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Apoptosis of hemocytes is associated with the infection process of white spot syndrome virus in *Litopenaeus vannamei*

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ABSTRACT

Previous studies have demonstrated that white spot syndrome virus (WSSV) could induce hemocytes apoptosis in shrimps, however the inter-relationship between apoptotic process and the WSSV infection status is still currently underexplored. In the present work, the apoptosis and the viral proliferation in hemocytes of *Litopenaeus vannamei* were simultaneously investigated post WSSV infection by two-color immunofluorescence flow cytometry and real-time quantitative PCR. The apoptotic hemocytes of WSSV-infected shrimp was significantly increased at 12 h post infection (hpi), whereas underwent a slight decline at 24 hpi subsequently. Since 24 hpi, the apoptotic rate of hemocytes in the WSSV-infected shrimp exhibited a rapid and significant increase, and reached the peak level at 48 hpi with the ratio of 18.1 ± 2.0%. Meanwhile, the percentage of WSSV-infected hemocytes and WSSV copies in hemocytes significantly increased at 24 hpi and maintained at a high level afterwards. With the rapid increase of hemocytes apoptosis, hemocyte density in hemolymph decreased dramatically to less than 20% of the mean value of control. Co-localization assay showed that the apoptotic WSSV-infected hemocytes occupied the dominant proportion of total apoptotic hemocytes, which reached the peak at 48 hpi with 12.6 ± 1.5%. The expression profiles of seven pro-apoptotic genes and two apoptosis-inhibiting genes showed significant differential responses at different stages of WSSV infection, reflecting the interplay between the virus and the host immune response. Our results demonstrated that the apoptotic response of shrimp hemocytes could be significantly influenced by the WSSV infection process, which might provide an insight into deeper relationships between viral infection and apoptosis.

1. Introduction

Apoptosis, also called genetically programmed cell death, is a kind of conserved protective physiological mechanism that plays a critical role in surviving organisms. Apoptosis could be able to clear off the surplus, damaged or diseased cells caused by inner or outer destructive factors [1–4]. It is generally considered that apoptosis is a host defense mechanism against pathogen infections and has a key role in the maintenance of immune system homeostasis [5–7]. The physiological function and complexity of apoptosis showed considerable taxon-dependent variations. In mammals, classical apoptotic pathways include the death receptor-mediated pathway and the intracellular signals-mediated intrinsic pathway [8]. Although the apoptotic signal pathways in mammals are getting clearer, it is still vague in crustacean [9,10]. The debate is mainly focused on whether the extrinsic apoptotic pathway existed in invertebrates. For example, some molecules involved in the extrinsic apoptosis pathway in mammals were not found in *Caenorhabditis elegans*, and the homologs of mammalian death ligand and receptor (TNF and TNFR)in *Drosophila* lacks the structure for transducing death signaling [11,12]. Although the TNF and TNFR superfamily genes were both cloned in shrimp, however whose functions have not yet been investigated [13,14]. On the other hand, the mitochondria-mediated intrinsic apoptotic pathways have been confirmed to be an important way of crustacean apoptosis [15–17]. In recent years, various apoptotic molecules of intrinsic pathway have been cloned and their functions were explored in different crustacean species, including Caspases [18,19], apoptosis inducing factor (AIF), cytochrome *c* (Cyt-c) [20], tumor suppress protein p53 [21], inhibitor of apoptosis proteins (IAPs) [22] and tumor control transcriptional protein (TCTP, or Pm-fortlin) [23]. More and more evidences suggest that apoptosis plays an important role in the innate immunity of crustaceans [24,25].

White spot syndrome virus (WSSV) is the most devastating shrimp viral pathogen and responsible for the huge economic losses in the

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shrimp culture industry [26,27]. Previous works showed that apoptosis could be clearly detected in the organs and tissues of WSSV-infected shrimps [28–30], which indicated that apoptosis could be efficiently induced by WSSV infection and might play an important role in the non-adaptive immunity in WSSV-infected shrimp. Although the importance of apoptosis has been widely emphasized in viral infection, it is controverted that apoptosis in shrimp might be the factor that help the shrimp to survive the infection or lead to mortality [31–33]. As major proteins in response to apoptosis, Caspases have vital functions at various stages of the apoptotic process [34]. Five types of Caspase genes that are extremely sensitive to WSSV infection have been reported in *Litopenaeus vannamei*. And different Caspases appear to play different roles in WSSV-induced apoptosis. Silencing of *Lv*Caspase2, *Lv*Caspase3, and *Lv*Caspase5 resulted in increased WSSV replication, indicating that *Lv*Caspase2/3/5 possessed antiviral roles against WSSV [19,35], which was also supported by the researches in other shrimps [36,37]. On the contrary, knocking down *Lv*Caspase1 reduced mortality in *L. vannamei* challenged with WSSV, suggesting that *Lv*Caspase1-mediated apoptosis contributes to viral pathogenicity [38]. Similarly, silencing of Dap-1 of *Marsupenaeus japonicas* decreased apoptosis of WSSV-infected shrimp and was helpful for amplifying WSSV in shrimp [39]. Thus, it still requires more sufficient data to clearly elucidate the roles of hemocytes apoptosis in WSSV infection.

Hemocytes are one of the major immune cells of shrimp that could execute both humoral and cellular immune responses to defend against pathogens [40–42], which were also found to be the target cells for viral infection [43–45]. In the present work, the TUNEL assay combined with flow cytometry was adopted to detect the apoptotic hemocytes during the process of WSSV infection, and real-time qPCR were used to determine the expression of nine apoptosis-related genes. Meanwhile, the infected hemocytes ratio, viral copies in hemocytes and accumulative mortality of WSSV-infected shrimp were also investigated. The resultant data would provide an insight into deeper relationship between the apoptosis of hemocytes and WSSV infection.

2. Materials and methods

2.1. Shrimp and WSSV infection

The apparently healthy shrimp, *L. vannamei* (13–15 cm), was purchased from the aquaculture farm in Qingdao, China, and confirmed by nested PCR to be WSSV-free according to the method described previously [46]. Then shrimps were temporarily maintained at 23 °C in tanks containing aerated filtered seawater with salinity of 31 for one week before experiments. A total of 500 healthy shrimps were equally divided into two groups. In the WSSV infection group, one hundred shrimps were used for the sampling, and 150 shrimps were equally divided into three groups and used for calculating the cumulative mortality. The control group was treated with the same amount of PBS.

WSSV inoculum was prepared as described by previous method with a slight modification [47]. Briefly, the gill tissue (1 g) from natural heavily WSSV-infected *L. vannamei* was homogenized in 10 mL sterile phosphate buffered saline (0.01 M, pH 7.4, PBS), and then centrifuged at 600 × *g* for 20 min at 4 °C. The supernatant was filtered through a 450 nm membrane to obtain the crude extract of WSSV(CE-WSSV). CE-WSSV was centrifuged at 55000 × *g* for 1.5 h at 4 °C. The supernatant was determined by absolute quantitative PCR [48]. After acclimation for one week, the shrimps were intramuscularly injected on the flanks of the second abdominal segment with 100 μL WSSV inoculum (diluted 1:500 with PBS, containing 10^4 copies/μL). The shrimp in the control group were injected with 100 μL sterile PBS. The number of dead shrimps was recorded during the process of infection.

2.2. Sampling of hemocytes

Hemolymph was withdrawn from the pericardial cavity using a syringe containing modified cold Alsever solution (27 mM Na citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.2, AS) as anticoagulant. The hemocytes were collected from 7 shrimp at 0, 6, 12, 18, 24, 36, 48, 60, 72 h post infection (hpi) in the control and infection groups, respectively. Afterwards, hemocytes were pelleted by centrifuging at 400 × *g* for 5 min at 4 °C and rinsed with sterile prawn homoiosmotic phosphate buffered saline (377 mM NaCl, 2.70 mM KCl, 8.09 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4, 780 mOsm/L, PHPBS), then pelleted and suspended with PHPBS once again. The total homogeneous hemocytes were divided into six groups for subsequent experiments. All trials repeated in triplicate for each sample. In addition, total hemocytes count (THC) was performed with a little modification on methods mentioned previously [30]. Briefly, 10 μL of hemocyte suspension was diluted for 10 times in 90 μL Alsever solution, then the density of the diluted hemocyte suspension was measured by Neubauer hemocytometer.

2.3. Detection of apoptotic hemocytes by flow cytometry and immunofluorescent assay

Since DNA fragmentation is widely regarded as one of the important biochemical hallmarks of apoptosis [28], here we adopted TUNEL method combined with microscopic observation and flow cytometry to qualitatively and quantitatively detect the apoptotic hemocytes. Apoptotic hemocytes were detected by employing Apo-BrdU-RedTM In Situ DNA Fragmentation Assay Kit (Biovision, USA) in combination with immunofluorescence flow cytometry assay, and all procedures were conducted following the manufacturer's instructions with an optimization of some pretreatment procedures [49,50]. Briefly, hemocytes for TUNEL assay were all fixed by 2% (w/v) paraformaldehyde in PHPBS for 15 min on ice, and then resuspended in PHPBS containing 0.1% (w/v) TritonX-100 for 15 min. The cell suspension was pelleted and rinsed with PHPBS twice. For flow cytometry assay, approximately 10^6 cells of the pretreated hemocytes were suspended in 51 μL of DNA fragmentation labeling solution, a mixture of 32.25 μL ddH_2O, 10 μL TdT Reaction Buffer, 8 μL Br-dUTP and 0.75 μL TdT Enzyme, and then incubated at 37 °C for 1 h with gentle shaking. The non-TdT-Enzyme labeling solution was used as the control. Following three rinses, the hemocytes were incubated in 100 μL antibody solution (5 μL of Anti-BrdU-Red antibody in 95 μL PHPBS) for 30 min in the dark at room temperature (RT), then diluted with 0.5 mL PHPBS for another 30 min incubation. TUNEL positive hemocytes were analyzed using flow cytometer (Accuri C6, BD Biosciences, USA). For microscopic immunofluorescence assay, the healthy and WSSV-infected (48 hpi) hemoyctes were chosen for use, and approximately 10^5 fixed cells in total were settle onto glass slides. The settled cells were covered over with 25 μL of DNA fragmentation labeling solution or PHPBS, and incubated at 37 °C for 1 h. After three rinses with PHPBST (PHPBS containing 0.5‰ Tween-20, Solarbio, USA), 25 μL of the antibody solution was added at RT for 45 min incubation. Following rinse steps, the cells were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for locating the cell nucleus. Finally, the hemocytes were observed under a fluorescence microscope (Olympus DP70, Japan).

2.4. Detection of WSSV-infected hemocytes by flow cytometric immunofluorescence assay

The WSSV-infected hemocytes was stained using mouse anti-WSSV monoclonal antibodies prepared previously by our laboratory [51]. For this trial, the fixed and permeabilized hemocytes (containing about 10^6 cells in total) was incubated with the anti-WSSV Mabs (1:1000 diluted in PHPBS) at 37 °C for 1 h. After three rinses with PHPBS, goat anti-mouse IgG Alexa Fluor®488 antibody (Invitrogen) was added for 45 min incubation at 37 °C, and washed as described above. Then, the hemocytes were suspended in 1 mL of PHPBS and analyzed by Accuri C6 flow cytometer (BD, USA). The myeloma culture supernatant instead

of anti-WSSV Mabs were used as control.

2.5. Double immunofluorescence flow cytometric analysis

For the hemocytes sampled at 12, 24, 36, 48 hpi, colocalization assay of apoptosis and WSSV infection was conducted. Briefly, approximately 2×10^7 cells of the pretreated hemocytes were incubated with the anti-WSSV Mabs for 1 h at 37 °C. After three rinses, the cells were incubated with the goat anti-mouse IgG Alexa Fluor®488 for 45 min. After rinse as above, the hemocytes were incubated with the TUNEL labeling solution as described above. The samples were analyzed using the flow cytometer and fluorescence microscopic assay.

2.6. Real-time qPCR for quantification of gene expression and WSSV copies in hemocytes

The expression profiles of nine apoptosis-related genes including initiator Caspase (ICaspase), effector Caspases 2 (ECaspase-2), effector Caspases 3 (ECaspase-3), cytochrome *c* (Cyt-c), high-temperature requirement protein (HtrA2), apoptosis inducing factor (AIF), tumor suppressor protein p53, translationally controlled tumor protein (TCTP) and inhibitor of apoptosis protein 1 (IAP1) were detected by RT-qPCR with specific primers (shown in Table 1). Total RNA was extracted from each hemocyte sample using trizol reagent and its quality and quantity was examined by a Nanodrop 8000 spectrophotometer (Thermo Scientific, MA, USA). Single-strand cDNA was synthesized from 1 μg total RNA by the PrimeScriptTM RT reagent Kit with gDNA Eraser (Takara, Japan) according to the manufacturer's instructions. The RT-qPCR was performed using SYBR Green I Master mix (Roche, Basel, Switzerland) in a LightCycler®480 II Real Time PCR System (Roche, Basel, Switzerland). Each optimized reaction system contained 10 μL of SYBR Green I Master mix, 0.6 μL each of forward and reverse primers (10 mM), with 100 ng of cDNA added and RNase-free water up to a volume of 20 μL. The real time qPCR assay here was performed in triplicate using18S rRNA gene as the reference gene, procedures were described as following: 1 cycle of 95 °C for 5 min and 40 cycles of 95 °C for 5 s, annealing at specific temperature for 20 s, and 78 °C for 1 s. Indication of specific amplification is subsequently obtained by analysis of the melting curve of the PCR amplicons The fold change for the relative gene expression was determined by the $2^{-\triangle\triangle t}$ method [52].

The number of WSSV copies in hemocytes were measured according to the method developed by Yuan [48]. Briefly, serial dilutions of WSSV recombinant plasmid were prepared as standards for quantification. Then the total genomic DNA of hemocyte samples were extracted using the DNA extraction kit (Takara, Japan) and quantified by the Spectrophotometer. An equal quantity of 50 ng DNA was added into qPCR premix mentioned above with the WSSV detection primer pairs (QVP28F and QVP28R, each 1 μL) for amplification, the absolute quantification PCR was performed in triplicate as following: 1 cycle of 95 °C for 5 min and 40 cycles of 95 °C for 5 s, 57 °C for 20 s, and 78 °C for 1 s. Results were analyzed and calculated according to the standard curve established before. The primers used in qPCR were listed in Table 1.

Table 1
Names and sequences of primer pairs used in this study.

Primer name	Sequences (5′-3′)	Primer source (or Genbank No.)
QVP28 F	AAACCTCCGCATTCCTGTGA	Yuan et al., 2010
QVP28 R	TCCGCATCTTCTTCCTTCAT	
LvICAP F	AGTTAGTACAAACAGATTGGAGCG	Wang et al., 2013
LvICAP R	TTGTGGACAGACAGTATGAGGC	
LvECAP3 F	CGGACTCAGCGAGCGTTAC	Designed; DQ988351.1
LvECAP3 R	GATTTCGGCGTCGTGGG	
LvECAP2 F	ATGGCTCGTGGTTCATTCAG	Wang et al., 2013
LvECAP2 R	CATCAGGGTTGAGACAATACAGG	
LvTCTP F	CAATGGACCCTGATGGC	Wu et al., 2013
LvTCTP R	GCTTCTCCTCTGTTAGACCGTAT	
LvIAP1 F	TCCCGCACTGTCCATTTATC	Designed; JX392326.1
LvIAP1 R	CCATGTGCCTTGACGTTCC	
LvHtrA2 F	ACAGCGGCGATATTTAGGCG	Peepim et al., 2016
LvHtrA2 R	TTGCGTACCACTACCATAACC	
LvAIF F	AACGGGAAAGAAAGGAGCAT	Designed; KX096891.1
LvAIF R	CCTCCTGTAGCAATAAGGCACT	
LvCyt-c F	CGTCCAGAAGGGAAAGAAGC	Designed; KX096890.1
LvCyt-c R	GACTTGTTGGCGTCGGTGT	
Lvp53 F	CGAATCCCCACATCCACG	Qian et al., 2014
Lvp53 R	GGCGGCTGATACACCACC	
18sRNA F	ACAATGGCTATCACGGGTAACG	Li et al., 2014
18sRNA R	CTGCTGCCTTCCTTAGATGTGGTA	

2.7. Statistical analysis

All of the data was expressed as the mean ± SD. Three technical repeat assays were undertaken from each biological sample. Statistical comparisons between groups were made using one-way ANOVA with Duncan's multiple range tests. Statistical analysis was performed using IBM SPSS Statistics 19. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Time-course changes of apoptotic hemocytes ratio

According to the results of TUNEL assay combined with flow cytometry, the apoptotic hemocytes of WSSV-infected shrimp was significantly increased at 12 hpi compared with control group ($p < 0.05$), with an apoptotic rate of 3.8 ± 0.9%. However, subsequently, the apoptotic rate of total hemocytes in the WSSV-infected shrimp underwent a slight decline, and there was no significant difference between the WSSV-infected and PBS injected shrimp at 24 hpi ($p > 0.05$). Since 24 hpi, the apoptotic rate of hemocytes in the WSSV-infected shrimp exhibited a rapid and significant increase, and reached peak level with an apoptotic rate of 18.1 ± 2.0% at 48 hpi ($p < 0.05$), then underwent a decline afterwards (Fig. 1). The hemocytes from the WSSV- or PBS-injected shrimp at 48 hpi were further selected for observation under fluorescence microscopy, the positive apoptosis signals (bright red) could be obviously observed in the nuclei of hemocytes from WSSV-infected shrimp, which exhibited to be granular, however no positive signal was found in the hemocytes of PBS-injected shrimp (Fig. 2).

3.2. WSSV proliferation

The ratio of WSSV-infected hemocytes to total hemocytes were determined by flow cytometry using the mixed anti-WSSV monoclonal antibodies as the probe, and the results showed that the WSSV-infected hemocytes significantly increased since 12 hpi ($p < 0.05$), and reached peak level with an infection rate of 38.6 ± 6.5% at 36 hpi, then maintained at a high level afterwards (Fig. 3A). Meanwhile, the absolute qPCR was employed to determine the change of WSSV copies in the WSSV-infected hemocytes, and the results showed that the WSSV copies did not show significant increase within 18 hpi ($p > 0.05$), but which significantly increased since 24 hpi and reached the peak at 48 hpi, then maintained at a high level afterwards (Fig. 3B).

3.3. The change of hemocyte density and shrimp accumulative mortality

Post WSSV infection, the density of total hemocyte in hemolymph showed no significant decrease within 12 hpi, however which exhibited a sharp decrease to only about 20% from 18 to 48 hpi, and then underwent a slight decrease afterwards. At 72 hpi, the density of total hemocytes was extremely low with $0.8 \pm 0.32 \times 10^6$ cell/mL (Fig. 4A). Meanwhile, the accumulative mortality of *L. vannamei* post

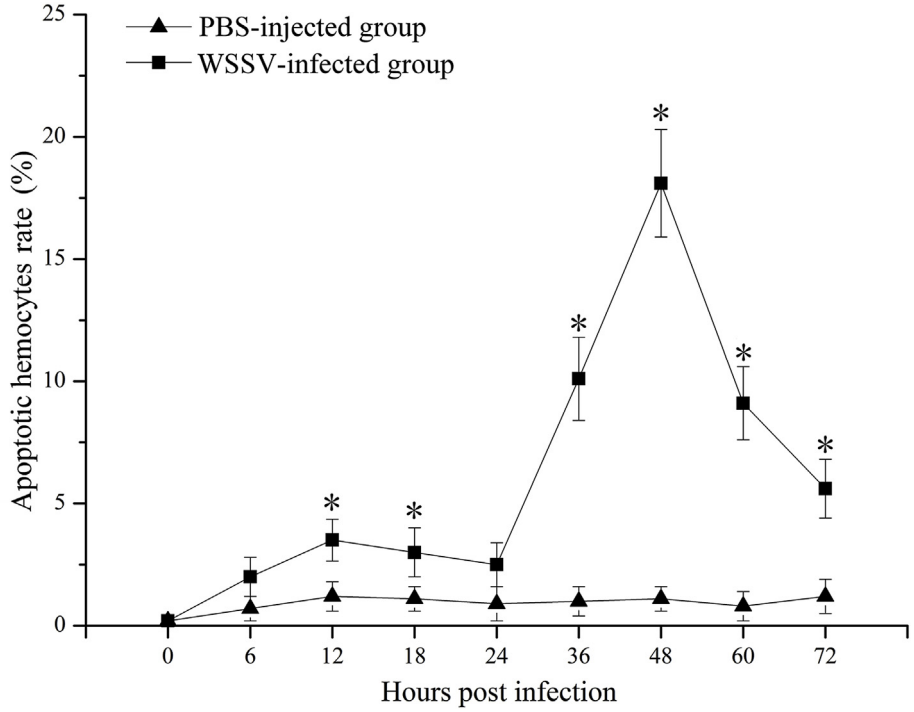

Fig. 1. The changes of apoptotic hemocytes rates in *L. vannamei* post WSSV infection. The asterisk indicates significant difference when compared with the control ($p < 0.05$).

WSSV infection was recorded and shown in Fig. 4B. In the PBS injected group, no mortality of shrimp was found during the whole experimental period. In contrast, the WSSV-injected shrimp began to die at 18 hpi, and a slight increase of mortality was observed within 36 hpi, however a sharp increase of mortality was observed afterwards with 80% at 72 hpi (Fig. 4).

3.4. The change of apoptotic hemocytes in WSSV-infected shirmp

According to the experimental results of two-color flow cytometric assay, the apoptotic rate of total hemocytes in WSSV-infected shrimp was significantly increased at 12 hpi, but underwent a slight decline by 24 hpi, and then followed by a rapid increase and reached to a high value of 18.1 ± 2% at 48 hpi. Interestingly, the apoptotic rates of

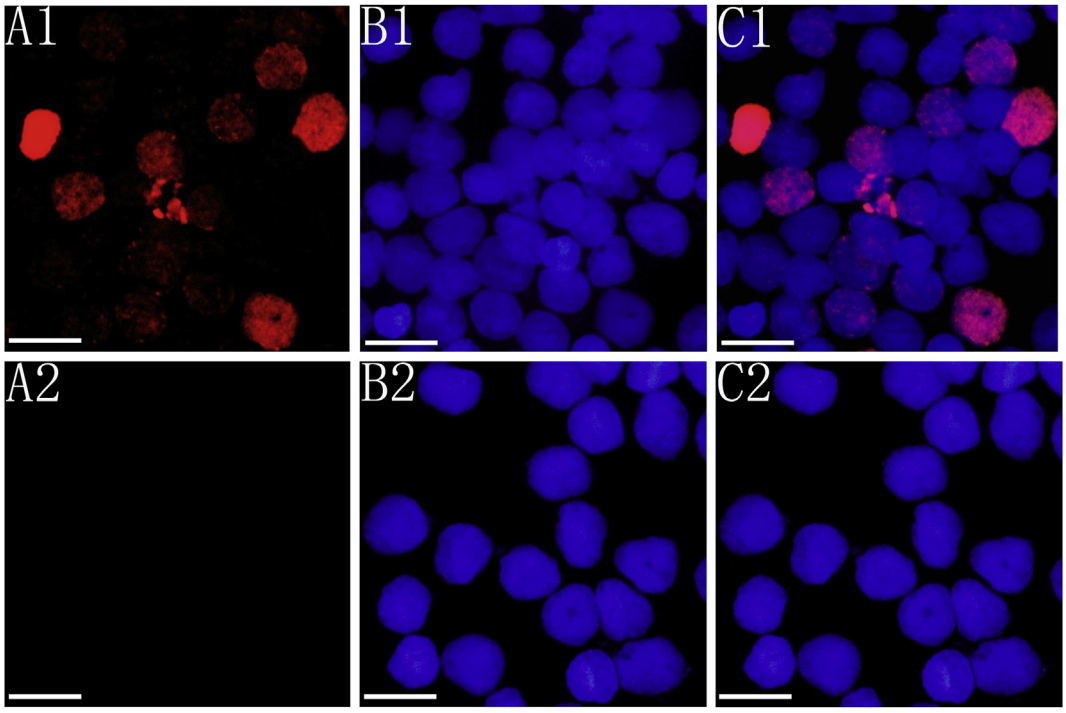

Fig. 2. Detection of apoptotic hemocytes by TUNEL staining combined with microscopic immunofluorescence assay. (**A1**) TUNEL staining of the WSSV-infected hemocytes at 48 hpi; (**A2**) TUNEL negative control; **(B)** the nuclei of hemocytes were stained with DAPI; **(C1)** and (**C2**) the merges of **A1** and **B1**, **A2** and **B2**, respectively. Bar = 10 μm.

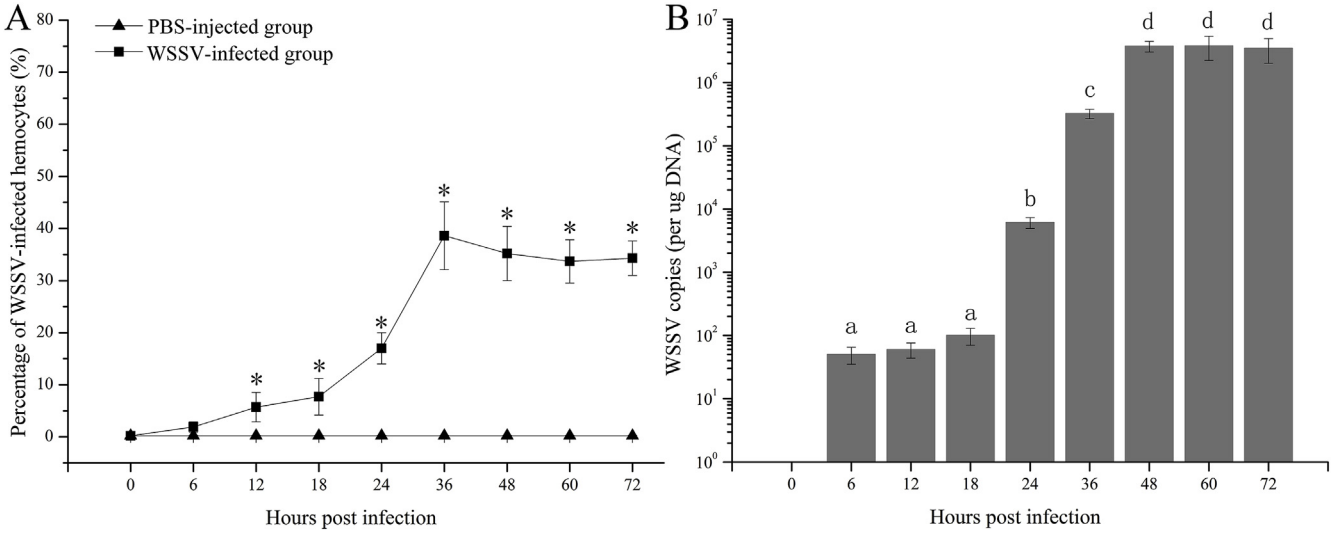

Fig. 3. The changes of the percentage of WSSV-infected hemoyctes **(A)** and the WSSV proliferation **(B)** in hemocytes in *L. vannamei* post infection by flow cytometric immunofluorescence assay and absolute qPCR. The asterisk indicates significant difference when compared with the control ($p < 0.05$).

uninfected and infected hemocytes were both significantly increased at 12 hpi, whereas there was no significant difference between them ($p < 0.05$). At 24 hpi, the apoptotic rate of uninfected hemocytes decreased, which was significantly lower than that of infected hemocytes ($p < 0.05$). Since 24 hpi, the apoptotic rate of infected hemocytes showed a rapid increase, which was significantly higher than that of uninfected hemocytes ($p < 0.05$). At 48 hpi, a high percentage of apoptotic WSSV-infected hemocytes with about 12.6 ± 1.5% was detected (Fig. 5). When the double labeled hemocytes sampled at 48 hpi were observed under fluorescence microscope, the hemocytes of WSSV-infected shrimp underwent different degrees of apoptosis, partial cells exhibited to be apoptotic and WSSV-infected with green and red fluorescent signals, whereas partial apoptotic hemocytes appeared to be WSSV negative (Fig. 6).

3.5. Expression profile of nine important apoptosis-related genes

The expression profile of nine apoptosis-related genes in *L. vannamei* was shown in Fig. 7. Overall, the expression levels of nine genes in the WSSV-infected shrimp varied significantly in comparison with the PBS-injected shrimp. Within 24 hpi, the expressions of apoptosis-inducing genes including AIF, HtrA2 and Cyt-c were significantly downregulated (Fig. 7A, B and C). However, these three genes began to upregulate after 24 hpi, and reached their peak levels at 36–48 hpi, which were significantly higher than the control level ($p < 0.05$). At the late stage of infection (60–72 hpi), the expression levels of AIF and HtrA2 were significantly lower than the control level ($p < 0.01$). Apoptosis Caspase enzymes initiator ICaspase began to significantly upregulate since 12 hpi ($p < 0.01$, Fig. 7D), while ECaspase-3 and ECaspase-2 began at 18 and 24 hpi, respectively (Fig. 7E and F). The expression level of ICaspase was significantly higher than the control level during the period of 12–72 hpi, while ECaspase-3 was significantly higher during 18–48 hpi, and ECaspase-2 was significantly higher during 24–72 hpi. p53 began to significantly upregulate since 24 hpi, and maintained significantly higher expression compared with the control during the period of 24–48 hpi ($p < 0.01$, Fig. 7G).

The expressions of two apoptotic inhibitors TCTP and IAP1 were significantly higher than the control at 24 hpi and 36 hpi, respectively (Fig. 7H and I). Of which, TCTP expression was significantly higher than the control at 24–36 hpi ($p < 0.01$), while IAP1 maintained significantly higher compared with the control at the period of 36–60 hpi. However, the expression of TCTP was significantly lower the control

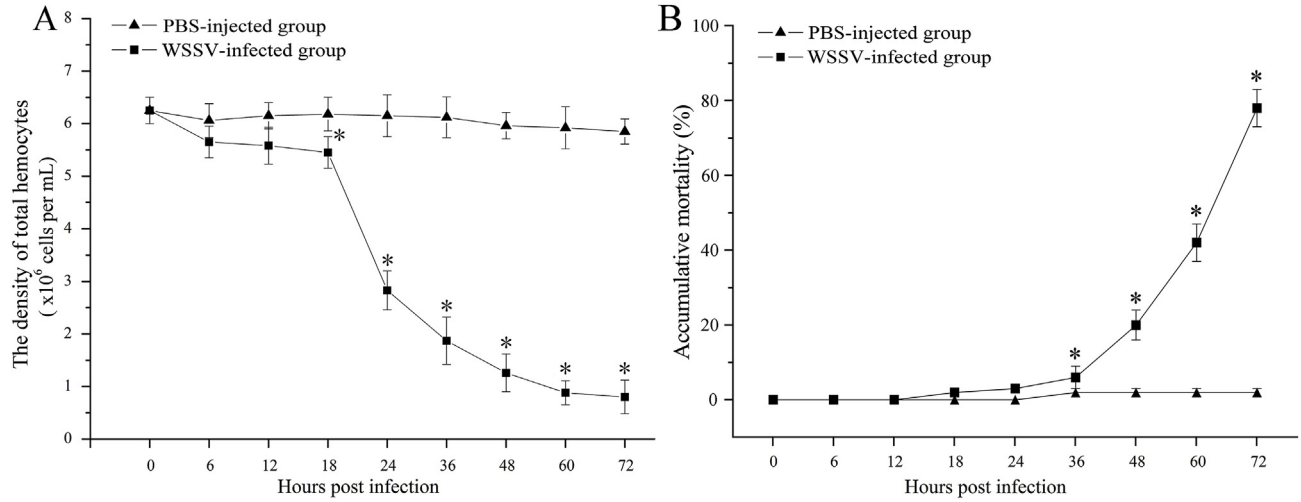

Fig. 4. The change of the total hemocytes density in hemolymph **(A)** and accumulative mortality **(B)** of *L. vannamei* post WSSV infection. The asterisk indicates significant difference when compared with the control ($p < 0.05$).

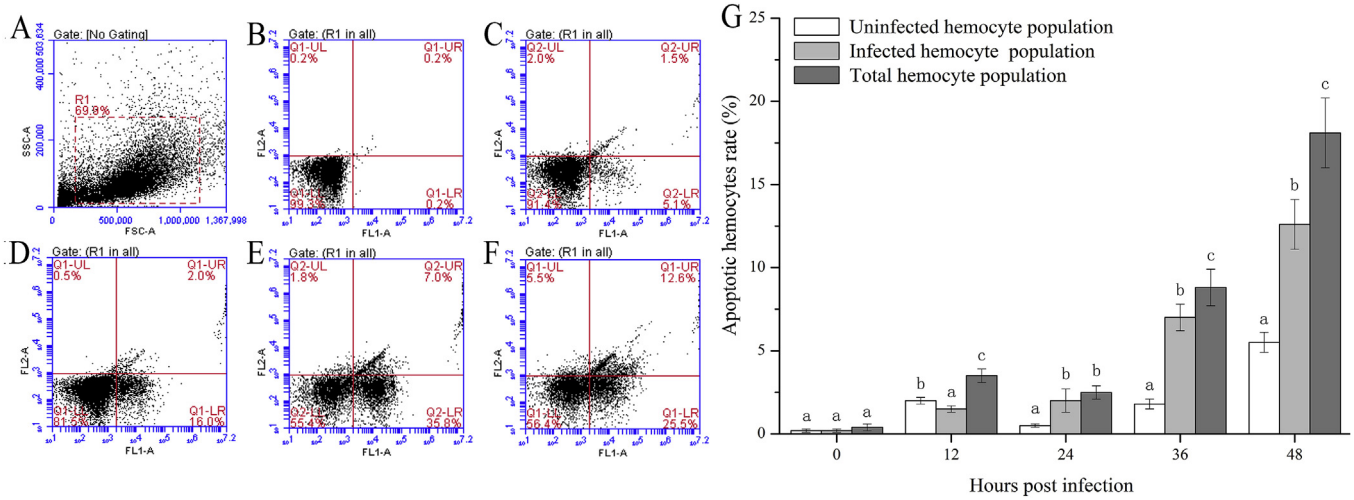

Fig. 5. The changes of apoptotic hemocytes post WSSV infection by double immunofluorescence flow cytometric assay. **(A)** FSC/SSC dot plots of *L. vannamei* hemocytes. **(B)-(F)** The fluorescence dot plots represent apoptotic and/or WSSV-positive hemocytes sampled at 0, 12, 24, 36 and 48 hpi, respectively. **(G)** Apoptotic rates of uninfected hemocytes, infected hemocytes and total hemocytes. The different letters represent statistically significant difference among different groups at each time point ($P < 0.05$).

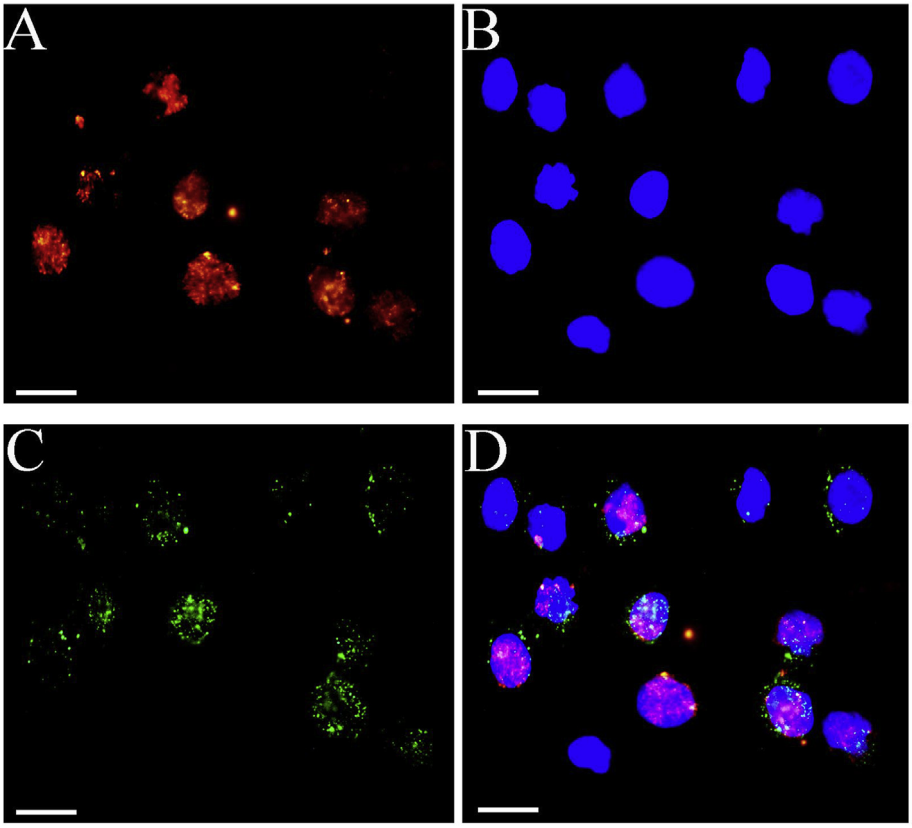

Fig. 6. Microscopic observation of apoptotic and/or WSSV-infected hemocytes sampled at 48 h post WSSV infection. (**A**) TUNEL stained hemocytes; (**B**) DAPI stained hemocytes; (**C**) WSSV-positive hemocytes stained with Alexa Fluor®488; (**D**) The merge of **A**, **B** and **C**. Bar = 10 μm.

group at 72 hpi ($p < 0.01$).

4. Discussion

Viral infections often elicit apoptosis as part of the host defense system or as a component of the survival strategy of the virus [53]. In crustacean, studies have shown that apoptosis occurred in tissues to eliminate damaged cells and has been proposed as the main anti-viral mechanism [28,54,55]. Previous studies have evidenced that gross signs of apoptosis occurred in quite a number of cells in WSSV infected shrimp tissues, accompanied with high level of DNA fragmentation and effector Caspase activity [28–30]. In the present work, the hemocytes of *L. vannamei* exhibited obvious apoptotic characteristics post WSSV infection. According to the fluorescence signal in the WSSV-infected hemocytes by TUNEL method, the nuclei of hemocytes were fragmented into small pieces. Moreover, the apoptosis-inducing genes in hemocytes showed significantly upregulation at 24 h post WSSV infection, which was accompanied with an apparent increase of apoptotic rate of

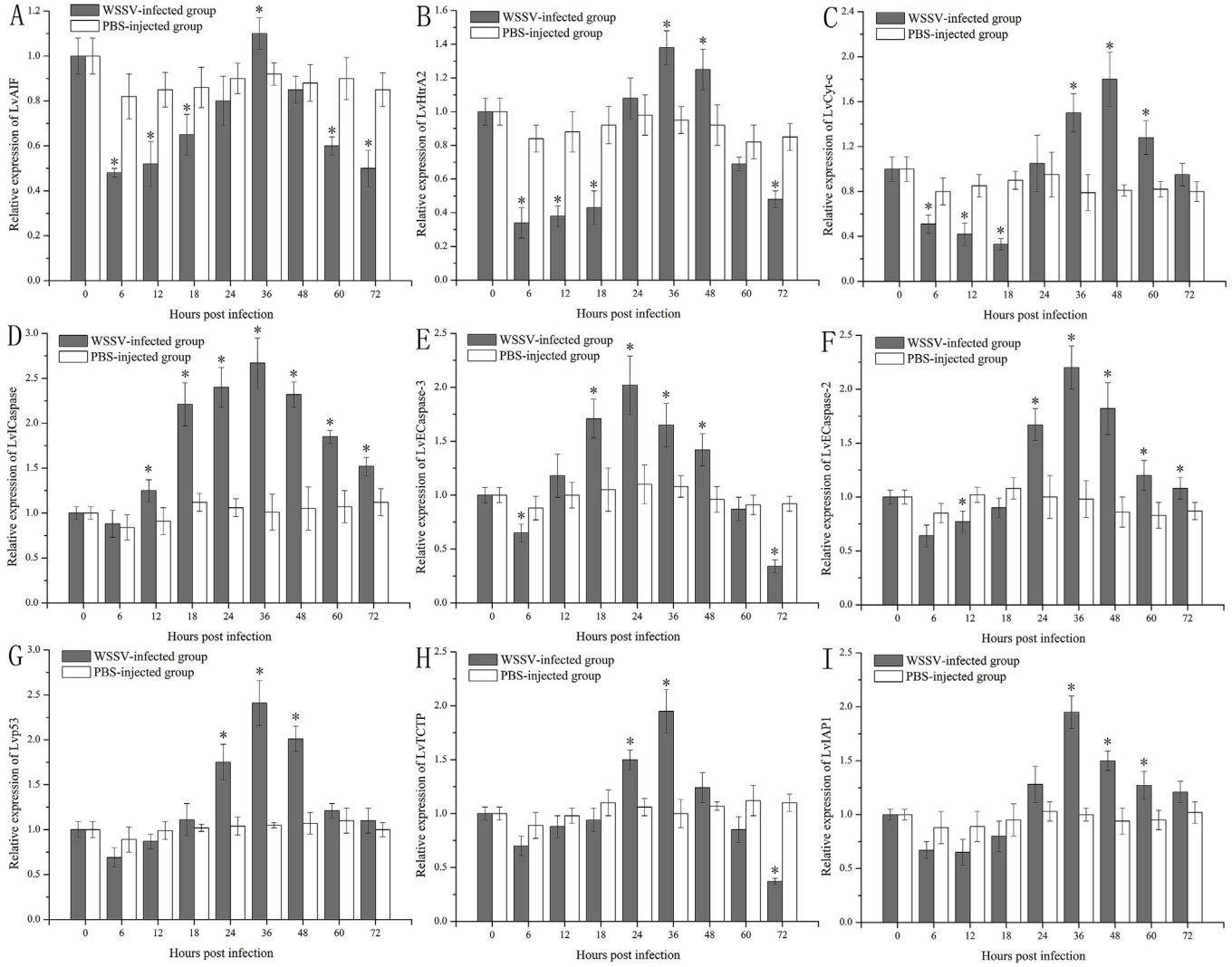

Fig. 7. The expression profiles of nine apoptosis-related genes in *L. vannamei* hemocytes post WSSV infection. The asterisk indicates significant difference when compared with the control ($p < 0.05$).

hemocytes, and then induced a rapid decrease of hemocytes intensity in hemolymph. Thus, we believed that WSSV infection could efficiently induce the apoptosis of hemocytes in *L. vannamei*. Similarly, a much higher apoptotic rate of hemocytes was detected in WSSV-infected *L. vannamei* at 48 hpi compared with our results, which was probably due to the difference of detection method, infection intensity and the physiological status of experimental shrimp [56]. Moreover, we interestingly found that the apoptotic rate of virus-positive hemocytes was significantly different from that of virus-negative hemocytes. At 12 hpi, the apoptotic rate of virus-negative hemocytes was much higher than that of virus-positive cells. As the infection progressed, the apoptotic rates of the virus-positive hemocytes increased, and the apoptotic rate of the virus-positive cells was much higher than that of the virus-negative cells since 24 hpi. Similar result was also found in kuruma shrimp post WSSV infection [57]. We speculated that early apoptosis of virus-negative hemocytes might play an important role in limiting the viral proliferation. It is also probably that the virus-negative hemocytes might be infected with WSSV, whereas the virions could not be detectable by monoclonal antibodies due to the low amounts of viral proteins [58,59].

Apoptosis could be used by the host to limit the production and dissemination of viruses, which could be also used by the infected virus to produce sufficient virus progeny or facilitate virus release [60,61]. When viral infections are detected, apoptosis is triggered to eliminate virus-infected cells, preventing the virus from diffusing and infecting adjacent cells [62,63]. The results of this study showed that the apoptotic rate of hemocytes increased to the first peak at 12 hpi, and the apoptotic hemocytes were mainly the virus-negative hemocytes. By this time, the initial Caspase (*Lv*ICaspase) also showed significant up-regulation. Similarly, the initial Caspases in *M. japonicus* and *L. vannamei* also exhibited markedly up-regulation post WSSV infection, which have been confirmed to have inhibiting effects on viral replication [19,64]. So, we speculated that early apoptosis of hemocytes might play a potential role in limiting WSSV proliferation [57]. In another hand, viruses can block apoptosis to prevent the death of infected cells, facilitating the viral progeny proliferation [65,66]. WSSV has also evolved some strategies to counter host antiviral apoptosis. For example, AAP-1 and WSV222 as viral proteins have been shown to inhibit the release of pro-apoptotic factors or disturb the activities of the Caspases to block apoptosis [67]. In this work, the results showed that the apoptotic rate of hemocytes exhibited a slight decline from 12 hpi to 24 hpi, and the pro-apoptotic factors like AIF, HtrA2 and Cyt-c were significantly down-regulated in the early stage of infection, indicating that WSSV is able to inhibit the expression of pro-apoptotic factors to block the apoptosis of hemocytes. At this stage, the effector Caspases were down-regulated first and then up-regulated. We speculated that WSSV exerted inhibitory effects on apoptosis and inhibits the expression of the effector Caspases [19]. And the later up-regulated expression

of the effector Caspases might be induced by the initial Caspase, which was up-regulated in response to WSSV infection. The apoptosis-inhibiting genes did not change significantly at this phase, which is consistent with *Penaeus monodon* post yellow head virus infection [68]. Overall, the apoptosis response in hemocytes could be efficiently activated post WSSV infection, which might play a potential role in inhibiting WSSV proliferation. However, WSSV would produce inhibitory effects on apoptosis during the early stage of infection to facilitate its replication.

Virus would intentionally induce the apoptotic program at the late stage of infection in order to facilitate the assembly, or to promote the spread of progeny virus in the host [64,69]. In crustaceans, apoptosis was also found to not only play a role in viral clearance but also as a major cause of dysfunction and death of the host at the late stage of infection [32,70,71]. WSSV has learned how to hijack apoptosis for its replication cycle, and WSSV protein ICP11 appears to have apoptosis-inducing activity [17]. In the present work, since 24 hpi, the proportion of apoptosis increased significantly, and the rate of virus-positive hemocytes and WSSV copies increased sharply. Meanwhile, the density of total hemocytes decreased rapidly, and a mass of shrimps died. These results suggested that during the late stage of infection of WSSV, extensive hemocytes apoptosis might cause the loss of physiological functions of shrimp, which ultimately leads to the death of the shrimp [72]. We also found that both the pro-apoptotic factors and the different caspases expression levels were up-regulated most significantly during late phase of viral infection. Combined with the significantly higher levels of virus-positive hemocytes rate and WSSV copies at this stage, we speculated that WSSV infection would induce apoptosis after completing its proliferation, thereby releasing progeny virus to infect other cells. This speculation could be supported by the evidence that the yellow-headed virus could aggravate infection by using apoptosis [10,54,73]. Interestingly, the apoptosis-inhibiting genes also showed significant up-regulation at late phase of viral infection, which seems to be the last fight for shrimp to resist apoptosis caused by WSSV. It was reported that the loss of apoptosis-inhibiting genes would cause the hemocytes apoptosis, allowing further propagation of the WSSV [74]. During the late stages of WSSV infection, apoptosis appears to no longer exhibit anti-WSSV effects, but rather a tool to release WSSV progeny virus. However, the mechanism by which WSSV manipulates apoptosis is still unknown [9], and more research are needed on the interaction between the WSSV pathogenesis and apoptosis in crustaceans.

To sum up, based on the dynamic changes of apoptotic rate of hemocytes and the expression profile of apoptosis-related genes, the hemocytes apoptosis had a close relationship with the infection process of WSSV in *Litopenaeus vannamei*. In the different stages of WSSV infection, the apoptosis might play different roles in hemocytes, which reflected the interplay between the virus and the host immune response. Our resultant data would provide an insight into deeper relationships between WSSV infection and apoptosis in shrimp hemocytes.

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