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Comparison of antiviral efficiency of immune responses in shrimp

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The antiviral effectiveness of three major immune responses including phagocytosis, apoptosis and proPO system in the shrimp *Marsupenaeus japonicus* was characterized. It was found that the suppression of apoptosis and phagocytosis in vivo by their corresponding inhibitors resulted in the increase of white spot syndrome virus (WSSV) copies and shrimp mortality, whereas the inhibition of phenol oxidase generated the least influence on WSSV infection and shrimp mortality. These results suggest that apoptosis and phagocytosis were the essential immune responses to protect shrimp from virus infection, while the phenol-oxidase-dependent proPO system plays a comparatively minor role in antiviral defense of shrimp.

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1. Introduction

The shrimp culture industry has been severely hampered by outbreaks of white spot syndrome virus (WSSV) worldwide [1]. The infection does not occur only in shrimp but also in other marine and freshwater crustaceans, including crabs and crayfish [2,3]. The virus has caused large economic losses and so far no adequate treatment is available [4]. Although considerable progress has been made in WSSV characterization [5] as well as crustacean immune responses against bacterial and fungal pathogens [6,7], the understanding of shrimp defense system in response to viral infection is still poor. Crustaceans have an innate immune system, characterized by lack of immunoglobulin and memory, but are efficient to protect and preserve themselves from all intruding pathogens [8]. The innate immune system is the first line of inducible host defense against bacterial, fungal and viral pathogens [9], and therefore essential for the survival and perpetuation of all multicellular organisms [10–12]. The innate defense system of crustaceans including shrimp comprises cellular and humoral responses, except for external barriers such as skin. The cellular immune responses include apoptosis, encapsulation, phagocytosis and nodule formation, whereas the humoral responses include the prophenoloxidase (proPO) system, the clotting cascade and the synthesis of a wide array of antimicrobial peptides and antiviral peptides [13,14].

Among the innate defense systems, the cellular immune system including apoptosis and phagocytosis and the proPO system are recognized to play central roles in shrimp immunity. Apoptosis is a tightly regulated process by which excess or damaged cells are eliminated in order to maintain tissue homeostasis. It is also a major defense mechanism for removal of unwanted and potentially dangerous cells such as virus-infected cells [15,16]. Phagocytosis, an actin-dependent process representing an essential branch of the immune system, is fundamental to the survival of a broad variety of organisms, which is an important process of eliminating microorganisms or foreign particles [17]. Animals have extended this phagocytic response for purposes of host defense against invading microbial pathogens and for the regulation of specific developmental pathways. The activation of proPO system can elicit phagocytosis and melanin synthesis. Phenol oxidase (PO) is the terminal enzyme in the proPO system and is activated by minute amounts of microbial cell wall components such as lipopolysaccharides (LPS) from Gram-negative bacteria and β-1,3-glucan from fungal cell walls [18]. In crustaceans, the components of innate immune system are localized mainly in hemolymph which contains three types of hemocytes: hyaline (agranular), semigranular (small granular) and granular (large granular) hemocytes [19]. It is believed that hyaline cells of crustaceans have a main function of phagocytosis [20], whereas granulocytes are involved in apoptosis [21,22] and prophenoloxidase (proPO) activation [23].

Abbreviations: WSSV, white spot syndrome virus; proPO, prophenoloxidase; THC, total hemocyte count.

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In recent years, substantial insights have been gained into the innate immune pathways of invertebrates at the biochemical and molecular level [16,17,24,25]. However, the relative importance of immune responses to defense against viral infections is still unknown. The purpose of this study was to evaluate the cellular responses (apoptosis and phagocytosis) and humoral response (proPO system) for efficiency against WSSV infections in shrimp. With the in vivo suppressions of apoptosis, phagocytosis and PO activity by specific inhibitors (cytochalasin B, z-VAD-FMK and allylthiourea, respectively), it was demonstrated that the antiviral activities of apoptosis and phagocytosis were higher than that of proPO system, suggesting that cellular responses played more important roles in the immune defense of invertebrates against virus infection.

2. Materials and methods

2.1. Shrimp culture

Marine shrimp *Marsupenaeus japonicus*, approximately 10 g and 10–12 cm each, were maintained in groups containing 20 individuals each in polyethylene tanks containing 100 L of aerated natural seawater at 20 °C. To rule out the presence of WSSV prior to experimental infection, gill tissues of three randomly selected shrimp from the same batch were tested by PCR with WSSV-specific primers.

2.2. Experimental infection of shrimp by WSSV

The WSSV-infected tissues from *M. japonicus* shrimp with a pathologically confirmed infection were homogenized in TN buffer (20 mM Tris–HCl, 400 mM NaCl, pH 7.4) at 0.1 g/ml. After centrifugation at $2000 \times g$ for 10 min, the supernatant was diluted with physiological saline and filtered through a 0.45 μm filter. Subsequently, the WSSV inoculum (3×10^6 viral copies determined by real-time PCR) was intramuscularly injected into healthy *M. japonicus* in the lateral area of the fourth abdominal segment using a syringe with a 29-gauge needle, resulting in 50–70% mortalities.

2.3. Inhibitory assays of shrimp immunity

In order to determine the optimum concentration of inhibitors, healthy shrimps were treated by intramuscular injection using 1-ml insulin syringes in the third abdominal segment with 50 μl physiological saline containing apoptosis inhibitor z-VAD-FMK [26] or phagocytosis inhibitor cytochalasin B [27] or phenol oxidase inhibitor allylthiourea [28] at various concentrations. It was found that the basal apoptosis level in healthy shrimp was very low (about 2–3%). To enable an accurate evaluation of z-VAD-FMK efficiency, the shrimp were infected by WSSV to enhance the apoptosis. After the injection of inhibitor, the shrimps were infected by the WSSV inoculum in 50 μl physiological saline containing 3×10^6 viral copies to assess the effect of z-VAD-FMK on apoptosis.

Based on the optimum concentrations of inhibitors, the effect of the inhibition of apoptosis, phagocytosis or phenol oxidase activity on WSSV infection was examined. The inhibitor-treated shrimp were infected by WSSV at 3×10^6 viral copies at 90 min after the injection of inhibitor. In the inhibitory assays, a negative control (physiological saline only), a positive control (WSSV only) or inhibitor only (z-VAD-FMK, cytochalasin B or allylthiourea) was included.

The hemolymph of three randomly selected shrimp were collected at 24, 48, 72 and 96 h post-infection. A 26-gauge needle attached to a 1-ml syringe preloaded with 100 μl anticoagulant (1500 U/ml heparin sodium salt) was used to withdraw hemolymph from the ventral sinus of shrimp. The freshly collected hemolymph was subjected to apoptosis, phagocytosis and phenol oxidase activity assays. At the same time, the shrimp mortality was monitored. All the assays including the collection of shrimp samples, apoptosis, phagocytosis, phenol oxidase activity assays and the mortality assay were repeated three times.

2.4. Phenol oxidase activity of shrimp hemolymph

Phenol oxidase activity of shrimp hemolymph was spectrophotometrically determined as described previously with minor modifications [29]. Briefly, freshly sampled shrimp hemolymph was kept on ice, then processed by low-speed centrifugations at $500 \times g$ for 3 min to remove cells and tissue debris. Subsequently, 10 μl of the cell-free hemolymph was transferred to a tube containing 90 μl of L-Dopa buffer (3 mM L-Dopa in 10 mM Tris–HCl, pH 7.2). After incubation at 37 °C for 20 min, the amount of dopachrome produced in the reaction mixture was determined as the optical density at 490 nm (OD_{490}). The optical density of the shrimp's phenol oxidase activity for all test conditions was expressed as dopachrome formation in 10 μl of cell-free hemolymph. The L-Dopa buffer only was used as a control.

2.5. Apoptosis assay of shrimp hemocytes

Shrimp hemolymph was placed onto a poly-L-lysine-coated glass slide and stood for 30 min at room temperature. Hemocytes were fixed with 4% paraformaldehyde for 30 min at room temperature, washed once with 4,6-diamidine-2-phenylindole dihydrochloride (DAPI) working solution (1 μg/ml in methanol), and then stained with DAPI working solution for 15 min at 37 °C inside a small humidified chamber covered with aluminum foil. The solution was discarded and cells were washed with phosphate-buffered saline (PBS) to remove the excessive dye. The glass slide was air-dried and mounted with anti-fade solution (Beyotime, China) to assess nuclear morphology by fluorescence microscopy. Hemocytes with condensed or fragmented nuclei were recognized as apoptotic cells. For each treatment, 1000 cells were counted to determine the percentage of apoptotic hemocytes.

2.6. Detection of phagocytic activity

Phagocytic activity of shrimp hemocytes was measured following the method described previously [17]. Briefly, 100 μl of hemolymph was incubated with FITC-labeled WSSV at 28 °C for 30 min. The mixture was smeared onto a poly-L-lysine-coated glass slide and allowed to fix for half an hour at room temperature. Subsequently, the hemocytes were stained with propidium iodide (PI, Sigma) at the final concentration of 20 μg/ml for 1 min. Then, the nonadherent cells were removed with PBS and the Trypan blue stain was added to quench any free floating and adherent WSSV. After incubation for 15 min, the cells were washed once with PBS and one drop of anti-fade solution (Beyotime, China) was added before mounting to examine phagocytic activity under a fluorescence microscope. A total of 200 hemocytes was examined and phagocytic activity was expressed as the % of cells showing fluorescence.

2.7. Quantitative real-time PCR

Real-time PCR was conducted to measure WSSV copies in shrimp that were injected with inhibitors and virus inoculum. The viral DNA was extracted as described before [17] and subjected to real-time PCR with primers 5′-CCACCAATTCTACTCATGTACCAAA-3′ (forward) and 5′-TCCTGCAATGGG CAAAATC-3′ (reverse). The probe was 5′-FAM-TGCTGCCGTCTCCAA-TAMRA-3′. A 1.1 kb DNA fragment from WSSV genome was cloned into phagemid vector pBluescript II KS (+/−) (Stratagene, CA, USA). The recombinant phagemid was

purified and subjected to *Eco*RI digestion. Linear phagemid was quantified by UV spectroscopy (2.28×10^{11} copies/μl). To generate standard curves for quantitative determinations and to access amplification efficiency, replicate serial 10-fold dilutions of the pBluescript II KS (+/−) WSSV phagemid were prepared. The results (C_t value) of viral quantity would be converted into copy number of virus based on the standard curve. The reaction mixture consisted of 20–50 ng of shrimp DNA template, 1× *Taq* buffer, 1.5 U *Taq* polymerase (Genescript), 800 μM of dNTPs, 40 pmol of each primer, 40 pmol of probe and nuclease-free water to a total volume of 25 μl. PCR profile consisted of initial denaturation at 94 °C for 4 min, 45 cycles at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. The WSSV quantity of each sample was determined by Rotor-Gene 3000 Fluorescence Cycler (Corbett Research). Fluorescent data were acquired during each annealing phase. Each sample was tested in triplicate.

2.8. Shrimp mortality assay

For the monitoring of mortality, 20 shrimp for each group were used. After injections with WSSV and/or immune inhibitors, the shrimp mortality was recorded daily over a 12-day period. The inhibitors only were included in the injections. The shrimp mortality assay was repeated three times.

2.9. Statistical analysis

The numerical data from three independent experiments were analyzed by one-way ANOVA to calculate the mean and standard deviation of triplicate assays.

3. Results

3.1. Dosages of shrimp immune inhibitors

The results showed that the WSSV-induced apoptosis, the phagocytosis against WSSV and the phenol oxidase activity of shrimp were significantly decreased by 2 mg/kg z-VAD-FMK, 10 μg/kg cytochalasin B and 200 mg/kg allylthiourea at 48 h after inhibitor injection (data not shown). To determine the inhibitor concentration required for the inhibition of shrimp immunity, the effects of three inhibitors with various concentrations on the immune responses were examined at 48 h after inhibitor injection. As indicated in Fig. 1, the inhibitory effects of z-VAD-FMK, cytochalasin B and allylthiourea exhibited a dosage-dependent manner. The inhibition efficiency increased with increasing inhibitor concentrations. The maximum inhibition ability was obtained at 10 mg/kg for z-VAD-FMK, 100 μg/kg for cytochalasin B and 100 mg/kg for allylthiourea, respectively, over which dosage the inhibition ability was stabilized (Fig. 1).

3.2. Effects of inhibitions of apoptosis, phagocytosis or phenol oxidase on WSSV infection

To examine the effects of inhibition of shrimp immunity, the shrimp hemocyte samples at four time intervals (days 1–4) from the control group and WSSV-challenged groups treated or untreated with inhibitors were, respectively, analyzed for the intensities of immune responses (Fig. 2). The results showed that when used alone, the three inhibitors (z-VAD-FMK, cytochalasin B or allylthiourea) had no cytotoxicity on shrimp (data not shown). By DAPI staining, the number of condensed and fragmented nuclei in hemocytes of WSSV-infected shrimp was significantly increased as the virus infection progressed (Fig. 2A). For the treatment with inhibitor z-VAD-FMK, the percentage of apoptotic hemocytes induced by WSSV was significantly lower than that of the treatment

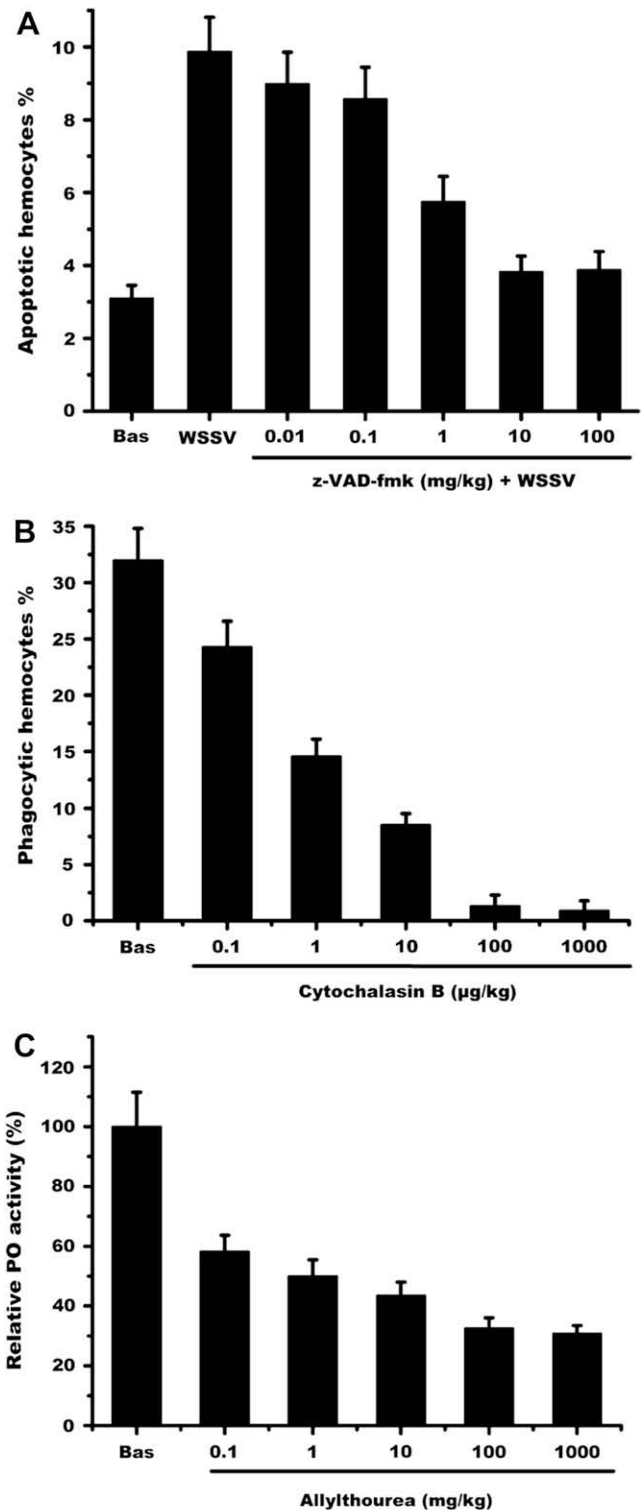

Fig. 1. Dosages for apoptosis inhibitor z-VAD-FMK (A), phagocytosis inhibitor cytochalasin B (B) and phenol oxidase inhibitor allylthiourea (C). The healthy shrimp were treated by intramuscular injection with physiological saline containing z-VAD-FMK or cytochalasin B or allylthiourea at various concentrations. For the apoptosis inhibition assay, the shrimp were simultaneously infected by WSSV. The hemolymph of three randomly selected shrimp were collected at 24 h after the last injection and subjected to apoptosis, phagocytosis and phenol oxidase activity assays. Each column represents the mean of triplicate assays with standard deviation. Bas (basal level) indicates the control group injected with physiological saline.

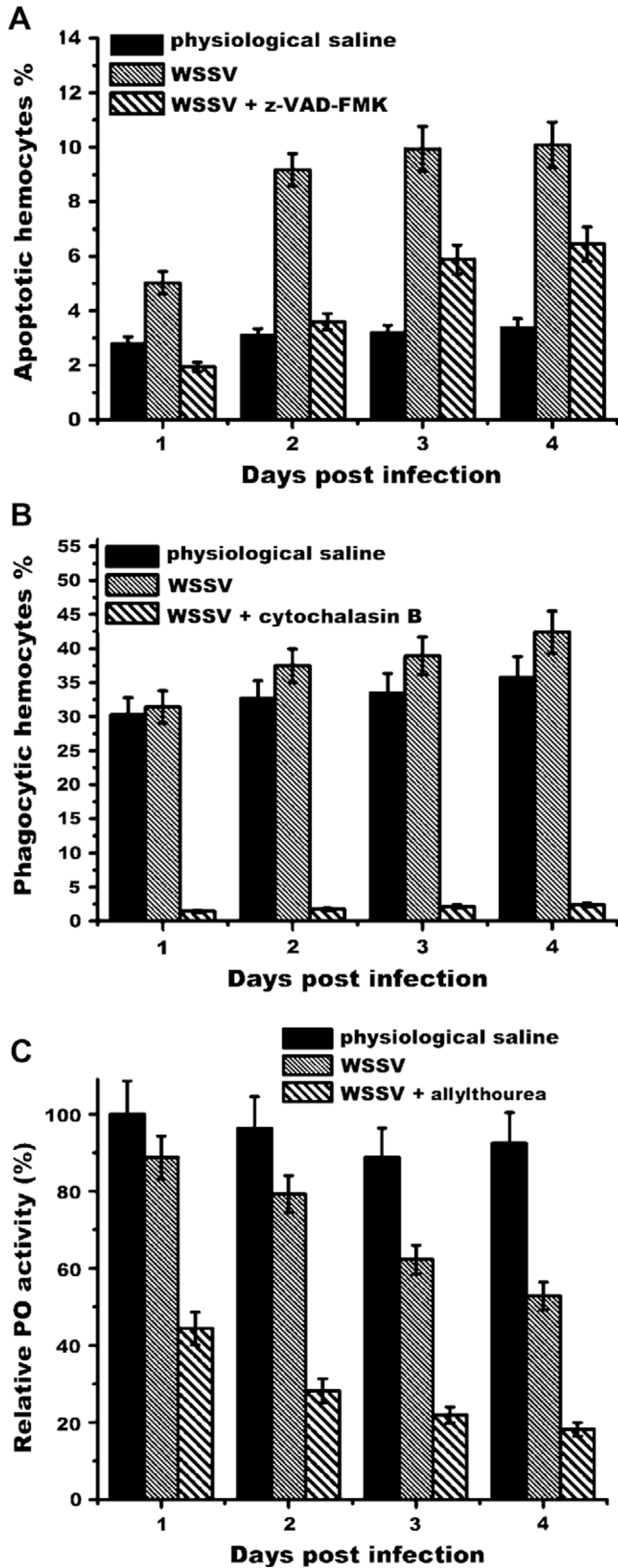

Fig. 2. Inhibition of the WSSV-induced apoptosis (A), the phagocytosis against WSSV (B) and the phenol oxidase activity (C) by inhibitors z-VAD-FMK, cytochalasin B and allylthiourea, respectively. The treatments are shown on the top. Each column represents the mean of triplicate assays with standard deviation.

with WSSV only, indicating that the WSSV-induced apoptosis was inhibited by z-VAD-FMK (Fig. 2A). As shown in Fig. 2B, the treatment with cytochalasin B resulted in a significant suppression of the phagocytosis against WSSV, demonstrating that the shrimp phagocytosis was inhibited by cytochalasin B. It was found that the treatment with allylthiourea led to a significant decrease of phenol oxidase activity (Fig. 2C). However, the treatment with WSSV alone also resulted in the inhibition of phenol oxidase activity.

In an attempt to compare the relative importance of the three immune responses including apoptosis, phagocytosis and proPO system on WSSV infection, the WSSV copies in shrimp were quantified for the WSSV-challenged shrimp with or without inhibitors at 1, 2, 3 and 5 days post-infection. The results showed that the apoptosis inhibitor z-VAD-FMK and the phagocytosis inhibitor cytochalasin B significantly affected the replication of WSSV, while the phenol oxidase inhibitor allylthiourea had only a slight effect on virus infection (Fig. 3). At day 2 post-infection, the WSSV copies with treatments WSSV + z-VAD-FMK and WSSV + cytochalasin B were 100 and 10 times higher than that of the control (WSSV only).

3.3. Effects of inhibitors of apoptosis, phagocytosis or phenol oxidase on shrimp mortality

The cumulative mortalities of WSSV-challenged shrimp with or without immune inhibitors are shown in Fig. 4. The results showed that when used alone, the inhibitors (z-VAD-FMK, cytochalasin B or allylthiourea) had no effect on shrimp mortality (data not shown). The shrimp mortality was very low for the control group injected with physiological saline, whereas the shrimp injected with WSSV only displayed 60% mortality at day 12 post-infection. When the shrimp were treated with immune inhibitors, the shrimp mortalities were significantly increased. For the treatment with WSSV + allylthiourea, 75% mortality was reached at day 12 post-infection. However, 100% of the shrimp treated with WSSV + z-VAD-FMK and WSSV + cytochalasin B died within 9 and 12 days, respectively (Fig. 4).

4. Discussion

The efficiency of the antiviral activities of apoptosis, phagocytosis and proPO system in the innate immune defense of

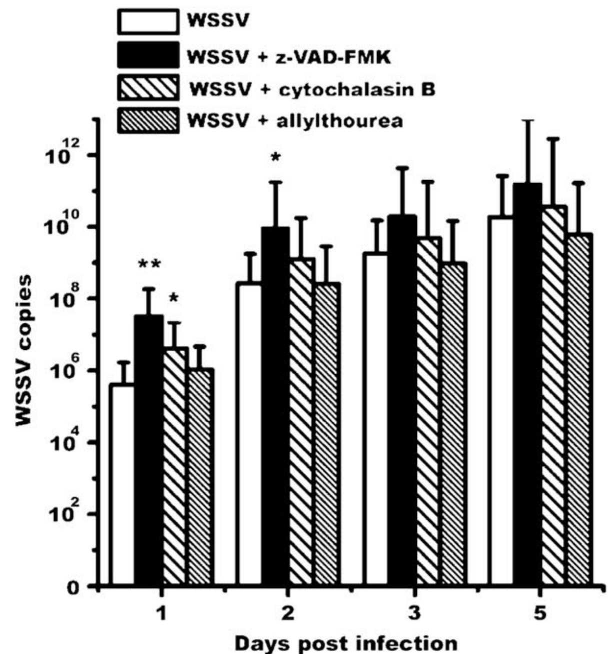

Fig. 3. Real-time PCR detection of WSSV from virus-challenged shrimp with or without immune inhibitors. The solutions used for injections are shown on the right. Each column represents the mean of triplicate assays with standard deviation. Asterisks denote significant differences (*$P < 0.05$, **$P < 0.01$) between samples.

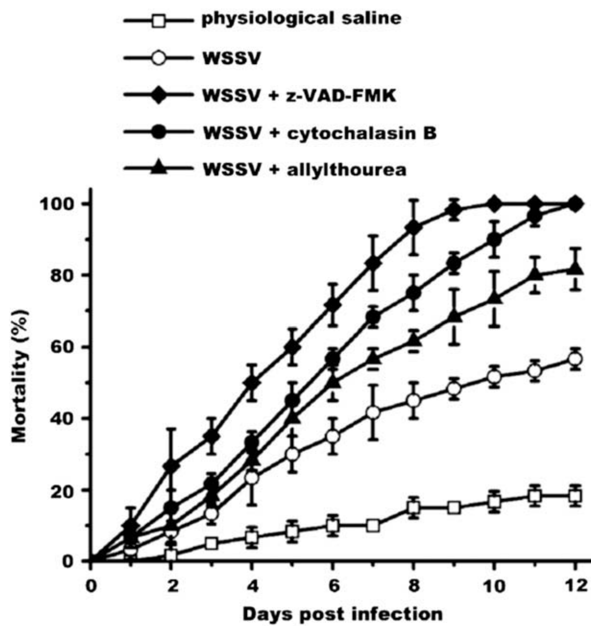

Fig. 4. Cumulative mortalities of WSSV-challenged shrimp with or without immune inhibitors. The solutions used for injections are shown on the right. Each point represents the mean of triplicate assays with standard deviation.

invertebrates has not been well characterized. In an attempt to address this issue, the present study was carried out to determine the relative importance of apoptosis, phagocytosis and proPO system against virus infection in *M. japonicus*. The inhibitor dosage assays revealed that z-VAD-FMK, cytochalasin B and allylthiourea exhibited dosage-dependent inhibitory effect on apoptosis, phagocytosis and phenol oxidase, respectively. Samples taken over 4 days following administration of inhibitors under optimum concentration indicated that the inhibitory effects were stably maintained throughout the time course. The results displayed that the inhibition of apoptosis caused the most pronounced increase of WSSV copies, followed by the inhibition of phagocytosis, whereas the inhibition of phenol oxidase generated the least influence on WSSV infection. The monitoring of shrimp mortality caused by WSSV yielded essentially the same results. These results suggest that the apoptosis and phagocytosis were the essential immune responses to protect shrimp from WSSV infection, while the phenol-oxidase-dependent proPO system played a comparatively minor role in antiviral defense of shrimp. Our observations were consistent with the report by Sarathi et al. [8], but contradict that described by Jiang et al. [30] who found the phagocytic percentage decreased after WSSV infection. The discrepancy might come from different infection approaches used. In this context, the cellular responses including apoptosis and phagocytosis might play very important roles in the innate immunity against virus infection in shrimp as well as in invertebrates.

Our study suggested that cellular responses exhibited powerful antiviral activity in shrimp when compared with the proPO system, one of the important humoral responses. It is known that the proPO system in invertebrates participates in the host defense by enhancing phagocytosis, initiating nodule or capsule formation, mediating coagulation and producing fungistatic substances. This system is reported to function in the anti-fungal and anti-bacterial responses of invertebrates. As demonstrated in this study, however, it provided a very limited contribution to the host antiviral immune defense. It is believed that viruses require an intact and functioning cell during the initiation of infection. When the cell suicide by apoptosis occurs, the replication and spread of virus can be efficiently suppressed [31]. Meanwhile, the phagocytosis is important in eliminating virus particles. Interestingly, both phagocytosis and apoptosis can lead to the decrease of total hemocyte count. On one hand, the demise of cells can be a primitive viral defense response and provide a mechanism for preventing viral replication in host cells, on the other hand, severe cell death will also lead to host death [32]. Our study showed that the inhibition of apoptosis or phagocytosis resulted in the reduction of shrimp survivals, indicating that the two cellular responses functioned to protect the infected shrimp against mortality, instead of causing death of shrimp. Therefore, the results of this study could provide insights into the improvement of the disease management practices of crustaceans.

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