shrimp infected with WSSV at 10^3-10^7 copies were from 40% to 100%, whereas, the shrimp final cumulative mortalities, infected with WSSV at 10^0-10^2 copies, were 15%–30%. We observed the typical clinical symptoms at 10^3-10^7 copies in the 3rd day after WSSV injection that the moribund shrimp cannot swim at all and can only shake their pleopods slightly. The results suggested there existed a closely correlated regression line "$y = 15.476x - 1.667$, $R^2 = 0.9673$" in semi-logarithm graph between shrimp mortalities and WSSV doses (Fig. 2B). It was calculated that 50% lethal dose (LD_{50}) of the viral isolate to shrimp was $10^{3.338}$ copies WSSV per shrimp.

As a first step to determine if catehlicidin 5, as the host defense peptides, protects shrimp to virus infection, we have conducted the survival and transcript expression analysis of the WSSV V28 genes after cathelicidin 5 treatment during WSSV challenge. Kaplan-Meier survival curves of caridean shrimp infected with/without WSSV or cathelicidin 5 were shown in Fig. 2C. The survival rate was greatly improved by shrimp treated with cathelicidin 5 in which 40% reduction (84% survival rate in cathelicidin 5 treatment compared to 44% in WSSV treatment at the 1st day) was detected. In addition, approximately 80% of shrimp co-stimulated with cathelicidin 5 and WSSV died at the 3rd day, whereas only 32% of the shrimp pre-stimulated with cathelicidin 5 died during the challenge. This suggested that cathelicidin 5 is more efficient in immunization rather than in remedy.

The tissue of experimental shrimp *E. modestus* with the highest amount of WSSV was gill and the lowest WSSV-containing tissue was the hemolymph (Fig. 2D–G). Consistent with the mortality, the viral load of cathelicidin 5 treated group in the tested tissues were significantly lower than that in non-cathelicidin 5 treated group. *T* test analysis showed that significant difference of WSSV amount existed between the cathelicindin 5 treated groups and control group. Theses result suggested that cathelicidin 5 could inhibit WSSV infection *in vivo*.

3.3. Cathelicidin 5 attenuated the hepatopancreatic tissue injury induced by WSSV

The hepatopancreatic tissue sections of *E. modestus* challenged with WSSV were shown in Fig. 3. The hepatopancreas of control group showed well-organized glandular tubular structure including blasenzellen (B-cell), fibrillazellen (F-cell), restzellen (R-cell) and embryonalzellen (E-cell) (Fig. 3A). Nevertheless, shrimp infected with WSSV showed severe hepatopancreatic changes including abnormal lumen, reduced B-cells and R-cells, epithelial cells lysis, and cell necrosis (Fig. 3B). Compared with WSSV group, shrimp had less histopathological damage with cathelicidin 5 treatment (Fig. 3C and D). Furthermore, the pre-stimulated with cathelicidin 5 group (Fig. 3C) showed better organized glandular tubular structure than it of the co-stimulated

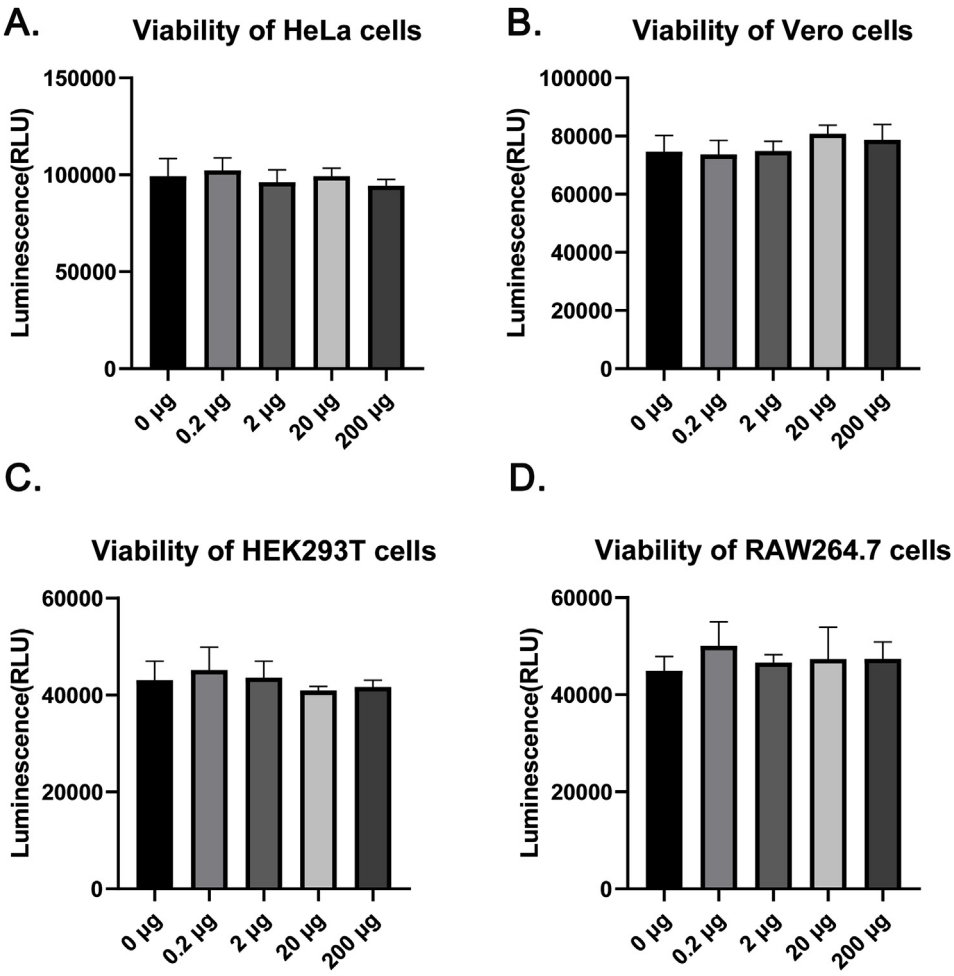

Fig. 1. No cytotoxicity of cathelicidin 5 to mammalian cells.
(A) Stimulated with cathelicidin 5 showed no cytotoxic effect to HeLa cells (Cell Viability assay, Promega). (B) Stimulated with cathelicidin 5 showed no cytotoxic effect to Vero cells. (C) Stimulated with cathelicidin 5 showed no cytotoxic effect to HEK293T cells. (D) Stimulated with cathelicidin 5 showed no cytotoxic effect to RAW264.7 cells.

Table 2
The toxicity of cathelicidin 5 on shrimp lethality test *in vivo*.

Groups	Cathelicidin 5	Total	% Death			
	(μg/g)	shrimp	24 h	48 h	72 h	96 h
I	0	60	0	0	0	0
II	1	60	0	0	0	0
III	10	60	0	2	2	22
IV	20	60	8	17	17	38
V	40	60	30	30	57	88
VI	80	60	62	100	100	100

with cathelicidin 5 and WSSV group (Fig. 3 D).

3.4. Effect of cathelicidin 5 on immune characteristics in E. modestus

In the present study, the ACP, AKP, POD, and LZM activities in the hemolymph of shrimp were measured to evaluate the stimulation effect of cathelicidin 5 on shrimp immune system. As shown in Fig. 4, the activities of ACP, AKP, POD, and LZM were increased with a dose increase with cathelicidin 5. Briefly, the ACP activity increased in each treatment group and reached a maximum at 24 h (Fig. 4A). At the 48 h, the shrimps presented significantly higher AKP activity than that at 24 h, and got a peak activity at the concentration of 20 μg/g (Fig. 4B). There was no significant difference between the 0 h and 24 h in POD activity with concentrate of 1 μg/g, whereas, the concentration of 10 μg/g had a dose increase on the treatment time points (Fig. 4C). For LZM, the significant activity appeared at the time point of 24 h with 1 μg/g, 10 μg/g, and 20 μg/g (Fig. 4D).

3.5. Expression of immune-related genes in tissues of the shrimp

As to determine if immune-related genes are responsive to WSSV infection, we have conducted transcript expression analysis of JAK, Relish, proPO, Dorsal, CAT, SOD, and Hsp70 after infection with WSSV. Fold change was calculated relative to the corresponding timed, PBS-injected control. In addition to hemolymph and hepatopancreas, expression profiles of the immune genes were also determined in gill due to its important role in respiration and a direct contact to the aquatic environment. Expression of the immune genes showed varied changes in the tested tissues following challenge. Shrimp that were infected with WSSV had a better immune response than the controls (Fig. 5). The transcript level of tested immune genes increased dramatically with WSSV infection, and reached their peaks in cathelicidin 5 treated groups, except for the SOD and Hsp70 in gill, and JAK in hepatopancreas. These results show that expressions of most tested immune genes are significantly activated by cathelicidin 5 treatment.

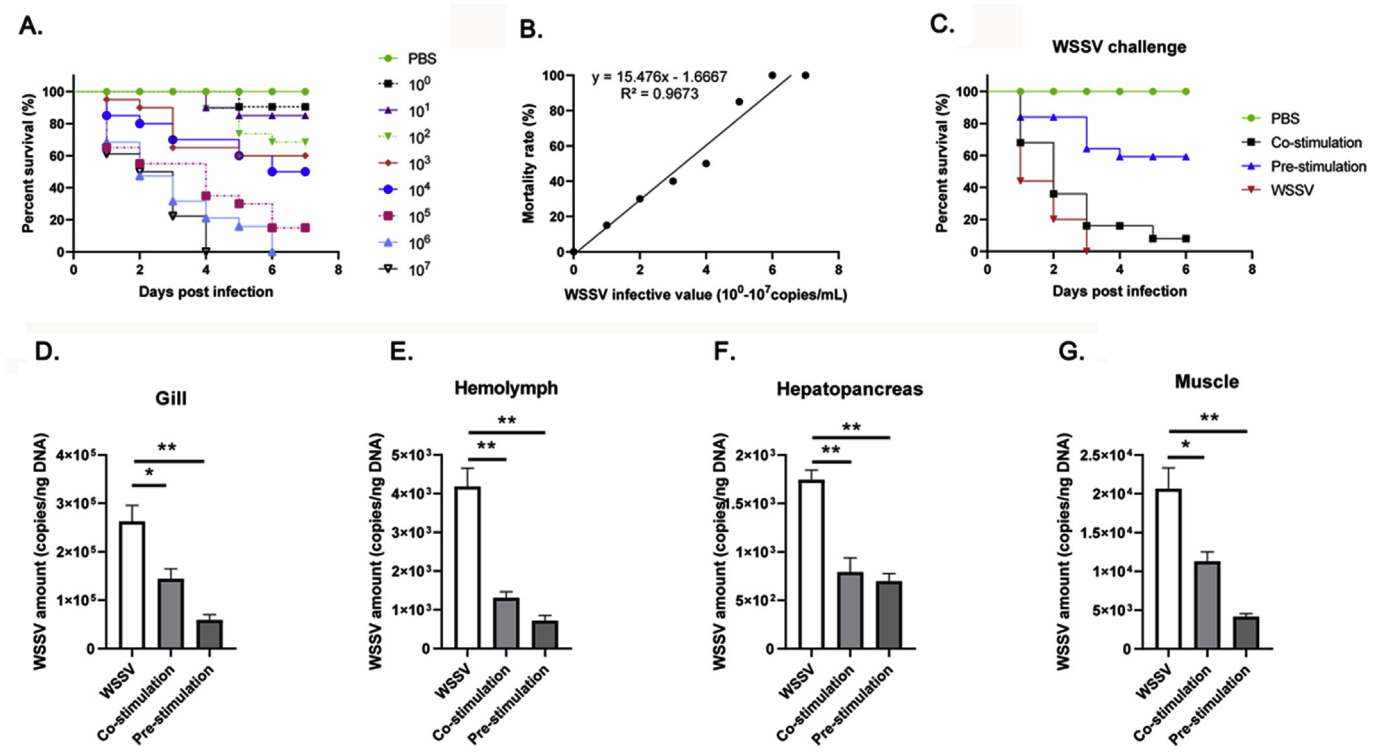

Fig. 2. Cathelicidin 5 exhibited anti-WSSV infection in *E. modestus*.
(A) Survivorship curves of *E. modestus* intramuscularly injected with WSSV at 10^{0-7} copies/mL per shrimp. (B) Correlation of shrimp mortalities and infected WSSV doses. The broken line indicates a regression line for mortality rate and infected WSSV dose. (C) Survivorship curves of shrimp intramuscularly injected with 10^4 copies/mL WSSV and 2.5 μg cathelicidin 5 per shrimp. (D–G) The tissue distribution of WSSV in shrimp gill (D), hemolymph (E), hepatopancreas (F), and muscle (G). Each value was represented as mean ± SEM of three independent experiments. $*p < 0.05$, $**p < 0.01$ (*t*-test).

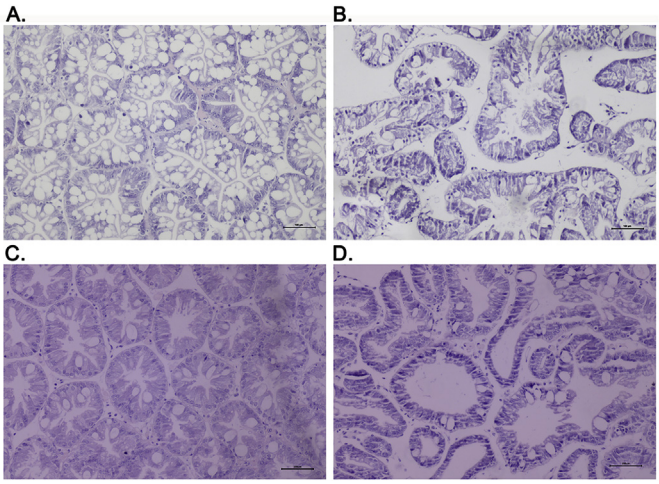

Fig. 3. H&E staining of shrimp hepatopancreas tissues with different treatments.
Transverse section of the hepatopancreas of the shrimp intramuscularly injected with PBS (A), WSSV (B), pre-stimulated with 2.5 μg cathelicidin 5 and 24 h later 10^4 copies/mL WSSV (C), and co-stimulated with 2.5 μg cathelicidin 5 and 10^4 copies/mL WSSV (D). Magnification 200×, Scale bars, 100 μm.

4. Discussion

WSSV is the main pathogens of shrimp. The present study showed that the mortality of WSSV infected shrimp was significantly higher than that of the control group ($p < 0.05$). Simultaneously, the WSSV was detected in several tissues of the shrimp, which revealed that the significant high mortality was due to the replication of WSSV. A common practice in shrimp farming is the excessive use of antibiotics and artificial antimicrobials, which cause pathogen resistance to drugs [18,19], so it is advisable to use natural products that improve the resistance of shrimp to viral diseases [18,20,21].

Here we have explored the potential use of cathelidicin 5 as an immunostimulant to enhance shrimp immune defenses against viral infections. Survival is one of the main parameters in the commercial production of cultured shrimp. Indeed, the cathelicidin 5 improved shrimp survival rate against the WSSV from 44% to 84%. This finding is particularly interesting given the potential antiviral protection conferred by this host defense peptide for 24 h post-WSSV-challenge. This is a reflection of the benefit of cathelicidin 5 in the fight against disease in aquatic organisms, as discussed by Guo et al. [22], who show that used cathelicidin 5 to fight against bacterial infections in crabs and found that cathelicidin 5 have great antibacterial capacity and immunostimulating properties, which greatly improve the survival of crabs in the presence of some pathogens. Considering the similar living environment and relatively conserved innate immune system between the shrimp and crab, we speculated that cathelicidin 5 may also work as immunostimulants to protect shrimps from aquatic diseases attack.

Shrimps mainly depend on innate immune system to resist microbial infections, as they have no adaptive immune memory cells to produce immunoglobulins. The immune response in shrimp is an important parameter for measuring the immune-stimulating effect of certain compounds [23]. Another benefit of the use of cathelicidin 5 in shrimp culture is the improvement in the immune response and greater enzymatic activity in response to oxidative stress, which are products of the fight against pathogenic organisms. Lysozyme, acid phosphatase, alkaline phosphatase, and peroxidase are important immune parameters in crustaceans, which have been widely used as indicators for shrimp health and stress tolerance [24–26].

AKP and ACP are important components of the lysosome system of

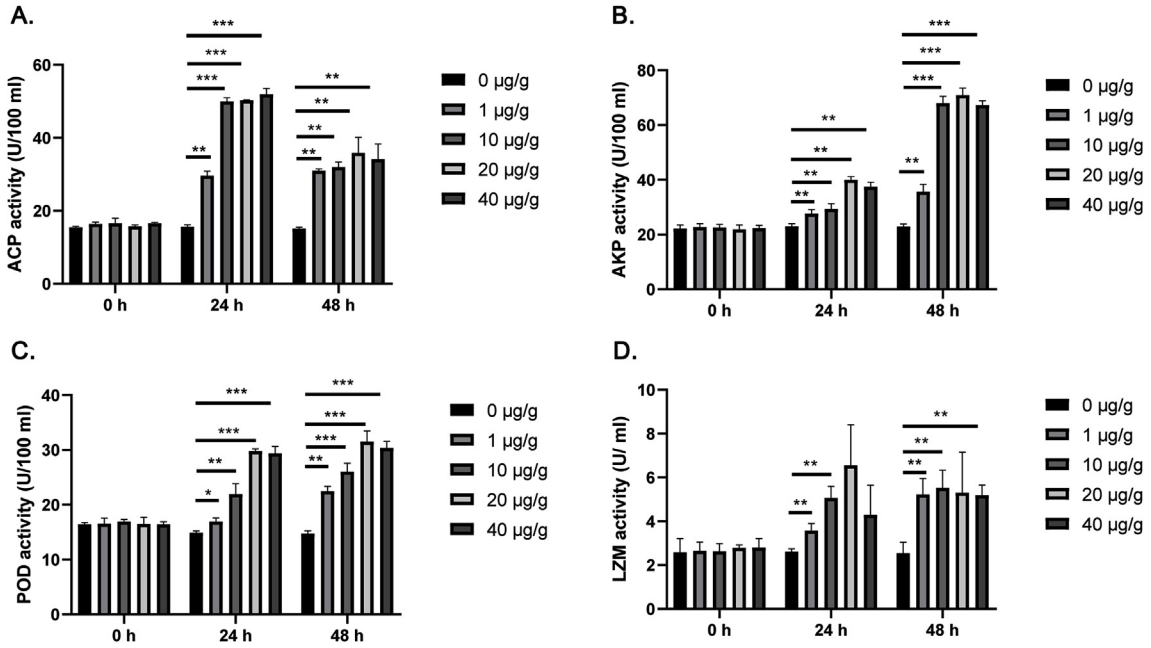

Fig. 4. The immune enzyme activities in hemolymph of shrimp after injection with catehlicidin 5.
(A) Acid phosphatase (ACP) activity of shrimps induced by cathelicidin 5. (B) Alkaline phosphatase (AKP) activity of shrimps induced by cathelicidin 5. (C) Peroxidase (POD) activity of shrimps induced by cathelicidin 5. (D) Lysozyme (LZM) activity of shrimps induced by cathelicidin 5. Data are mean ± SEM value of three separate experiments. $*p < 0.05$, $**p < 0.01$ significantly different compared to control group.

the body. They are directly involved in a series of physiological metabolic activities, such as catalysis, metabolism and hydrolysis of the phosphate group. They not only effectively detoxify pollutants and toxicants invading the crustaceans, but also play a positive role in the immune system as parts of the lysosomal enzyme, which is important in immune defense of shrimp [27]. The ACP and AKP activities of disease shrimp are significantly enhanced in *Fennopenaeus chinesis* [28] and *Macrobrachium rosenbergii* [29], which may indicate the emergency response of the immune system's to the pathological invasion [30]. In this study, the ACP activity of the treatment groups reached the

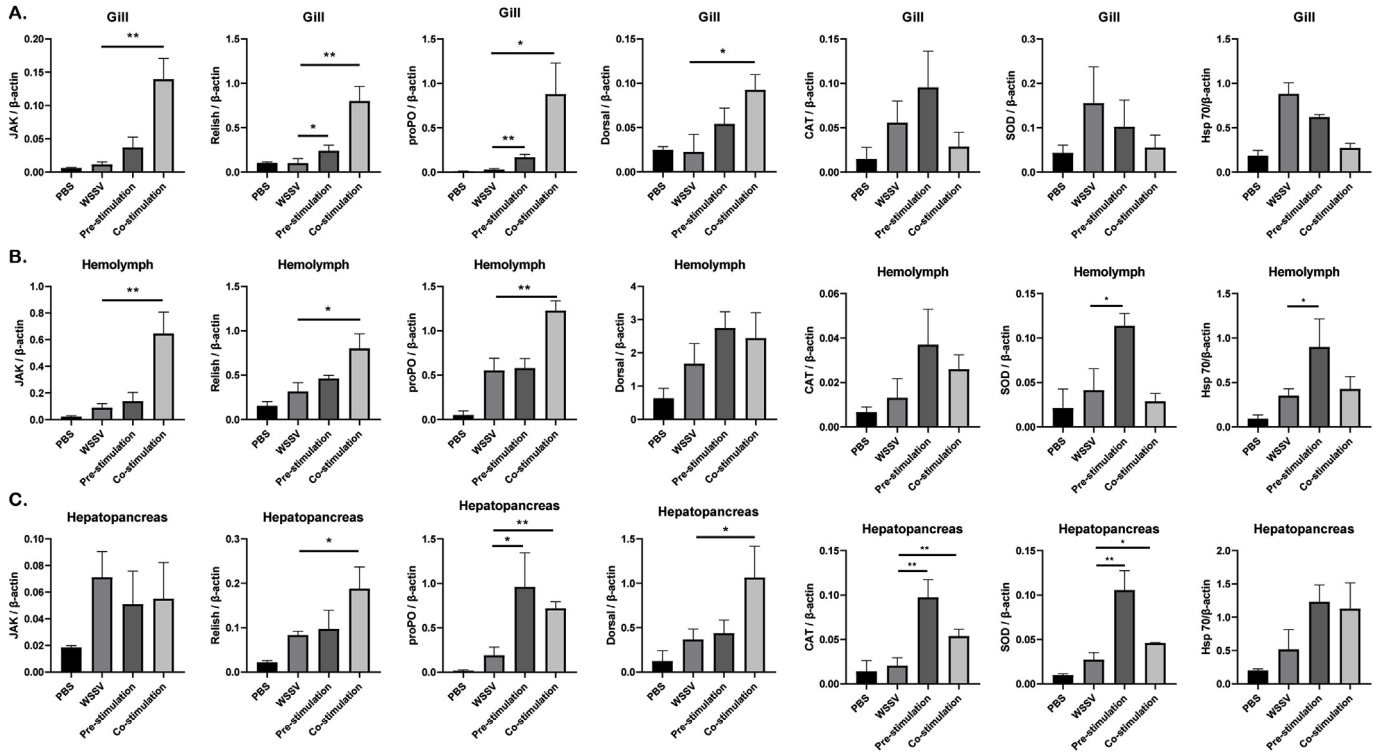

Fig. 5. Expression profiles of immune-related genes in tissues of shrimp after infection with WSSV or cathelicidin 5.
Immune-related genes of Janus Kinase (JAK), Relish, prophenoloxidase (proPO), Dorsal, Catalase (CAT), heat shock protein 70 (Hsp 70), and superoxide dismutase (SOD) in gill (A), hemolymph (B), hepatopancrease (C). Normalized to shrimp β-actin. Results were expressed as the mean + the SEM. $*p < 0.05$ and $**p < 0.01$ (*t*-test). Representative results from at least three independent experiments.

maximum level at 24 h which suggested an immediate response of the body due to stress. Unlike the ACP activity, the AKP activity increased at 48 h after injection of cathelicidin 5. When compared the variance of AKP activity between the treatment groups, we can conclude that the shrimp ACP and AKP activity are most sensitive in high dose of cathelicidin 5.

POD is produced by peroxisomes, which are primarily responsible for the removal of excess H_2O_2 produced in the respiratory burst [31]. In the present study, the POD activity of *E. modestus* was significantly enhanced by cathelicidin 5 stimulating at 24 h and increased slightly at 48 h. The surge of POD activity is most likely to reduce the damage of free radicals in normal cells, thereby enhancing immune function and detoxification ability of the body to resist disease infection [32].

The lysozyme mainly stems from neutrophils, monocytes and macrophages, and acts as the first line of defense in the immune system [33], as is an important component of the shrimp's innate immune defense system [34,35]. It can promote the hydrolysis of the bacterial cell wall and cause cell disintegration, and plays a major role in defending against pathogens and oxidative stress [36]. Here, cathelicidin 5 stimulation caused a slight increase in lysozyme activities at 24 h, while long time stimulation of cathelicidin 5 showed analogous pattern with it at 24 h.

Innate immune system is dominant immune system of invertebrates to defend against invading pathogens, because invertebrates are generally believed to lack of adaptive immunity [37–39]. The innate humoral immune response is mainly mediated by three immune signaling pathways: Toll pathway, IMD pathway and JAK/STAT pathway. In shrimp, the Toll pathway and IMD pathway are two distinct nuclear factor-κB (NF-κB)-signaling pathways, which are proved to regulate the expression of anti-microbial peptide genes to counter invading microbes [8]. The JAK/STAT signaling pathways are conserved in evolution and mediate diversity immune responses to virus infection [40,41].

In the present study, the mRNA expression level of JAK was prominently upregulated after the WSSV challenges and cathelicidin 5 treatment. Dorsal is an NF-κB transcription factor of shrimp Toll pathway and the shrimp homolog of mammalian RelA. Shrimp NF-κB pathway played a vital role in defense against invading pathogenic microorganisms. NF-κB family proteins Dorsal and Relish expressions are significantly activated by WSSV challenge in *L. vannamei* [42]. In our studies, expressions of Dorsal and Relish were significantly activated by WSSV challenge and cathelicidin 5 treatments. The prophenoloxidase (proPO) activating system serves an important role as a no self-recognition system that participates in the innate immune responses through accompanying with the cellular responses via phagocytosis, melanization, and cytotoxic reactant production [43]. Pathogenic microorganisms can stimulate the proPO system, which produces active phenoloxidase (PO). PO is presented with a form of zymogens in crustaceans and plays an important role in host recognition, defense and cell-to-cell communication, which can participate in the body immune response [44]. The role of proPO in systemic innate immunity has been well documented in several microbial challenges to different penaeid shrimps. In this study, the proPO gene is high expressed in the hemocytes (Fig. 5). It was in line with the study that the proPO transcript was used as a hemocyte marker to monitor hemocyte infiltration into different tissues [45].

To a certain extent, the antioxidant enzyme activity and immune-related genes reflect the immune response status of the shrimp. As in the experiment in which shrimp were pre-stimulated with cathelicidin 5 and were infected with WSSV had a better immune response and activity of antioxidant enzymes, such as SOD and CAT, than the controls.

SOD is a major antioxidant enzyme, which played important parts in the self-defense system, and is responsible for scavenging reactive oxygen species and protecting mechanisms within tissue injury following the radical process and phagocytosis [46]. The pre-stimulated group presented higher of SOD, which suggested the catelicidin 5 might have induced an increase in SOD to neutralize oxidative stress-induced damage, the higher SOD value is, the more superoxide radicals need to be reacted. HSPs are a suite of highly conserved proteins well known for their quick responses to environmental stresses, which can assist to repair and to protect cellular proteins from stressor-induced damage [47]. Previous studies indicated that extreme conditions represent high-stress levels that can lead to the induction of genes involved in the cellular response such as heat-shock proteins [48,49]. The expression level of HSP mRNA was significantly up-regulated during WSSV infection than PBS, which suggests HSP paly key role in shrimp immune response following virus infection. Whereas, the HSP expression in catelicidin 5 treatment group was significantly higher than that of shrimp with WSSV infected group, which proved that cathelicidin 5 could partially alleviate the virus challenge stress response.

Noxious substances could cause damage to tissue structure in shrimp, which ultimately influenced the physiological functions of shrimp [50]. As a vital organ of crustacean, hepatopancreas could adjust nutrient metabolism, detoxification of xenobiotics, antioxidant and immune responses [51,52]. The hepatopancreas is composed of many tubules, which consisting of different epithelial cell types, namely E-cell, R-cell, F-cell and B-cell [53]. Histological analysis of the hepatopancreas has been reckoned as one of the important means for reflecting health status in shrimp [54,55]. In the present study, structures of hepatopancreas in shrimp with cathelicidin 5 stimulation did not cause serious damage to the hepatopancreas compared with WSSV group. This suggested that WSSV infection led to severe damage to hepatopancreas, which could be slightly mitigated by cathelicidin 5. R-cells and vacuolated cells can be regarded as reliable biomarkers of toxic injury [56]. Thus, less reduced numbers of R-cells, and slight increased numbers of vacuolated cells in cathelicidin 5 treatment groups would protect the detoxification functions of the hepatopancreas compared with WSSV group.

Notably, prophylactic treatment is one of the major bottlenecks for the use of immunostimulants in aquaculture and the identification of broad-spectrum and low-cost products is still the main goal to be achieved [57]. These results bring further support to the application of cathelicidin 5 for the prevention of different infectious diseases in shrimp aquaculture.

5. Conclusion

Cathelicidin 5 plays an active and essential role in the innate immune system of shrimp. Of the peptide studied in the immunostimulation bioassays infected with WSSV, a better immune response was obtained from treated organisms compared to the controls, suggesting that cathelicidin 5 provides bioactive compounds, as expressed in the quantification of soluble protein and activity of antioxidant enzyme and immune-related genes, to improve the immune response through production of hemocytes in response to virus infection.

Author contributions

Tingting Feng designed the experiments and analyzed the data. Qing Xie, Yang Liu, Fangmei Luo, Qingqing Yi, Yipeng Wang, Lei Deng, Jianfeng Dai performed the experiments. Qing Xie and Tingting Feng wrote the manuscript with all the authors contributing to writing, discussion and agreeing with the conclusion presented in the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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