Fish and Shellfish Immunology 98 (2020) 236–244

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

The role of *Astakine* in *Scylla paramamosain* against *Vibrio alginolyticus* and white spot syndrome virus infection

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ARTICLE INFO

Keywords:
Scylla paramamosain
Astakine
Vibrio alginolyticus
White spot syndrome virus
Hemocytes
Immunization

ABSTRACT

Astakine is a crucial factor in the proliferation and differentiation of hematopoietic stem cells and is directly involved in hematopoiesis in crustaceans. To assess the role of Astakine in the innate immune system of *Scylla paramamosain*, the immune responses in healthy and Astakine-inhibited *S. paramamosain* were investigated in the present study. The RNA transcripts of *Astakine* were widely distributed in all examined tissues, with significantly higher levels of expression in hemocytes of both healthy and challenged *S. paramamosain* with *Vibrio alginolyticus* and WSSV. When *Astakine* was knocked down by RNA interference technology, immune-related genes, including *Janus kinase*, *prophenoloxidase*, *hemocyanin*, *β-actin*, *myosin II essential light chain-like protein*, *signal transducer and activator of transcription*, *Relish*, and *C-type-lectin*, were significantly down-regulated in hemocytes. The levels of phenoloxidaseactivity (PO), total hemocyte counts (THC) and hemocyte proliferation decreased significantly in hemocytes of Astakine-dsRNA treated *S. paramamosain*. After being challenged with *V. alginolyticus* and WSSV, the THC decreased significantly and the levels of hemocyte apoptosis increased significantly in Astakine-dsRNA treated *S. paramamosain* in comparison with those in infected groups without Astakine-dsRNA treatment. After being challenged with WSSV, the WSSV copies were significantly lower in Astakine-dsRNA treated groups than those in the WSSV infection group, which suggested that knockdown of *Astakine* was not conductive to WSSV replication and this might be associated with the decreasing THC. The results of survival analysis showed that the survival rate of *V. alginolyticus* or WSSV infected *S. paramamosain* decreased significantly following *Astakine* knockdown. These results suggested that RNA interference of *Astakine* might weaken the resistance of *S. paramamosain* to *V. alginolyticus* or WSSV infection. The weaken resistivity after knockdown *Astakine* might be related to the changes of important immune-related gene expression, THC, PO activity, proliferation and apoptosis of hemocytes.

1. Introduction

Scylla paramamosain is an economically important crab species in southern China and other Asian countries due to its high nutritional value, fast growth, and high levels of adaptability [1,2]. As all invertebrates, crustaceans (including *S. paramamosain*), lack a true adaptive immune response system. Instead they rely on an efficient innate immune system for protection against invading microorganisms, in which hemocytes play a key role [3,4]. Some innate immune responses are conducted by or originate from the hemocytes [2]. Hemocytes are involved in the synthesis of different antimicrobial peptides, lectins, proteinase inhibitors, and opsonins such as the cell adhesion protein peroxinectin, in addition to participating in immediate immune responses such as clotting, melanization, phagocytosis, and encapsulation [5–7]. Infection or injury leads to a loss of free circulating hemocytes and recovery is mainly facilitated by the rapid synthesis and release of new hemocytes [8]. In crustaceans, the continuous formation of new hemocytes is tightly regulated by factors released from circulating hemocytes, such as Astakine [9,10].

Astakine is a hematopoietic cytokine in crustaceans, which is a crucial factor in the proliferation and differentiation of hematopoietic stem cells and is directly involved in hematopoiesis [9–11]. In 2005, *Astakine* was first identified in the freshwater crayfish (*Pacifastacus leniusculus*). It can induce a strong hematopoiesis response in live animals and is necessary for new hemocyte synthesis and release [9]. Two different *Astakines* have been detected in freshwater crayfish. *Astakine*-1 is essential for stimulating the proliferation and differentiation of hematopoietic tissue cells, while *Astakine*-2 plays a role in granular cell

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https://doi.org/10.1016/j.fsi.2020.01.024
Received 1 November 2019; Received in revised form 9 January 2020; Accepted 13 January 2020
Available online 15 January 2020

differentiation [9,12]. In recent years, *Astakines* have also been identified from other invertebrates, including *Eriocheir sinensis* [10], *Penaeus monodon* [13], and *Crassostrea gigas* [14]. After being challenged with *Vibrio anguillarum*, there was a significant increase in the mRNA expression of *Astakine* in the hemocytes of *E. sinensis* and *C. gigas* and there was also a significant increase in total hemocyte counts (THC) after injecting Astakine recombinant protein [10,14]. These studies indicate that Astakine is probably involved in the defense against the invasion of foreign pathogens by modulating the hematopoietic process.

In crab culture systems, the challenge posed by increasing outbreaks of infectious disease in *S. paramamosain* farming have become severe due to viral, bacterial and parasitic diseases. Vibriosis caused by *Vibrio alginolyticus* and white spot syndrome virus (WSSV) infection are two of the most serious diseases in crab culture, and have resulted in large economic losses to the industry globally [15,16]. A better understanding of the immune defense mechanism is essential for the sustainable development of the *S. paramamosain* farming industry. The role of Astakine in hematopoiesis has been reported for several crustaceans [9,10,13], but the effects of Astakine expression on the immune responses of *S. paramamosain* against *V. alginolyticus* and WSSV infection are still unclear. Therefore, the objective of the present study was to determine the role of Astakine in the innate immune system of *S. paramamosain*.

2. Materials and methods

2.1. Crabs and tissue preparation

The healthy adult *S. paramamosain* (approximately 100 g) were obtained from a seafood market of Zhejiang province (China). All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang **Agriculture and Forestry** University (Zhejiang, China). The muscles, hepatopancreas, gills, heart, gonad, and hemocytes were collected from health or challenged *S. paramamosain*. All samples were used immediately for RNA extraction, in order to prevent total RNA degradation. WSSV (GenBank accession no. AF332093.1) was purified and used in challenge experiments as described previously [17]. *V. alginolyticus* was cultured and used to challenge *S. paramamosain* according to the previous report [18].

2.2. The real-time quantitative PCR

Samples used to quantify gene expression included various tissues of healthy adult *S. paramamosain* and hemocytes of *S. paramamosain* challenged by intramuscular injection of 100 μL of WSSV (10^6 copies/mL) or *V. alginolyticus* (10^6 CFU/mL) for different time. We extracted total RNA from the collected samples, determined nucleic acid quality and concentration, synthesized cDNA, and performed real-time quantitative PCR (RT-qPCR) to quantify gene expression as described previously [19]. The relative expression levels of *Astakine* and immune-related genes, including *Janus kinase* (*JAK*), *prophenoloxidase* (*proPO*), *hemocyanin*, *β-actin*, *myosin II essential light chain-like protein* (*Myosion*), *signal transducer and activator of transcription* (*STAT*), *Relish*, *C-type-lectin* (*CTL*), and *Toll-like receptor* (*TLR*), were determined using RT-qPCR. *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) was chosen as the internal standard [19,20]. All RT-qPCR primers were designed using Primer Premier 5.0 software based on the gene sequences available in GenBank (Table 1). Each sample was run in triplicate. Dissociation-curve analyses were performed and showed a single peak in all cases. The relative expression of each gene was determined using the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen [21].

2.3. Prokaryotic expression and purification of Astakine-dsRNA

The primers (shown in Table 1) with specific restriction sites (*Hind* III in the forward primer and *EcoR* I in the reverse primer) were designed from the known nucleotide sequence of Astakine (GenBank: HF952165.1). PCR product digested with *Hind* III/*EcoR* I was subcloned into LITMUS 38i Vector (NEB, MA, USA) digested with the same enzymes to gain plasmid L38i-Astakine. The constructed L38i-Astakine was verified by restriction enzyme digestion and DNA sequencing. The recombinant plasmid L38i-Astakine was transformed into HT115 (DE3) cells. Single colonies of the above engineering bacteria were subsequently inoculated to 5 mL of LB medium containing ampicillin (100 μg/mL), cultured at 37 °C with shaking at 220 rpm/min for 12–16 h (OD600 ≈ 0.6), and added with IPTG (Isopropyl β-D-Thiogalactoside, 0.8 mmol/L) to induce the expression for 4 h. After purifying with mirVana miRNA Isolation Kit (Ambion, USA), the double-stranded RNAs were annealed and precipitated with 5 M sodium acetate and anhydrous alcohol [18,22]. The EGPF-dsRNA was used as the control [10,22].

2.4. Knock down of astakine by RNAi and challenge experiments

Total RNA was extracted using the RNApure Tissue & Cell Kit (Beijing ComWin Biotech Co. Ltd., China) following the manufacturer's instructions. 100 μL of Astakine-dsRNA (125 μg/crab; the injection dose was determined by a preliminary experiment) was immediately injected intramuscularly into the fourth walking leg of each *S. paramamosain*, and *Astakine* mRNA expression levels were detected by RT-qPCR at different time post Astakine-dsRNA injection or WSSV or *V. alginolyticus* challenge.

2.5. Determination of immune parameters after RNAi

After treatment with Astakine-dsRNA or pathogen, the immune parameters including total hemocyte count (THC), phenoloxidase (PO) and superoxide dismutase (SOD) activities were determined. Hemolymph of *S. paramamosain* was collected using a syringe from the fourth walking leg and mixed with anticoagulant solution (ACD-B: 4.8 g/L citric acid, 13.2 g/L sodium citrate, 14.7 g/L glucose, 1.2 g/L NaCl, pH 4.6) at ratio of 1:1. To determine THC, 100 μL of hemolymph mixture was immobilized with 100 μL of 10% formalin in 0.45 M NaCl, and it was performed using a hemocytometer and defined as number of cells per milliliter [23]. 100 μL of hemolymph mixture wasused to determine PO activity based on the formation of dopa chrome from the substrate L-3, 4-dihydroxyphenylalanine (L-DOPA), as described previously [24]. 300 μL of hemolymph mixture was used to determine SOD activity by optimized NBT (nitro-blue tetrazolium) photoreduction method [25].

2.6. Proliferation of S. paramamosain hemocytes

Hemolymph mixed with anticoagulant solution was centrifuged at 2000 rpm at 4 °C for 10 min to collect hemocytes. Separated hemocytes were re-suspended with L-15 medium, and a 100 μL amount of hemocytes suspension was immediately seeded into 96-well transparent plates at a density of 5×10^4 cells/well. Hemocytes were adherent cultured for 24 h at 28 °C, and then supernatant was replaced with equal volume of L-15 medium. A 100 μL amount of Astakine-dsRNA (10 pmol) or PBS was added into each well. The plate was incubated for another 24 or 48 h at 28 °C. The proliferation levels of hemocytes were subsequently examined by Cell Counting Kit-8 (Dojindo Laboratories, Japan), following the manufacturer's instructions.

2.7. Apoptosis of S. paramamosain hemocytes

After 24 h post treatment with Astakine-dsRNA or pathogen, hemocytes were collected as described earlier. Then hemocytes were suspended in PBS (pH 7.4), counted and adjusted to a density of 1×10^6 cells/mL with PBS. The cells were stained using an Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, New Jersey, USA), and

Table 1
Universal and specific primers used in this study.

Name	Nucleotide Sequence (5′–3′)	Purpose	Accession NO.
Astakine-dsRNA-F	GCCTACCCTTCCCTGATTAC	for Astakine RNAi	HF952165.1
Astakine-dsRNA-R	GCTGTACCCACGCCCTGGCC		
Astakine-real time-F	CACCAGGTAGTAATCAGGGA	for Astakine expression	
Astakine-real time-R	AAGGCACCCAACTTCTCA		
GAPDH-real time-F	ACCTCACCAACTCCAACAC	for GAPDH expression	JX268543.1
GAPDH-real time-R	CATTCACAGCCACAACCT		
JAK-F	ATTGCTGAGGGGATGGATT	for JAK expression	KC711048.1
JAK-R	GCCCATCACATTCCCAAA		
proPO-F	ATGAAAGAGGAGTGGAGATG	for proPO	KP710954.1
proPO-R	GTGATGGATGAGGAGGTG	expression	
Hemocyanin-F	AACCCTGAACAAAGAGTTGCCTAT	for Hemocyanin expression	KM276088.1
Hemocyanin-R	AACGGACGGTAAGTTGATGATGT		
β-actin-F	ACCACTGCCGCCTCATCCTC	for β-actin expression	KC795683.1
β-actin-R	CGGAACCTCTCGTTGCCAATGG		
Myosin-F	GCCGAGATAAGTGTAGAGGAA	for Myosin expression	
Myosin-R	AGTGGGGTTCTGTCCAAG		
STAT-F	GACTTCACTAACTTCAGCCTCG	for STAT expression	KC711050.1
STAT-R	GAGCTGAGTCTGTCTTAATGTTATCC		
Relish-F	CAGGTACACCTTTGTGACCGT	For Relish expression	MH047674.1
Relish-R	CCTTCTACTTAGGGCATTTCG		
C-type-lectin-F	ACTGAGGGGAAAGTAGCC	for C-type-lectin expression	KC757381.1
C-type-lectin-R	TGCCCGTGTTTATTCATC		
Toll-like receptor-F	TGTTGCCAGAGCAGAAGGT	for Toll-like receptor expression	JQ327142.1
Toll-like receptor-R	TTCCGTGAATGAACGAAGG		

Note: Myosine gene has been cloned by our lab, but the sequence has not been upload to Gene Bank.

assessed by flow cytometry [26]. The cell numbers on quadrant 4, with low PI and high annexin V staining, were considered as apoptotic.

2.8. Quantitative analysis of WSSV

S. paramamosain were injected with WSSV or mixture of WSSV and Astakine-dsRNA. The whole-genome DNA was extracted from *S. paramamosain* hemocytes collected at 0, 24, 48, and 72 h post injection using DNA extraction Kit (Generay Bio. Co. Ltd., Shanghai, China), following the manufacturer's instructions. TaqMan real-time PCR for analysis of WSSV copies was conducted in a Light Cycler 480 Real Time System (Roche, Switzerland) with Perfect Real Time Premix (Takara, Japan) containing a high-performance Taq antibody. The TaqMan probe was synthesized and labeled with the fluorescent dyes 5-carboxyfluorescein (FAM) (5′-FAM-TGCTGCCGTCTCCAA-TAMRA-3′). PCR amplification was performed for 4 min at 50 °C, followed by 45 cycles of 45 s at 95 °C, 45 s at 52 °C and 45 s at 72 °C with two WSSV specific primers (5′-TTGGTTTCATGCCCGAGATT-3′ and 5′-CCTTGGTCAGCCC CTTGA-3′). Standard curve was made based on previous experiment [27].

2.9. Kaplan-Meier survival analysis

For the pathogen challenge, healthy *S. paramamosain* were randomly distributed into six groups (n = 10 per group, three repeats). Each group of *S. paramamosain* was injected with 100 μL of PBS or Astakine-dsRNA (125 μg/crab) or WSSV (10^6 copies/mL) or *V. alginolyticus* (10^6 CFU/mL) or the mixture of pathogen and Astakine-dsRNA. The six groups were administrated as follows: PBS, Astakine-dsRNA, WSSV, *V. alginolyticus*, WSSV + Astakine-dsRNA, *V. alginolyticus* + Astakine-dsRNA. All *S. paramamosain* were reared under the same condition. After every 24 h, the number of alive and dead *S. paramamosain* was counted. The survival data were arranged and analyzed in Microsoft GraphPad 5.0.

2.10. Statistical analysis

Quantitative data were expressed as means ± standard deviation (SD). Each group in the present experiment was performed in triplicate and data were analyzed by SPSS 19.0 software (IBM, USA). Statistical differences were estimated using one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. The differences between the different treatments were analyzed by Student's *t*-test. The results were considered as significant at $p < 0.05$.

3. Results

3.1. Distribution of astakine mRNA in tissues

Expression profiling of *Astakine* genes in different tissues of *S. paramamosain* was conducted by RT-qPCR.RNA transcripts of *Astakine* were detected in all tissues examined, including hemocytes, hepatopancreas, gill, muscle, gonad, and heart tissue (Fig. 1). *Astakine* mRNA expression was highest in hemocytes, compared to the other tissues. The lowest level of *Astakine* mRNA expression was in the heart tissue.

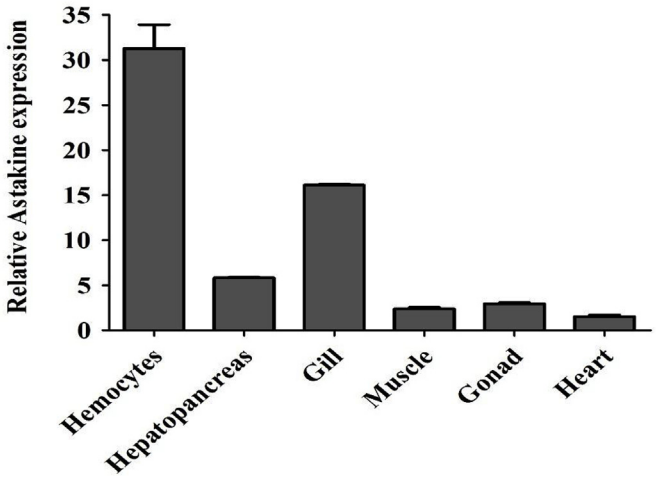

Fig. 1. Expression characterization of Astakine in various tissues from healthy *S. paramamosain* revealed by RT-qPCR. The amount of Astakine mRNA was normalized to the GAPDH transcript level. Data are shown as means ± SD of the tissues of three separate individuals.

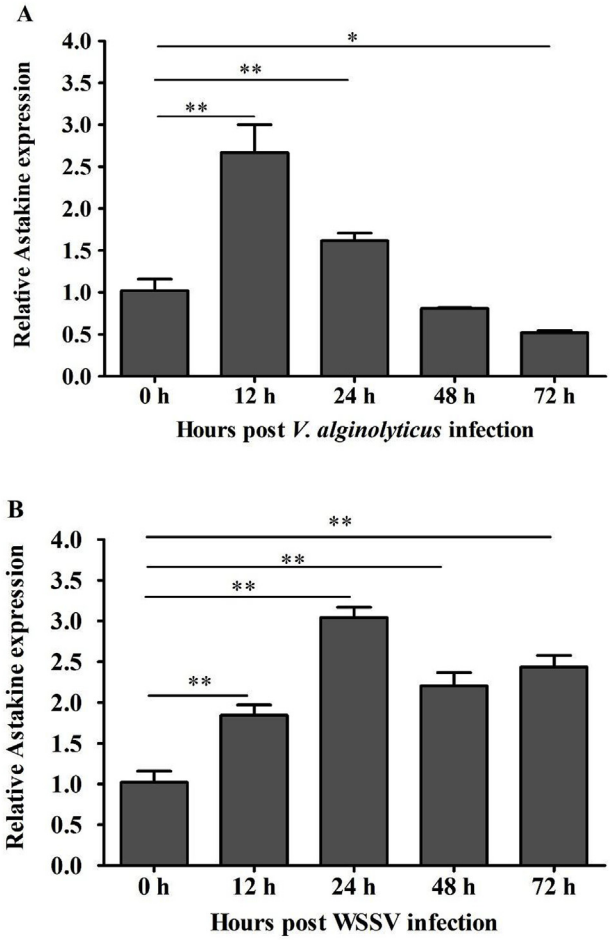

Fig. 2. (A) RT-qPCR analysis of Astakine expression in the hemocytes of *S. paramamosain* challenged with *V. alginolyticus* (A) or WSSV (B). The amount of Astakine mRNA was normalized to the GAPDH transcript level. Data are shown as means ± SD of three separate individuals in the tissues. Asterisks indicate a significant difference between two samples (*$p < 0.05$; **$p < 0.01$).

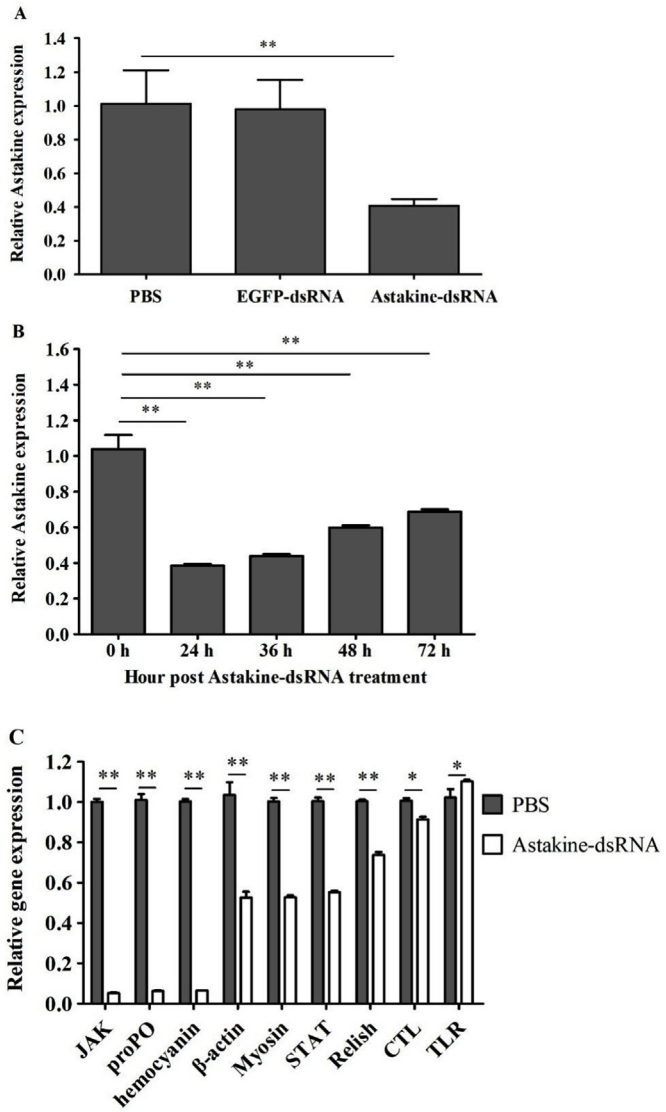

Fig. 3. RT-qPCR analysis of Astakine and immune-related gene expression. RT-qPCR analysis of Astakine expression in the hemocytes of *S. paramamosain* treated with Astakine-dsRNA at 24 h (A)or different time (B) post treatment. (C) RT-qPCR analysis of nine immune genes (Janus kinase (JAK), prophenoloxidase (proPO), hemocyanin, β-actin, Myosion, STAT, Relish, C-type-lectin (CTL), Toll-like receptor (TLR)) expression in the hemocytes of *S. paramamosain* treated with Astakine-dsRNA at 24 h post treatment. The amount of Astakine mRNA was normalized to the GAPDH transcript level. Data are shown as means ± SD of tissues in three separate individuals. Asterisks indicate a significant difference between two samples (*$p < 0.05$; **$p < 0.01$).

Astakine expression in hemocytes was 5.37-, 1.94-, 13.09-, 10.58- and 20.18-fold greater than that in hepatopancreas, gill, muscle, gonad, and heart tissue, respectively.

3.2. Astakine expression following V. alginolyticus or WSSV challenge

The alternation of *Astakine* mRNA expression in *S. paramamosain* hemocytes after challenges with *V. alginolyticus* or WSSV was investigated. The expression levels of *Astakine* mRNA at 12 and 24 h post challenge with *V. alginolyticus* were significantly higher than at 0 h ($p < 0.01$), and decreased to normal levels at 48 h (Fig. 2A).The lowest level of *Astakine* mRNA expression was at 72 h post challenge with *V. alginolyticus* ($p < 0.05$). After challenge with WSSV, the expression levels of *Astakine* mRNA at12 (1.81-fold), 24 (2.98-fold), 48 (2.16-fold), and 72 h (2.39-fold) were significantly higher than that at 0 h ($p < 0.01$; Fig. 2B). These results suggested that *Astakine* may play an important role in the innate immune system of *S. paramamosain* following infection with *V. alginolyticus* or WSSV.

3.3. Effects of astakine knockdown on expression of immune-related genes

The effect of Astakine-dsRNA on mRNA expression was determined by RT-qPCR. *Astakine* mRNA expression in *S. paramamosain* hemocytes was significantly knocked down by Astakine-dsRNA ($p < 0.01$; Fig. 3A). The effect of Astakine-dsRNA on *Astakine* gene expression in hemocytes at different times post Astakine-dsRNA treatment was determined. The results showed that Astakine-dsRNA inhibited the expression of *Astakine* mRNA in hemocytes from 24 to 72 h post Astakine-dsRNA treatment (Fig. 3B). The relationship between *Astakine* expression and the expression of other immune-related genes was also investigated by analyzing the effects of Astakine-dsRNA on the expression levels of important immune genes in hemocytes. All the examined immune-related genes except for *TLR*, including *JAK*, *proPO*, *hemocyanin*, *β-actin*, *Myosin*, *STAT*, *Relish* and *CTL*, were significantly down-regulated at 24 h post Astakine-dsRNA treatment ($p < 0.05$; Fig. 3C).

3.4. Determination of humoral immune-related parameters

To evaluate the effect of Astakine-dsRNA on humoral immune-related parameters, PO and SOD activities in *S. paramamosain*

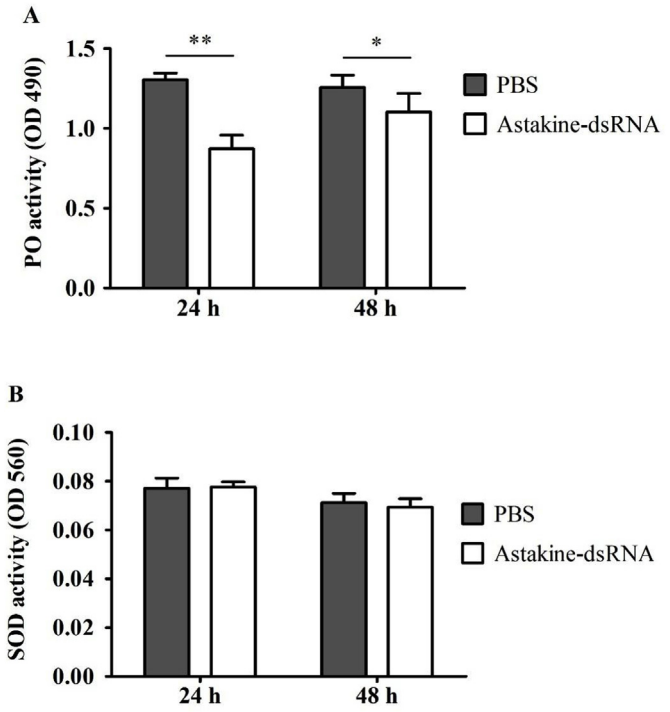

Fig. 4. PO (A) and SOD (B) activities of *S. paramamosain* treated with Astakine-dsRNA. Data are shown as means ± SD of tissues in three separate individuals. Asterisks indicate a significant difference between two samples (*$p < 0.05$; **$p < 0.01$).

hemolymph were examined at 24 and 48 h post Astakine-dsRNA treatment. At 24 and 48 h post Astakine-dsRNA treatment, PO activities were both significantly decreased in comparison with the PBS groups ($p < 0.05$; Fig. 4A). However, there was no obvious difference in SOD activities between Astakine-dsRNA and PBS groups (Fig. 4B).

3.5. Influence of Astakine knockdown on total hemocyte count

THC in the Astakine-dsRNA groups was significantly decreased in comparison to the PBS groups at 24 and 48 h post-treatment ($p < 0.01$; Fig. 5A). THC in *V. alginolyticus* groups increased significantly in comparison to the PBS groups at both 24 and 48 h post challenge ($p < 0.01$; Fig. 5B). However, THC in Astakine-dsRNA + *V. alginolyticus* groups both decreased significantly in comparison with the PBS and *V. alginolyticus* groups at 24 and 48 h post challenge ($p < 0.01$). Astakine-dsRNA had a similar effect on the THC of WSSV-infected *S. paramamosain* (Fig. 5C). These results suggested that Astakine may regulate hemocyte proliferation in *S. paramamosain*.

3.6. Effect of astakine on hemocyte proliferation

To evaluate the effect of Astakine-dsRNA on hemocyte proliferation, *S. paramamosain* hemocytes were cultured in 96-well transparent plates and subsequently treated with PBS or Astakine-dsRNA. At 24 and 48 h post-treatment, the levels of hemocyte proliferation significantly decreased in both Astakine-dsRNA groups in comparison with the PBS groups ($p < 0.05$; Fig. 6). These results suggested that THC might be lower in Astakine-dsRNA groups than those in PBS groups at either 24 or 48 h. However, the apoptosis levels of hemocytes should be investigated further.

3.7. Effect of astakine on apoptosis

The role of Astakine in the apoptosis of *S. paramamosain* hemocytes

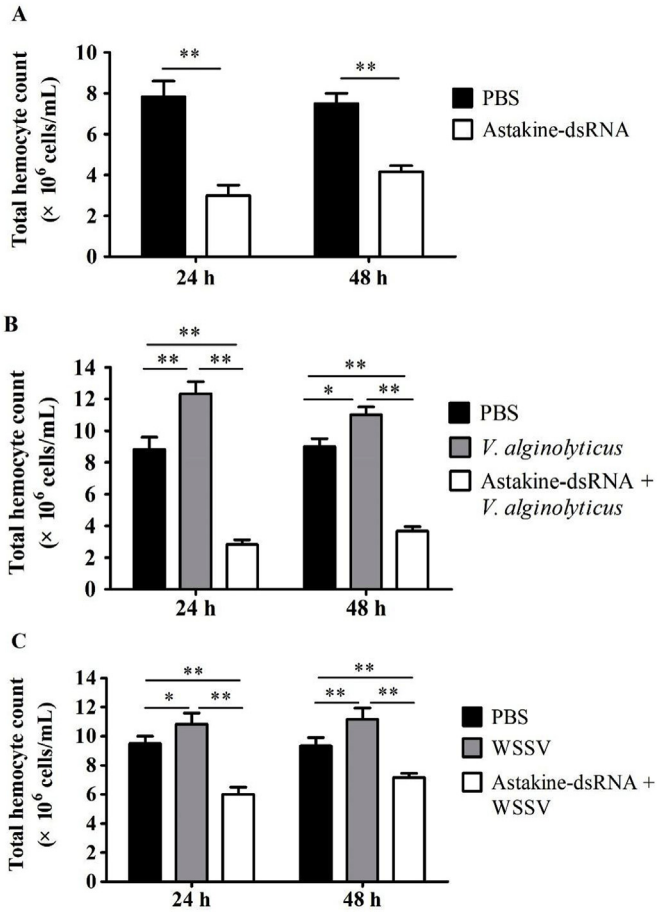

Fig. 5. Influence of Astakine knockdown on total hemocyte count (THC). (A) THC of *S. paramamosain* treated with Astakine-dsRNA. (B) THC of *V. alginolyticus* challenged *S. paramamosain* treated with Astakine-dsRNA. (C) THC of WSSV challenged *S. paramamosain* treated with Astakine-dsRNA. Data are shown as means ± SD of tissues in three separate individuals. Asterisks indicate a significant difference between two samples (*$p < 0.05$; **$p < 0.01$).

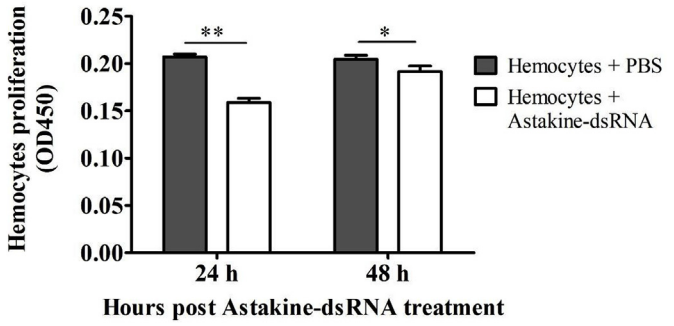

Fig. 6. Influence of Astakine knockdown on proliferation of hemocytes treated with Astakine-dsRNA at different time post treatment. Data are shown as means ± SD of tissues in three separate individuals. Asterisks indicate a significant difference between two samples (*$p < 0.05$; **$p < 0.01$).

at 24 h post-treatment was investigated. The apoptosis rates significantly increased in the Astakine-dsRNA groups in comparison with the PBS groups at 24 h post challenge with *V. alginolyticus* or WSSV ($p < 0.01$; Fig. 7). After *V. alginolyticus* challenge, the Astakine-dsRNA + *V. alginolyticus* group showed higher apoptosis rate than the *V. alginolyticus* group significantly ($p < 0.01$; Fig. 7G). Similarly, the apoptosis rate increased significantly in the Astakine-dsRNA + WSSV group compared with the WSSV group ($p < 0.01$; Fig. 7H). These

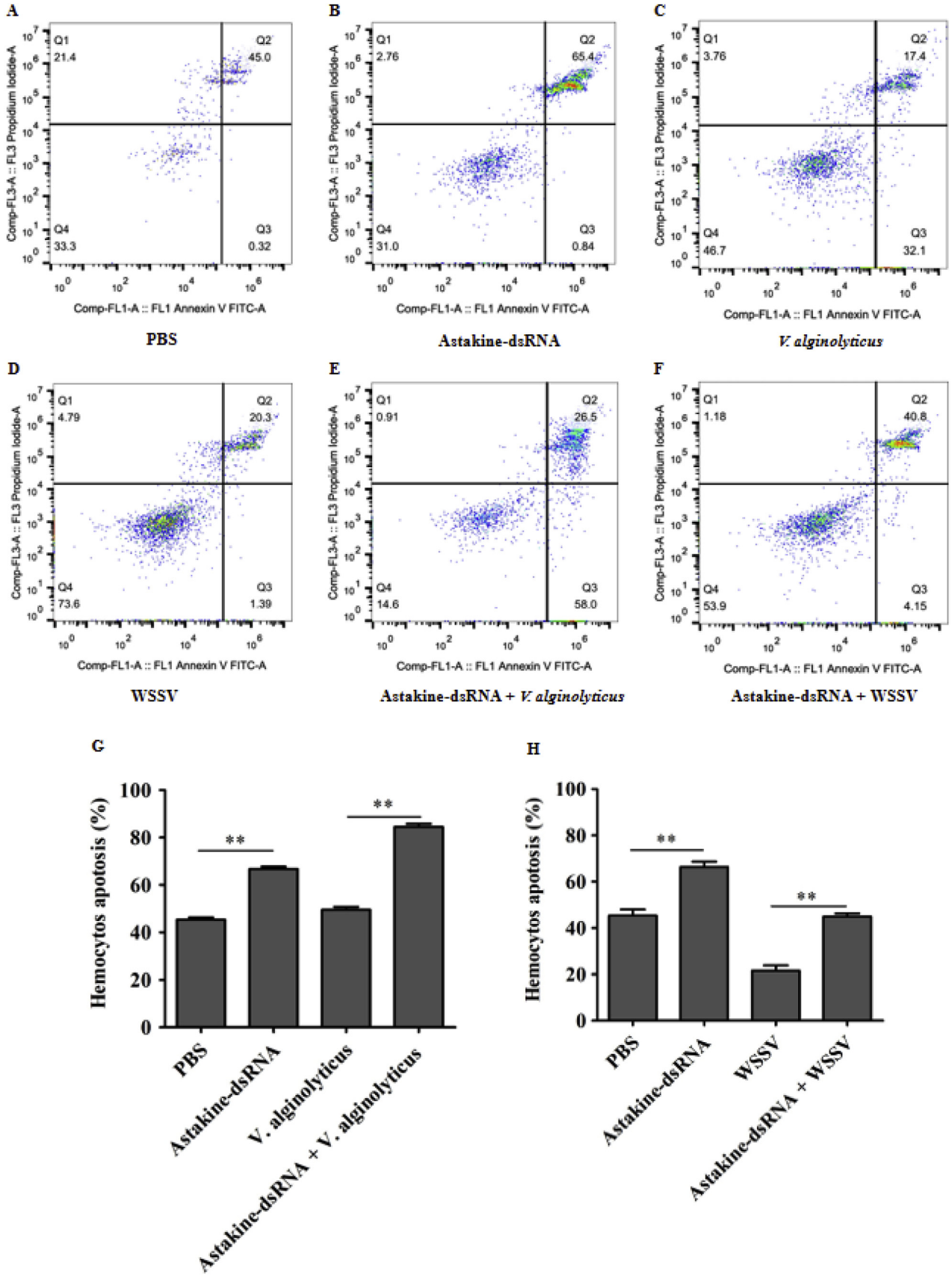

Fig. 7. Flow cytometry assay of hemocyte apoptosis. (A) PBS; (B) Astakine-dsRNA; (C) *V. alginolyticus*; (D) WSSV; (E) Astakine-dsRNA + *V. alginolyticus*; (F) Astakine-dsRNA + WSSV; (G) Bar graph of apoptosis due to *V. alginolyticus*; (H) Bar graph of apoptosis due to WSSV. Asterisks indicate a significant difference between two samples (**$p < 0.01$).

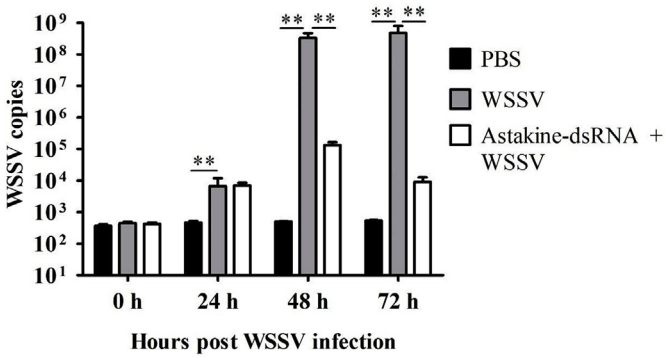

Fig. 8. Detection of WSSV copies in hemocytes of *S. paramamosain* at different time post WSSV infection. Asterisks indicate a significant difference between two samples (**$p < 0.01$).

results suggested that the knockdown of *Astakine* mRNA expression enhanced hemocyte apoptosis in both healthy *S. paramamosain* and those challenged with *V. alginolyticus* or WSSV.

3.8. Effects of astakine knockdown on WSSV copies and survival of challenged S. paramamosain

After the challenge, WSSV copies in hemocytes of WSSV infected *S. paramamosain* increased as the duration of infection increased (Fig. 8). The copy numbers in WSSV infected groups and mixed WSSV and Astakine-dsRNA treatment groups were significantly higher than those in PBS groups at each infection time ($p < 0.01$). However, the copy numbers in the mixed WSSV and Astakine-dsRNA treatment groups decreased significantly in comparison with those in the WSSV group at 48 and 72 h post-challenge ($p < 0.01$).

The results of survival analysis showed that the Astakine-dsRNA + *V. alginolyticus* group had a significantly lower survival rate at 168 h post-challenge ($p < 0.01$; Fig. 9A). The negative control showed a similar mortality to the Astakine-dsRNA group, indicating that Astakine-dsRNA itself was non-toxic in *S. paramamosain*. Astakine-dsRNA had a similar effect on the survival of WSSV-infected *S. paramamosain*. The survival rate in the Astakine-dsRNA + WSSV group was significantly lower than that in the WSSV group at 216 h post-challenge ($p < 0.01$; Fig. 9B). Overall, these results indicated that *Astakine* knockdown decreased the survival rate of *S. paramamosain* infected with *V. alginolyticus* or WSSV.

4. Discussion

The hard carapace and the external cuticle of crabs not only provide an effective physical barrier, but also produce immune factors to protect crabs against the attachment and penetration of pathogens, and are the first line of defense for crabs [2]. Once pathogens break through the hard shell and enter the hemolymph, a series of immune responses are stimulated, which involve different types of hemocytes [6]. The regulation of hemocyte homeostasis is of utmost importance for the health of crustaceans. There is increasing evidence to suggest that Astakine is an important invertebrate cytokine containing a prokineticin domain homologous to prokineticin in vertebrates and is involved in hematopoiesis in invertebrates [9–11]. To assess the potential role of Astakine in the innate immune system of *S. paramamosain*, *in vivo* and *in vitro* experiments, including the effects of Astakine-dsRNA on the expression levels of immune-related genes, hemocyte counts and functions, and the survival of challenged *S. paramamosain* with pathogens, were carried out in the present study.

The present study showed that the RNA transcripts of *Astakine* were widely distributed in all examined tissues, with significantly higher expression rates in hemocytes of both healthy and challenged *S. paramamosain*. As Astakine is known as one of the most important hematopoietic factors in crustaceans [10,11], the high expression levels of *Astakine* mRNA in hemocytes indicated that Astakine may play a role in the immune responses of *S. paramamosain* challenged with *V. alginolyticus* or WSSV by regulating hematopoiesis. RNA interference (RNAi) is a phenomenon of post-transcriptional gene silencing specifically mediated by dsRNA sequences *in vivo*, and this technology has been used to investigate the function of certain target proteins in many invertebrates [28–30]. In the current study, the expression of *Astakine* in *S. paramamosain* hemocytes was successfully inhibited with specific double-strand RNA (Astakine-dsRNA), providing a practical way to investigate the role of Astakine in the humoral and cellular immunity of *S. paramamosain*.

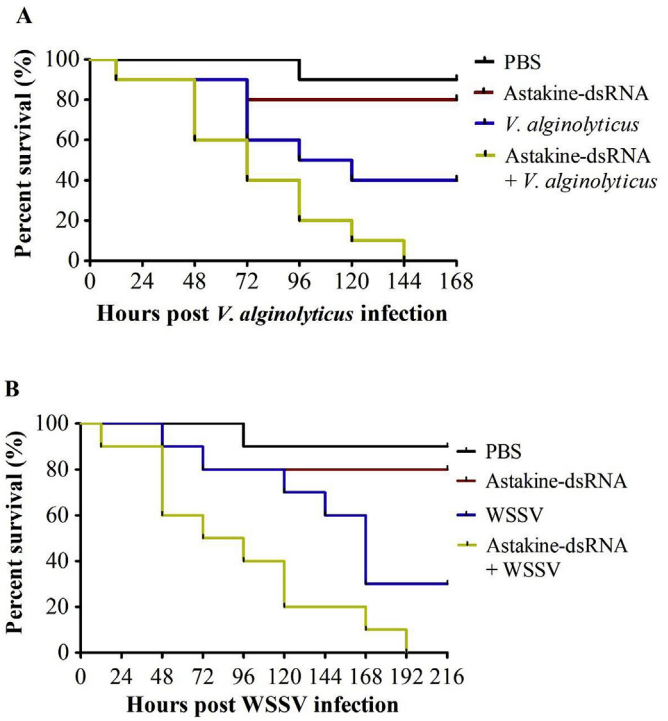

Fig. 9. The survival analysis of *V. alginolyticus* (A) or WSSV (B) challenged *S. paramamosain* treated with Astakine-dsRNA. Each group consisted of nine individuals, respectively.

The molecule cascades of the innate immune system can be rapidly activated within a few hours when hosts are at risk of infection [31]. The risk level of infection is initially recognized by pattern recognition receptors (PRRs). These then initiate downstream signal pathways, such as JAK/STAT and Toll signaling pathways; while on another level, the hosts fight the infection through cellular immune responses and in parallel, various immune factors including cytokines, antimicrobial peptides (AMPs) and antioxidant enzymes are produced [2,32,33]. In recent years, many immune-related genes involved with PRRs and signaling pathways have been identified providing further understanding of the immune response of *S. paramamosain* to pathogens [32–38]. The important roles of these core members, including JAK [32], proPO [33], Relish [34], C-type lectin B [35], scavenger receptor B [36], Toll-like receptors [37,38], and Myosin [39], have been demonstrated in the host's defense against pathogen invasion. For example, JAK might protect *S. paramamosain* from reovirus infection by activating the JAK/STAT signaling pathway, which is similar to the antiviral defense process in mammals and *Drosophila* [32,40,41]. The proPO system is one of the most efficient and important immune recognition and defense systems in crustaceans, and hemocyanin exhibits PO activity [42,43]. Myosin is involved in cell skeleton construction and motility processes, which was as a WSSV interacting protein to take

part in antiviral defense in shrimp [39]. Relish, a key transcription factor for AMPs, was found to affect the PO and SOD activities and the apoptosis in *S. paramamosain* following *V. alginolyticus* and WSSV infection [34]. In this study, knocking down the expression levels of *Astakine* mRNA resulted in significant down-regulation of several typical innate immune molecules and signal transduction factors, including *JAK*, *proPO*, *hemocyanin*, *β-actin*, *Myosin*, *STAT*, *Relish* and*CTL*, which suggested that Astakine might be involved in the innate immunity of *S. paramamosain* via the interaction between immune factors.

To further investigate the effects of Astakine on immune responses, a variety of functional parameters were evaluated in Astakine-dsRNA treated *S. paramamosain*. It was found that the PO activities decreased significantly in hemocytes of *S. paramamosain* treated with Astakine-dsRNA, which suggested that the proPO system might be activated when the expression levels of *Astakine* mRNA were knocked down [42,43]. Since SOD isan important antioxidant in the non-specific immune system and plays vital roles in reactive oxygen species (ROS) transfer, protecting both membranes and DNA from damage [44], SOD activities without obvious changes might be associated with other unknown factors, such as Astakine to promote hemocyte production in a ROS-dependent way in *E. sinensis* [10]. Apart from enzyme activities, the levels of THC decreased significantly when the expression levels of *Astakine* mRNA were knocked down in both healthy *S. paramamosain* and *S. paramamosain* challenged with *V. alginolyticus* and WSSV, suggesting that *Astakine* was involved in hemocyte release from hematopoietic tissues of *S. paramamosain*. In parallel, hemocyte proliferation and apoptosis might be affected by decreasing THC levels, as the present study showed that the levels of hemocyte proliferation decreased significantly and the levels of hemocyte apoptosis increased significantly in *Astakine* mRNA treated *S. paramamosain*. After being challenged with pathogens, the levels of hemocyte apoptosis also increased significantly in *Astakine* mRNA treated *S. paramamosain*. These results suggested that the resistance of *S. paramamosain* to *V. alginolyticus* and WSSV infection might be weakened when the expression levels of *Astakine* were knocked down. The weaker immune responses were also indicated by higher mortality rates when Astakine-dsRNA treated *S. paramamosain* were challenged with *V. alginolyticus* or WSSV. Though WSSV copies in the mixed WSSV and Astakine-dsRNA treatment groups decreased significantly in comparison with those in the WSSV group at 48 and 72 h post-challenge, there was still a negative correlation with mortality. The decrease in the copy numbers of WSSV might be related to the decrease in the levels of THC, which might be not conductive to WSSV replication [45]. In parallel, the weakened resistance of *S. paramamosain* to *V. alginolyticus* and WSSV might be closely related with the changes in hemocyte functions including proliferation and apoptosis after knocking down the expression level of *Astakine* mRNA.

In conclusion, our results indicated that Astakine plays a key role in the innate immunity of *S. paramamosain*. RNAi of Astakine could weaken the resistance of *S. paramamosain* to *V. alginolyticus* or WSSV infection, and the weaken resistivity might be related to the change of important immune-related gene expression, THC, PO activity, proliferation and apoptosis of hemocytes.

Acknowledgements

This work was supported by National Key Research and Development Program of China (2018YFD0500300), and PhD Research Startup Foundation of **Zhejiang Agriculture and Forestry University (2034020036)**.

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