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Hesperetin protects crayfish *Procambarus clarkii* against white spot syndrome virus infection

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**ARTICLE INFO**

**Keywords:**
- Hesperetin
- WSSV
- Innate immunity
- *Procambarus clarkii*

**ABSTRACT**

Hesperetin is a natural flavanone compound, which mainly exists in lemons and oranges, and has potential antiviral and anticancer activities. In this study, hesperetin was used in a crayfish pathogen challenge to discover its effects on the innate immune system of invertebrates. The crayfish *Procambarus clarkii* was used as an experimental model and challenged with white spot syndrome virus (WSSV). Pathogen challenge experiments showed that hesperetin treatment significantly reduced the mortality caused by WSSV infection, while the VP28 copies of WSSV were also reduced. Quantitative reverse transcriptase polymerase chain reaction revealed that hesperetin increased the expression of several innate immune-related genes, including NF-kappaB and C-type lectin. Further analysis showed that hesperetin treatment plays a positive effect on three immune parameters like total hemocyte count, phenoloxidase and superoxide dismutase activity. Nevertheless, whether or not infected with WSSV, hesperetin treatment would significantly increase the hemocyte apoptosis rates in crayfish. These results indicated that hesperetin could regulate the innate immunity of crayfish, and delaying and reducing the mortality after WSSV challenge. Therefore, the present study provided novel insights into the potential therapeutic or preventive functions associated with hesperetin to regulate crayfish immunity and protect crayfish against WSSV infection, provide certain theoretical basis for production practice.

1. Introduction

*Procambarus clarkii*, commonly known as crayfish, is a species of freshwater crayfish, native to northern Mexico, and southern and southeastern United States [1]. This species had been introduced into many areas of China and had become an important economic species of freshwater aquaculture [2]. With the rapid development of crayfish pond culture and rice shrimp comprehensive breeding industry, the white spot disease of crayfish has become increasingly prominent. White spot disease is one of the most disastrous in shrimp culture, having reduced shrimp production extensively worldwide. Mortality rates are usually very high and cumulative mortality can reach 100% within 3–10 days from the onset of visible gross signs [3,4]. The clinical symptoms of white spot syndrome include loss of appetite, lethargy, and the appearance of white spots on the exoskeleton [5]. White spot disease had been reported from several Asian countries since the early 1990s, and then from South America and from the south-eastern coast of the USA [6–9].

White spot syndrome virus (WSSV) is a rod-shaped crustacean virus with a wide geographic distribution and host range, which belongs to the genus *Whisposivirus* of the Nimaviridae family [10,11]. All the major species of cultivated penaeid shrimp can be naturally infected by this virus [6–9]. In addition to penaeid species, both natural and experimental infections have also been reported in caridean shrimp (*Macrobrachium Rosenbergii*), crayfish (*Orconectes punctimanus*), wild crabs (*Calappa lophos, Portunus sanguinolentus, P. pelagicus, Charybdis sp., Helice tridens and Scylla serrata*), wild lobsters (*Panulirus sp.*, palaeomonid pest shrimp, krill (*Acetes sp.*), planktonic copepods and pupae of an ephydridian insect [12–14]. WSSV replicates in the host cells after infecting the host. Virus could not be eliminated by conventional drugs without damaging the host cells, there is no specific treatment for the viral disease. During the breeding process, disease prevention and control measures are mostly based on prevention. Once the white spot disease breaks out, the crayfish’s mortality rate is extremely high, which leads to the farmers’ blood loss [5]. Therefore, there is an urgent need to develop a therapeutic drug that is less harmful to crayfish cells and could effectively reduce the mortality rate after infected WSSV.

By consulting a large amount of literature, we found that hesperetin has multiple effects, such as anti-inflammatory, anti-cancer, anti-virus and so on. Hesperetin is a type of natural flavonoid that occurs mainly
in the form of glycoside hesperidin, which is the main flavonoid in lemons and oranges. It is known that hesperitin has an anti-oxidation effect. It was selected as a biological antioxidant to protect osteoblasts and was used as an auxiliary preparation for melanin in the treatment of type I diabetic bone disease [15]. Additional, hesperitin has an anti-inflammatory effect and can reduce the production of sebum, thereby effectively inhibiting the growth of acne. Hesperitin can also be used to protect the cardiovascular, protect the nerves, and reduce triglyceride content in the liver [16–19]. Hesperitin has an anti-cancer effect, and citrus flavonoid hesperitin and naringin have obvious inhibitory effects on the proliferation of human breast cancer cells in vitro [20]. Most importantly, hesperitin has antiviral properties, and has significant antiviral activity against the Chikungunya virus (CHIKV), reducing CHIKV replication efficiency and down-regulating the production of viral proteins involved in replication [21]. Hesperitin can reduce intracellular replication of herpes simplex virus type 1 (HSV-1), poliovirus type 1, parainfluenza virus type 3 (PF-3), and respiratory syncytial virus (RSV) [22]. Hesperitin showed its inhibitory activity on Sindbis virus infection in vitro and were not cytotoxic on Baby Hamster cells 21 clone 15 (BHK-21) [23]. The intake of hesperitin in laying hens diet may exhibit positive effects and increased the ratio of yolk weight/egg weight and the blood serum superoxide dismutase (SOD) activity [24]. Based on this, we would explore whether hesperitin could effectively inhibit viral replication and reduce mortality in crayfish.

To elucidate whether hesperitin could be used as an effective antiviral additive for crayfish, we detecting the immunological indicators before and after treatment with hesperitin, as well as the immunological activity, VP28 copies of WSSV, mortality and apoptosis in crayfish after WSSV infection. In the present study, the results indicated that hesperitin could regulate the innate immunity, inhibit viral replication and reducing mortality of crayfish.

2. Materials and methods

2.1. Crayfish, hesperitin and pathogens preparation

Healthy crayfish (approximately 15 g and 10 cm each) were purchased from a crayfish breeding case in Wenzhou, China. In order to adapt the crayfish to the new environment, the crayfish were bred for a week before the experiment, they were kept in tanks with fresh water and fed with commercial pellet feed at 5% of body weight per day, the room temperature and water temperature were set at 25 °C constantly. The body weight of randomly selected individuals was recorded to calculate the average crayfish weight. Hemolymph and gill tissues from cultured crayfish were subjected at random to PCR detection with WSSV-specific primers to ensure that the crayfish were WSSV-free. Hesperitin (purity ≥ 98%) was purchased from Shanghai Yuanye Biotechnology Co. Ltd. (Shanghai, China). The commercial pellet feed is specifically used for crayfish. The preparation method of hesperitin additive feed is as follows: firstly, commercial pellet feed is crushed by disintegrator; secondly, crushed pellet feed is mixed with hesperitin in a certain proportion, added super-pure water to knead into ‘dough’, which is formed in dough press; finally, that is dried in an oven (40 °C). Oral use, hesperitin was added to commercial pellet feed for crayfish at a dose of 40, 50, and 60 mg/kg, stored at room temperature and kept dry.

The virus isolate, WSSV, used in this study originated from infected crayfish and was reserved at −80 °C as described previously [25]. 10 crayfish were randomly selected for WSSV challenge from the tanks, which were infected with WSSV. Prepared WSSV extract was diluted with sterilized PBS to a density of 1 × 10^8 WSSV copies per mL, each crayfish would receive 100 μL of diluted injection. Approximately four or five days later, crayfish infected with WSSV started to display various clinical signs including lethargy, reduced food consumption, reduced preening activities, loosening of the cuticle, and discoloration of the hepatopancreas. Before the crayfish died, partial appendages of each crayfish were taken for virus testing, and then the infected crayfish were stored in an ultra-low temperature freezer. In the virus challenge experiment mentioned in this article, we took out the infected crayfish from the ultra-low temperature freezer, thawed it, took its muscles, and made the meat batter. In manner of feeding attack, each crayfish was fed with minced meat, which was about the size of rice grains.

2.2. Hesperitin treatment and pathogen challenge

Crayfish were randomly transferred into separate tanks as different groups. In the hesperitin treatment experiment, crayfish were either fed with hesperitin additive feed every 24 h as the hesperitin group or commercial pellet feed as the control group. To determine the influence of hesperitin on crayfish innate immune signal pathways, the hemolymph of each group was collected 24 h post feeding to analyze gene expression. To determine the influence of hesperitin in the pathogen challenge process, after 72 h of hesperitin treatment, crayfish in each group were fed with minced meat of WSSV-infected crayfish. After the feeding, crayfish fed commercial pellet feed only, 40 mg/kg hesperitin additive feed only, 50 mg/kg hesperitin additive feed only, 60 mg/kg hesperitin additive feed only, commercial pellet feed for crayfish + minced meat, 40 mg/kg hesperitin additive feed + minced meat, 50 mg/kg hesperitin additive feed + minced meat, 60 mg/kg hesperitin additive feed + minced meat were placed back into separated tanks for further research. Crayfish fed with commercial pellet feed alone were defined as the control group and those fed with 40, 50, or 60 mg/kg hesperitin additive feed alone were defined as the 40 mg/kg hesperitin, 50 mg/kg hesperitin, or 60 mg/kg hesperitin groups, respectively. Crayfish fed with commercial pellet feed and minced meat were defined as the WSSV group. Crayfish fed with either 40, 50, or 60 mg/kg hesperitin additive feed and minced meat were defined as the WSSV + 40 mg/kg hesperitin, WSSV + 50 mg/kg hesperitin, or the WSSV + 60 mg/kg hesperitin groups, respectively. Based on the requirements of different analysis, crayfish samples were collected at different times after hesperitin or pathogen treatment.

To analyze mortality, eight groups of crayfish were kept in the tanks for 12 days and the populations were counted every 24 h. Dead crayfish were removed immediately, and aquatic water was replaced with clean water every day. To ensure the statistical accuracy in mortality analysis, every group contained 20 individuals. The mortality data was arranged and analyzed in Microsoft GraphPad 5.0. In the analysis of WSSV copies, three crayfish (as a technical repeat) from the control or WSSV challenge groups were collected at 24, 48, 72, and 96 h post-challenge with WSSV. In immune parameter analyze, at least three crayfish (as technical repeats) from each group were collected at 24 and 48 h after the challenge with pathogens. Experiments described above were all repeated three times.

2.3. WSSV replication analysis

The detection of WSSV copies was based on previous studies [26,27]. Briefly, DNA was extracted from the crayfish hemocytes of each sample, and TaqMan real-time quantitative PCR was performed. WSSV protein VP28-specific primers (5′-TTGTTTTCAGCCGGAGATT-3′ and 5′-CCTTGGTCAGCCCTCTGA-3′) and TaqMan fluorogenic probe (5′-FAM-TGCTGCGGTCTCCAA-TAMRA-3′) were applied. Thermal cycling was performed on an iCycle IQ5 real-time PCR detection system (Bio-RAD, USA).

2.4. Expression analysis by real-time quantitative PCR

Crayfish hemocytes were collected from each group 24 h after feeding. Total RNA was extracted from the hemocytes using an RNApure Tissue & Cell kit (CWBio, China) according to the manufacturer’s protocol. In total, 200 ng RNA was applied to cDNA reverse transcription using a ReverTra Ace qPCR RT Master Mix with a gDNA
DOPA), as described previously [30]. SOD activity was quanti-
dopa chrome from the substrate L-3, 4-dihydroxyphenylalanine (L-
doped in the hemolymph mixture based on the formation of
challenged crayfish

Crayfish mortality and viral copy number were assessed to de-
Viral challenge experiments, crayfish death was observed at 24 h in all
ew groups. From day 4 to day 10, crayfish mortality in the WSSV group
increased rapidly to 94.667% (p = 0.0032), and at day 12 after infec-
tion, crayfish mortality in the WSSV group reached 100%. When suc-
cessively treated with 40 or 60 mg/kg hesperetin and WSSV, crayfish
mortality was higher than that of 50 mg/kg hesperetin and WSSV-in-
fected crayfish. Especially after 10 days, the mortality of 50 mg/kg
hesperetin and WSSV group showed a significant difference from that
observed in the control group (p = 0.0032), and the mortality reached
only 55% (Fig. 1). A follow-up study with a 50 mg/kg hesperetin dose
group was conducted based on the results of the crayfish mortality.

3.2. Effects of hesperetin on the WSSV copy number

The sampling time of the WSSV copy number experiment was de-
termined by the crayfish mortality rate, which began on the 5th day
after the pathogen challenge. Therefore, the sampling time was set at 0,
24, 48, 72 and 96 h. The WSSV copy number after WSSV treatment of
each group reached 1 × 10^7 in 96 h. The WSSV copy number of the
WSSV group was higher than that of WSSV + 50 mg/kg hesperetin
group at 24, 48 and 72 h. We suggest that hesperetin might have a
certain inhibitory effect on viral replication, but the inhibition of
crayfish mortality may not be achieved by the inhibition of the WSSV
(Fig. 2).

3.3. Effects of hesperetin on the expression of immune pathway genes

Real-time reverse transcription polymerase chain reaction (RT-PCR)
analyis of four immune-related genes showed that the expression of
Toll-like receptor, crustin 1, NF-kappa B and C-type-lectin were down-
regulated/up-regulated following treatment with 50 mg/kg hesperetin
when compared with the control group (p < 0.05) (Fig. 3). These re-
results suggested that hesperetin might induce certain immune pathways.

Table 1 Real-time quantitative PCR primer sequences of immune signal pathways
related genes in the hemocytes of Procambarus clarkii.

| Primer Name  | Primer Sequence (5’ to 3’) |
|-------------|--------------------------|
| β-Actin-F   | ACCAAGGCCTGCTACCTTC      |
| β-Actin-R   | CGGAACCTCTGGTGCGAATGG    |
| Toll-like receptor-F | TGGCTAGTGGTTGCGAAGC |
| Toll-like receptor-R | CTACGGTAAAGGAGCCGGATGG |
| NF-appa B-F | TATGCTGATGATGATGGCTT    |
| NF-kappa B-R | GCTGATTGAGGCGAAGAAA    |
| crustin 1-F | CCACAGATGGCAATGCGACTC   |
| crustin 1-R | AGGGAGACCAAGCGTAGGAAAGT |
| C-type-lectin-F | ACTCTGCTAAGGCAATGCCAC |
| C-type-lectin-R | CTACGGTCTACGAGCAAAC    |

Remover kit (Toyoobo, Japan). The cDNA of each group was applied to
SYBR Green real-time quantitative PCR immediately. A two-step RT-
qPCR method was performed using a Bio-Rad Two-Color Real-Time
PCR Detection System. The gene expression level was calculated with the 2^(-ΔΔCT)
method [28], the amplification cycle of β-actin was used as an internal control to calculate the relative expression level. Expression
levels of genes of the control group were used as index 1.

Four innate immune pathway-related genes, Toll-like receptor
(KP259728.1, NF-kappa B (KF662471.1), crustin 1 (GQ301201.1), and
C-type-lectin (KC857544.1), were selected to predict the potential in-
fluence of hesperetin treatment on the innate immune system. The
design and synthesis of the RT-qPCR primers were entrusted to the Genenar Shanghai Company. The primer sequences are listed in
Table 1.

2.5. Total hemocyte count, phenoloxidase activity, and superoxide
dismutase activity

Three immune parameters, total hemocyte count (THC), phenolox-
idase (PO) activity, and superoxide dismutase (SOD) activity were used to
analyze the condition and immunity of the experimental crayfish. At 24
and 48 h post-pathogen challenge, crayfish hemolymph was collected
each group at 24, 48, 72 and 96 h. The WSSV copy number after WSSV treatment of
samples were centrifuged at 800 × g for 5 min at 4 °C to collect hemocytes. After
washing with sterilized PBS, counted and adjusted to a density of
3–5 × 10^5 cells/ml. hemocytes were resuspended in ice-cooled
1 × binding buffer with Annexin-V FITC and propidium iodide (PI),
and incubated at room temperature for 15 min to stain. After staining, the
samples were centrifuged to remove residual dye and applied to flow
cytometry immediately to avoid cell death. The empty control, negative
control, and positive control for threshold values were prepared si-
multaneously with experimental samples. The data were presented as
means ± standard deviation (SD) derived from three independent ex-
periments.

2.7. Statistical analysis

Quantitative data were expressed as mean ± standard deviation
(SD). Data from three independent experiments were analyzed by one-
way analysis of variance to calculate the means and standard deviations of
the triplicate assays. Statistical differences were estimated using one-
way ANOVA followed by least-significant differences (LSD) and Duncan’s multiple range test. All statistics were measured using SPSS
software version 19 (IBM, USA). And the level of 0.01 was used to in-
dicate statistical significance (P < 0.01). All graphs were made using
Microsoft GraphPad 5.0.

3. Results

3.1. Effects of hesperetin on the cumulative mortality of pathogen-
challenged crayfish

After feeding commercial pellet feed and hesperetin additive feed for 24 h. Secondly, the apoptosis of the
hesperetin group was measured after feeding the commercial pellet feed
and hesperetin additive feed for 24 h. At 24 h post
pathogen challenge, the hemolymph was drawn using 2 ml syringe
with 20 mM of EDTA at a ratio of 1:1 and kept on ice. Samples were

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termined by the crayfish mortality rate, which began on the 5th day
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24, 48, 72 and 96 h. The WSSV copy number after WSSV treatment of
each group reached 1 × 10^7 in 96 h. The WSSV copy number of the
WSSV group was higher than that of WSSV + 50 mg/kg hesperetin
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regulated/up-regulated following treatment with 50 mg/kg hesperetin
when compared with the control group (p < 0.05) (Fig. 3). These re-
results suggested that hesperetin might induce certain immune pathways.

Microsoft GraphPad 5.0.
3.4. Effects of hesperetin on immunological parameters

THC, PO activity, and SOD activity were measured at 24 and 48 h in the WSSV group, WSSV + 50 mg/kg hesperetin group, control group, and 50 mg/kg hesperetin group. There was no significant difference in the THC of crayfish between the 50 mg/kg hesperetin group and the control group at 24 and 48 h (p = 0.162 at 24 h; p = 0.303 at 48 h) (Fig. 4a), and after the WSSV infection. The THC of the WSSV group was significantly higher than the control group at 48 h. When hesperetin was applied, crayfish THC increased to 1.41 × 10^6/mL at 24 h and 1.27 × 10^6/mL at 48 h, which were both higher than levels observed in the WSSV-infected groups (p = 0.0014 at 24 h; p = 0.0029 at 48 h) (Fig. 4b).

PO activity of 50 mg/kg hesperetin showed no difference when compared with that observed in the control group at 24 (0.8176 U) and 48 h (1.0598 U) (p = 0.3397 at 24 h; p = 0.1453 at 48 h) (Fig. 4c). In WSSV challenge experiments, PO activity decreased (0.5091 U) nearly 2-fold at 24 h (p < 0.0001), while PO activity increased at 48 h, but was still significantly lower than in the control group (p = 0.0055). When hesperetin was applied in WSSV challenge experiments, PO activity was significantly higher than that in the WSSV group (p = 0.0055 at 24 h; p = 0.0129 at 48 h) (Fig. 4d).

There was no significant difference in SOD activity between the 50 mg/kg hesperetin group at 24 h (1.1084 U) and the control group (1.0924 U). In addition, there was no significant difference in SOD activity at 48 h between the 50 mg/kg hesperetin group (1.2821 U) and the control group (1.2707 U) (p = 0.3911 at 24 h; p = 0.5304 at 48 h) (Fig. 4e). In the WSSV challenge experiment, there was no difference between the control group at 24 h (p = 0.3253), and the SOD activity decreased slightly from 1.0834 U to 1.0429 U at 48 h. When hesperetin was applied in the challenged groups, the SOD activity increased slightly in the WSSV challenge experiment, increasing to 1.1493 U at 24 h (p = 0.0386) and increasing to 1.0972 U at 48 h (p = 0.0244) (Fig. 4f).

3.5. Effects of hesperetin on the apoptosis of hemocytes

The hemocyte apoptosis rates in the presence or absence of 50 mg/
Fig. 4. The immune-related parameters measured in the challenge experiment. Crayfish were treated with formula feed (control), 50 mg/kg hesperetin, WSSV, or WSSV and 50 mg/kg hesperetin to compare the differences of three immune-related parameters, total hemocyte count (THC), phenoloxidase (PO) activity, and superoxide dismutase (SOD) activity before and after WSSV challenges, and the influence of hesperetin on crayfish immunity. For the co-treatment of WSSV and hesperetin, crayfish were challenged with WSSV 72 h after 50 mg/kg hesperetin treatment, that is, minced meat carrying WSSV was used instead of 50 mg/kg hesperetin. The variation of 50 mg/kg hesperetin on THC (a), proPO activity (c), and SOD activity (e) showed the influence of hesperetin treatment on healthy crayfish. (b), (d), and (f) show the influence of hesperetin on the WSSV infection process. Each treatment at each time point contained at least three crayfish individuals, data are represented by mean values and standard deviation. Significant differences are represented with asterisks (* = P < 0.05, ** = P < 0.01), and analyzed by a multiple t-test.
Fig. 5. Apoptosis analysis of hemocytes in hesperetin treated crayfish by flow cytometry. Crayfish were treated with formula feed or 50 mg/kg hesperetin, followed by a pathogen challenge of WSSV. At 24 h post pathogen challenge, the hemocyte samples of different treatments were collected and stained with Annexin-V FITC and propidium iodide (PI) for flow cytometry detection. Fluorescent 1-Annexin V (FL1-A5) represents apoptosis cells. Fluorescent 3-PI (FL3-PI) represents dead or damaged cells. The determination of the threshold was based on the empty control, negative control, and positive control. The percentage of Annexin V positive cells represents the apoptosis rate of the hemocyte sample. The apoptosis rate of control and hesperetin control (a), each column represents the mean value of three isolated repeats. (b) Apoptosis rate of challenged groups. Scatter plots of one of the challenged groups (c), Q1 represents cell fragments caused by centrifuge and re-suspension, Q2 represents the late stage apoptosis, Q3 represents early stage apoptosis, Q4 represents normal cells. The apoptosis rate of the sample was determined by the total fluorescent intensity of Q2 and Q3.
kg hesperetin treatment were detected using flow cytometry. The apoptosis rate in the control group was approximately 7.645%, and the apoptotic rate of the 50 mg/kg hesperetin group was 10.23% (Fig. 5a), which was significantly higher than that of the control group (p = 0.0377), indicating that the hesperetin was cytotoxic to the blood cells of the crayfish. In these experiments, the treated crayfish blood cells were very fragile. Due to centrifugation, liquid impact associated with pipetting, and subsequent resuspension. We attempted to do our best to reduce the damage from external sources. In the WSSV-infected groups, the apoptosis rate increased to an average of 35.45%, and upon hesperetin treatment, this rate increased to 59.35% and showed significant variation from the WSSV group (p < 0.0001) (Fig. 5b).

4. Discussion

To protect the host from virus invasion, the host gradually evolves a series of antiviral mechanisms in response to virus attack. For crustaceans, the innate immunity is an important way to protect them from viral infection. In the present study, the results directly indicated that hesperetin can regulate the innate immune system of crayfish. Although hesperetin had been shown to influence the process of multiple viral infection, such as inhibited cleavage activity of the 3C-like protease, which is the SARS-coronavirus 3C-like protease that mediates the proteolytic processing of replicase polyproteins 1a and 1b into functional proteins [32], inhibited enterovirus 17 infection [33], inhibited influenza A virus replication and so on [34]. This article firstly explored the possibility of hesperetin to resist WSSV infection, which is the main pathogen for most crustaceans. Based on the results of mortality, the mortality rate of hesperetin treatment groups was significantly lower than that of un-treated group, and the best dosage of hesperetin was 50 mg/kg. This result indicated that hesperetin could effectively reduce the mortality of crayfish after WSSV infection. The results of WSSV copy number showed that hesperetin could effectively inhibit viral replication, maybe by affecting the innate immunity of crayfish. Moreover, after feeding 50 mg/kg hesperetin additive for 24 h, we detected the expression levels of four important immune genes in crayfish, and found that the expression levels of Toll-like receptor and crustin 1 were significantly down-regulated, but the expression levels of NF-kappaB and C-type lectin were significantly up-regulated. Proteins containing a C-type lectin domain have multiple functions including cell-cell adhesion, immune response to pathogens, and apoptosis [35,36]. C-type lectin plays a central role in host defense, and many members of this family are involved in viral infection, primarily by identifying virions and affecting their entry into host cells. C-type lectin, LvCTL1, in Litopenaeus vannamei has been shown to protect shrimps from WSSV in vitro and in vivo, and protection may be related to the interaction of LvCTL1 with several viral proteins [37]. The knockdown of crustin-like through RNAi inhibits WSSV replication in shrimp, and the mortality, WSSV copy number and expressions of WSSV immediate early genes (IE1, IE2, DNA polymerase, VP28) were both decreased [38]. To discover the effect of hesperetin on the innate immunity of crayfish, the immunological activity of crayfish fed hesperetin before and after WSSV challenge was tested.

THC is the total number of hemocytes in crayfish. Hemocyte is a very important immune tissue in the innate immunity of crustaceans. It is involved in cellular immunity and humoral immunity, and can directly recognize and engulf foreign pathogens. The results showed that hesperetin treatment significantly increased the total hemolymph counts of crayfish after the WSSV challenge. After treatment with hesperetin, PO activity was significantly up-regulated at 48 h, and pathogen challenge experiments showed that PO activity in crayfish treated with hesperetin for 48 h was also increased, indicating that hesperetin treatment promoted the expression of the proPO non-self-identification system. PO is the terminal enzyme of the proPO pathway, which converts tyrosine to dihydroxyphenylalanine (DOPA) and then to dopaquinone and melanin synthesis [39]. PO activity is very important for crustacean innate immune defense against invading pathogens [40]. In the WSSV challenge experiment, the SOD activity of the 50 mg/kg hesperetin group was higher than the WSSV group, indicated that hesperetin treatment significantly increased the SOD activity, which is very important for the stability and health of crayfish. Reactive oxygen species (ROS) released during the innate immune response can destroy bacteria or viruses; however, large amounts of ROS can also attack host proteins, lipids, and DNA nonspecifically, leading to cell damage [41]. To avoid ROS self-injury, SOD is activated as part of the antioxidant defense to remove superoxide anions and maintain homeostasis during the immune response [42]. The above results indicated that hesperetin seemed to reduce crayfish mortality mainly by enhancing innate immunity, but did not directly act on WSSV, although it was reported to inhibit viral replication.

It is well known that innate immunity plays an essential role in immune responses against viral infection in which apoptosis and phagocytosis are two important components [43]. During viral infection, a series of signaling pathways can be triggered in the host, leading to apoptosis of infected cells [44,45]. Apoptosis is a process of cell death caused by pre-existing death procedures triggered by internal and external factors, and it is also called programmed cell death [46]. NF-kappaB is closely related to apoptosis, which is involved in the transcriptional regulation of a variety of apoptosis-related genes and has a two-way effect of inhibiting and promoting apoptosis [47]. Whether or not infected with WSSV, hesperetin increase a little on the total hemocyte counts, and significantly increase the hemocyte apoptosis rates in crayfish. Due to the complexity of crayfish immune system, hesperetin may promote hemocyte apoptosis but have no cytotoxic to crayfish hemocytes. The apoptosis rate of the WSSV + 50 mg/kg hesperetin group was significantly higher than that of the WSSV group, which may be related to the up-regulation of the NF-kappaB gene, and the WSSV copy number during this period is directly and effectively suppressed. These results showed that hesperetin treatment significantly increased apoptosis. This finding revealed that hesperetin may be an enhancer of apoptosis, although it could restrain mortality. In the study of hesperetin against CHIKV virus, hesperetin can effectively inhibit the replication of CHIKV in host cells through eliciting the inhibitory effect against the activity of Bluc marker expressed by CHIKV [21]. In this study, according to the virus copy number, hesperetin can effectively inhibit the replication of WSSV in crayfish cells. In this study, hesperetin treatment has an impact on some immune parameters, and inhibiting WSSV replication maybe by regulating apoptosis.

In conclusion, our results showed that hesperetin treatment promoted the innate immunity of crayfish, especially the cellular immunity following viral infection, and the activity of several innate immune pathways. Additionally, hesperetin treatment inhibited pathogen replication and reduced mortality.

Acknowledgments

This work was financially supported by National Key Research and Development Program of China (2018YFD0500300) and Qianjiang talent program (QJD1602023).

References

[1] D.J. Harris, K.A. Crandall, Intragenomic variation within ITS1 and ITS2 of fresh- water crayfishes (Decapoda: Cambaridae): implications for phylogenetic and microsatellite studies, Mol. Biol. Evol. 17 (2000) 284–291.
[2] D.M. Holdich, A review of astaciculture: freshwater crayfish farming, Aquat. Living Resour. 6 (1993) 307–317.
[3] H.Y. Chou, C.Y. Huang, C.H. Wang, H.C. Chiang, C.F. Lo, Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan, Dis. Aquat. Org. 23 (1995) 165–173.
[4] C. Wongteerarattaya, J.E. Vickers, S. Sirirairatanan, G.L. Nash, P. Kanchanaphum, V. Boonsang, et al., A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn Penaeus monodon, Dis. Aquat. Org. 21 (1995) 69–77.

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