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Epigallocatechin-3-gallate protects Kuruma shrimp *Marsupeneaus japonicus* from white spot syndrome virus and *Vibrio alginolyticus*

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ABSTRACT

Epigallocatechin-3-gallate (EGCG) is the most abundant catechin in green tea and exhibits potential antibacterial and anticancer activities. In this study, EGCG was used in pathogen-challenge experiments in shrimp to discover its effect on the innate immune system of an invertebrate. Kuruma shrimp *Marsupeneaus japonicus* was used as an experimental model and challenged with white spot syndrome virus (WSSV) and the Gram-negative bacterium *Vibrio alginolyticus*. Pathogen-challenge experiments showed that EGCG pretreatment significantly delayed and reduced mortality upon WSSV and *V. alginolyticus* infection, with VP-28 copies of WSSV also reduced. Quantitative reverse transcription polymerase chain reaction revealed the positive influence of EGCG on several innate immune-related genes, including *IMD*, *pro*PO, *QM*, *myosin*, *Rho*, *Rab7*, *p53*, *TNF-alpha*, *MAPK*, and *NOS*, and we observed positive influences on three immune parameters, including total hemocyte count and phenoloxidase and superoxide dismutase activities, by EGCG treatment. Additionally, results showed that EGCG treatment significantly reduced apoptosis upon *V. alginolyticus* challenge. These results indicated the positive role of EGCG in the shrimp innate immune system as an enhancer of immune parameters and an inhibitor of apoptosis, thereby delaying and reducing mortality upon pathogen challenge. Our findings provide insight into potential therapeutic or preventive functions associated with EGCG to enhance shrimp immunity and protect shrimp from pathogen infection.

1. Introduction

The Kuruma shrimp *Marsupeneaus japonicus* is one of the major marine products in China and other Asia-Pacific countries and offers high commercial benefits. When the marine fishing industry is unable to match market demand, artificial farming becomes the main source of Kuruma shrimp. The rise of the shrimp artificial-culture industry also introduces problems, with unhealthy and highly intensive farming methods providing an ideal proliferative environment for pathogens, including white spot syndrome virus (WSSV), which has a high and rapid mortality rate, and the bacterium *Vibrio alginolyticus*, an alkalinephilia pathogenic bacteria that can cause enteritis and inflammation in both humans and shrimp.

Since first discovered in Taiwan [1], WSSV has spread throughout Pacific rim countries, causing massive commercial losses in the shrimp farming industry and considered the main lethal pathogen of shrimp, with mortality reaching 100% in 3–7 days [2]. Diseased shrimp have obvious white spots on their carapace, indicative of the syndrome name [1]. WSSV is a double-strand DNA virus that belongs to the genus *Whispovirus* in the family *Nimaviridae*. In addition to shrimp, other arthropods, such as crabs, crayfish, insects, and some *Rotifera* spp., can also be infected. *V. alginolyticus* is a lethal pathogenic bacterium in the marine environment that threatens both wild marine creatures and artificially cultured species [3]. *Vibrio* spp. can also affect humans and are responsible for potent neurotoxins in marine products that cause wound infections and acute gastroenteritis. To protect shrimp from these diseases, research has focused on the structure and mechanism of the pathogen, as well as pathogen interaction with host protein/genetic material and/or the innate immune system of the host.

For decades, researchers have explored the mechanisms associated with invertebrate immunity. Unlike vertebrates, invertebrates have no adaptive immunity, with the humoral and cellular innate immune responses being the two major barriers against pathogens [4,5] and the major site of the innate immune responses located in the hemolymph [6]. In our previous study, we found that a catechin from green tea, epigallocatechin-3-gallate (EGCG), significantly inhibits WSSV replication in the mud crab (*Scylla paramamosain*) hemolymph and reduces crab mortality caused by WSSV infection [7]. Green tea is a popular beverage in China, with its positive effects gaining the attention of both customers and scientists. The anti-inflammation and antioxidant

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activities from green tea originate from polyphenols, also known as catechins, which are extracts of *Camellia sinensis*. EGCG comprises ~55%–70% of total polyphenols [8,9], and studies report that EGCG possesses antiproliferative, antimutagenic, antioxidant, antibacterial, and antiviral activities as well as chemopreventative activity in cancer cells [10]. EGCG also limits lipid absorption and lowers plasma lipid levels in rats, aids their weight loss [11], and is suggested as a potential anticancer factor in several studies [10,12].

In this study, we used EGCG as an antiviral and antibacterial treatment in Kuruma shrimp to discover its potential influence on the shrimp innate-immune system. The results of our study confirmed that EGCG improves shrimp immune-enzyme activity, inhibits WSSV replication, and reduces shrimp mortality.

2. Materials and methods

2.1. Shrimps, EGCG and pathogens preparation

Healthy juvenile shrimps (approximately 12–15 g each) were purchased from local seafood market in Hangzhou (China). Before experimental treatment, shrimps were kept in 35 L artificial sea water equipped with air pump, the room temperature and water temperature were set at 24 °C and 22 °C constantly. The body weight of randomly selected individuals was recorded to calculate an average shrimp weight. EGCG compounds were purchased from Sigma-Aldrich Shanghai Trading (China). For injection use, EGCG powder was dissolved in 18.2Ω pure water as a 1.0 mg/mL mother solution, stored temporarily at 4 °C. Based on the average shrimp body weight and the suitable treatment doze we obtained from a previous research in crab [7], the EGCG 1.0 mg/mL mother solution was diluted to 1.0 mg/kg (EGCG/shrimp weight) with sterilized high saline PBS (pH 7.4) as the injection solution.

The pathogens used in the research included a virus, WSSV, and a halophilic bacterium, *Vibiro alginolyticus*. WSSV was separated from sick shrimps and reserved at −80 °C as described before [13]. In challenge experiment, prepared WSSV extract was diluted with sterilized high saline PBS to a density of 1×10^5 virus copies per ml, each shrimp individual would receive 100 μL of diluted injection.

Vibrio alginolyticus (ATCC17749) was provided generously by Jimei University, Xiamen, China. Preserved *V. alginolyticus* was cultured at 32 °C overnight to recover pathogenicity, and the active vibrio was diffused and gradually diluted to 1.0×10^5 per ml. Each shrimp individual would receive 100 μL of diluted vibrio injection.

2.2. EGCG treatment and pathogen challenge

To treat shrimp with EGCG or pathogen solution, shrimps were randomly transferred into separate tanks as different groups, the solutions were injected into shrimp body from the soft tissue between second and third segment of abdomen. In EGCG treatment experiment, shrimps were treated with 1.0 mg/kg EGCG solution as EGCG group or sterilized high saline PBS as control group. To determine the influence of EGCG on shrimp innate immune signal pathways, hemolymph of each group was collected 2 h post injection for gene expression analyze. To determine the influence of EGCG in pathogen challenge process, after approximately 10min of EGCG treatment, shrimps of each group would receive a second injection of WSSV solution, *V. alginolyticus* solution, or high saline PBS, separately. After the injection of PBS only, EGCG only, PBS + WSSV, EGCG + WSSV, PBS + *V. alginolyticus*, EGCG + *V. alginolyticus*, shrimps were back into separated tanks for further research. Shrimps treated with PBS alone were defined as control. Shrimp treated with EGCG alone were defined as 1.0 mg/kg EGCG group. Shrimps treated with PBS and pathogens were defined as WSSV group or *V. alginolyticus* group. Shrimps treated with EGCG and pathogens were defined as WSSV + 1.0 mg/kg EGCG group or *V. alginolyticus* + 1.0 mg/kg EGCG group. Based on the requirement of different analysis, shrimp samples were collected at different time after EGCG or pathogen treatment.

In mortality analyze, 6 groups of shrimps were kept in the tanks for 120 h, the current populations were counted every 12 h. Dead shrimps were removed immediately, 1/3 of the sea water was replaced with clean sea water every day. To ensure the statistical accuracy in mortality analyze, every group contained at least 20 individuals. In WSSV copies analyze, at least three shrimps (as technical repeat) from control or WSSV challenge groups were collected at 5–10 min (immediate after injection), 2 h, 6 h, 12 h, 24 h, 48 h post WSSV challenge, separately. In immune parameter analyze, at least three shrimps (as technical repeat) from each group were collected at 24 h, 48 h after pathogen challenge, separately. Experiments described above were all biologically repeated for three times.

2.3. WSSV replication analysis

WSSV copies were detected based on previous study [14,15]. Briefly, DNA of shrimp hemolymph of each samples were extracted, and the TaqMan real-time quantitative PCR was performed. WSSV protein VP28-specific primers (5′- TTGGTTTCAG CCCGAGATT-3′ and 5′-CCTT GGTCAGCCCCTTGA-3′) and TaqMan fluorogenic probe (5′-FAM-TGCT GCCGTCTCCAA-TAMRA-3′) were applied.

2.4. Expression analysis by real-time quantitative PCR

Shrimp hemolymph of control or 1.0 mg/kg EGCG group was collected 2 h after injection. Total RNA was extracted from hemolymph using Easy spin tissue/cell RNA extra kit (Aidlab, China) according to manufactures protocol. 200 ng RNA was applied to cDNA reverse transcription using ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (Toyobo, Japan). The cDNA of each group was applied to SYBR Green real-time quantitative PCR immediately. RT-qPCR of two step method was performed using a Bio-Rad Two Color Real-Time PCR Detection System. Gene expression level was calculated with $2^{-\Delta\Delta}$CT comparative CT method, the amplification cycle of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as internal control to calculate relative expression level. Expression levels of genes of the control group were used as index 1.

Twelve innate immune pathway related genes, *MAPK* (mitogen-activated protein kinase), *myosin* (myosin light chain), *p53*, *IMD* (the immune deficiency pathway), *Rho* (Rho GTPase), *NOS* (nitric oxide synthase), *pro*PO (prophenoloxidase), *Rab7*, *QM* protein, *STAT* (Signal transducers and activators of transcription), *Toll-like receptor* and *TNF-α* (tumor necrosis factor), were selected to predict the potential influence of EGCG treatment on innate immune system. The design and synthesis of the RT-qPCR primers were entrusted to Generay Shanghai Company. Primer sequences were listed in Table 1.

2.5. Total hemocyte count (THC), phenoloxidase (PO) activity and superoxide dismutase (SOD) activity analysis

Three immune parameters, total hemocyte count (THC), phenoloxidase (PO) activity, and superoxide dismutase (SOD) activity, were detected to analyze the condition and immunity of experiment shrimps. At 24 h and 48 h post pathogen challenge, shrimp hemolymph of each group was collected 1:1 with anticoagulant (450 mM NaCl, 10 mM KCl, 10 mM EDTA-Na_2, 10 mM HEPES, pH 7.3) [16,17], the mixtures of hemolymph and anticoagulant were kept on ice constantly. THC detection method was described in a previous research [7]. PO extract method was described in a previous research [7]. SOD extraction and SOD activity detection method were described in a previous research [7].

Table 1
Real-time quantitative PCR primer sequences of immune signal pathways related genes in the hemocytes of *Marsupeneaus japonicus*.

Primer Name	Primer Seqeunce (5′ to 3′)
GAPDH-F	GGTGCCGAGTACATCGTTGAGTC
GAPDH-R	GGCAGTTGGTAGTGCAAGAGGC
MAPK-F	CGCATCACTGTTGAGGAGG
MAPK-R	GCAGGTCATCAAGTTCCATCT
myosin -F	GCCCAGGTCAAGAAGGACAAGGA
myosin -R	AAGACGCTCACCAAGGGACAGGA
p53-F	TTCCTGCCTGGCTGACTCTA
p53-R	CACCCAATCTTCCAACATCACAT
IMD-F	ATTCATCCGTCTACCTCCCTACA
IMD-R	GAGCTGAGTCTGTCTTAATGTTATCC
Rho-F	GTGATGGTGCCTGTGGTAAA
Rho-R	GCCTCAATCTGTCATAGTCCTC
NOS-F	CCAGGATCTTCTTGTTGGTGTTG
NOS-R	CCCTCATCTGTAGCATAAAGTTCTC
proPO-F	TTCTACCGCTGGCATAAGTTTGT
proPO-R	TATCTGCCTCGTCGTTCCTCAC
Rab7-F	TCATTAGGTGTTGCATTTTATCGC
Rab7-R	AGGCTTGAATTAGGAACTCGTC
QM-F	CGTCACAAGGAGCAGGTTATT
QM-R	GGGACCATGTTCAGGGAGA
STAT-F	TGGCAGGATGGATAGAAGACAAG
STAT-R	TGAATAAGCTGGGATACGAGGGA
Toll-like receptor-F	CCACCTAAAGTCATCATCGCCAGTA
Toll-like receptor-R	TCTTCATTCACCACAGCCCACAA
TNF-alpha-F	ACAGACGGTCCAGAGTCCCAAAG
TNF-alpha-R	GCGACGAAGTGAGCCACAGTAA

2.6. Apoptosis analysis

The apoptosis assay was conducted using Annexin V (Invitrogen, USA) according to a method optimized based on the manufacturer's protocol. Briefly, shrimps were treated with PBS or1.0 mg/kg EGCG, followed by a second treatment of PBS, WSSV or *V. alginolyticus* as pathogen challenge. 24 h post pathogen challenge, the hemolymph samples of different treatment samples were draw and kept on ice. Centrifuge at 800 g for 5 min at 4 °C to collect hemocytes. After washed by sterilized high saline PBS, hemocytes were re-suspended with ice-cooled prepared 1 × Binding buffer with Annexin-V FITC and PI (propidium iodide). Incubate at room temperature for 15 min to stain. After staining, the samples were centrifuged to remove residual dye and applied for flow cytometry analyze immediately to avoid cell death. The empty control, negative control, and positive control for threshold value define were prepared simultaneously with experimental samples.

2.7. Statistical analysis

Every experiment was performed three times as biological repeats to exclude variation from weather, individual difference, and anthropogenic operation. The data from three independent experiments were analyzed by one-way analysis of variance (ANOVA) to calculate the mean and standard deviation of the triplicate assays. The differences between the different treatments were analyzed by Multiple *t*-test method. Statistical significance was defined as alpha = 0.05, represented with asterisk.

3. Results

3.1. Effects of EGCG on the cumulative mortality of pathogen-challenged shrimps

Shrimp mortality and viral copy number were detected to demonstrate the immunity of different groups. Healthy shrimp under experimental conditions showed a ~20% mortality rate, with their death possibly caused by multiple sources, such as fishing stress, injection stress, or condition change. Other human activities, including sudden approaches and water changes, were minimized to decrease the mortality of the control group. EGCG-only treatment (1.0 mg/kg) did not affect shrimp mortality ($p = 0.437$; data not shown). In WSSV-challenge experiments, shrimp death was observed at 12 h in all groups. From 24 h to 60 h, shrimp mortality in the WSSV group increased rapidly to nearly 90% ($p = 0.0003$), and at 72 h post-infection, shrimp mortality in the WSSV group reached 100%. When successively treated with EGCG and WSSV, shrimp mortality was significantly lower than that of WSSV-infected shrimp, especially before 48-h post-infection, where mortality showed no difference from that observed in the control group ($p = 0.596$). However, after 72 h, shrimp mortality increased rapidly, similar to that observed in the WSSV group, ultimately reaching nearly 100% (Fig. 1a). WSSV copy number shortly (2 h) after WSSV treatment of each group increased rapidly, reaching 1×10^7 in only 12 h (Fig. 1c). EGCG treatment restricted WSSV copy number, with the number in the WSSV + EGCG group was approximately 10-fold lower than that in the WSSV group ($p < 0.001$). At 48 h post-WSSV injection, shrimp mortality in the WSSV group was approximately 4-fold higher than that in the WSSV + EGCG group; meanwhile, the virus copies number of WSSV group (1.520481e+008) was higher than that of WSSV + EGCG group (1.136506e+008) ($p = 0.0055251$) at 48 h. The variation of shrimp mortality was greater than the variation of virus replication. Based on the rapid increase in shrimp mortality after 48 h, we suggest that EGCG might affect WSSV replication by directly targeting the virus or indirectly enhancing host immunity. Our results were not sufficient to draw a definitive conclusion; however, we did observe that EGCG enhanced shrimp immunity to levels exceeding its impact on the virus (see Fig. 2).

In the *V. alginolyticus*-challenge experiment, the mortality of *V. alginolyticus*-infected shrimp increased over time, reaching 50% at between 48 h and 60 h, and mortality at 120 h was approximately 65%. When shrimp were successively treated with EGCG and *V. alginolyticus*, mortality decreased significantly relative to that observed in the *V. alginolyticus*-only group ($p < 0.0001$), reaching 50% at 96 h post-infection and approximately 55% at 120 h post-infection, which was about10% lower than the *V. alginolyticus*-only group ($p = 0.123$) (Fig. 1b).

The effect of EGCG treatment over the first 48 h of infection was efficient, as the mortality of shrimp in the pathogen-challenged groups showed no difference from the control; however, after 48 h and when the effect of EGCG began to attenuate, shrimp mortality increased and showed no difference from that of the infection groups, especially in the case of WSSV infection. The differential mortality rates associated with pathogen-challenge experiments in the presence or absence of EGCG treatment indicated that 1.0 mg/kg EGCG was able to non-permanently enhance shrimp immunity and delay shrimp death caused by pathogens.

3.2. Effects of EGCG on the expression of immune pathway genes

Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of 12 immune-related genes showed that the expression of *MAPK*, *myosin*, *p53*, *IMD*, *NOS*, *pro*PO, *Rab7*, *QM*, and *TNF-alpha* were upregulated following treatment with 1 mg/kg EGCG as compared with the control group ($p < 0.05$) (Fig. 3). Moreover, the expression of *STAT* and *Toll-like receptor* (*TLR*) remained unchanged. These results suggested that EGCG might induce certain immune pathways, such as the phenoloxidase (PO) or immune deficiency (IMD) pathways, and apoptosis in the hemocytes of *M. japonicus*.

3.3. Effects of EGCG on immunological parameters

Total hemocyte count (THC) and PO and superoxide dismutase (SOD) activities were measured at 24 h and 48 h after challenge with PBS, 1.0 mg/kg EGCG, pathogen (WSSV or *V. alginolyticus*), or the

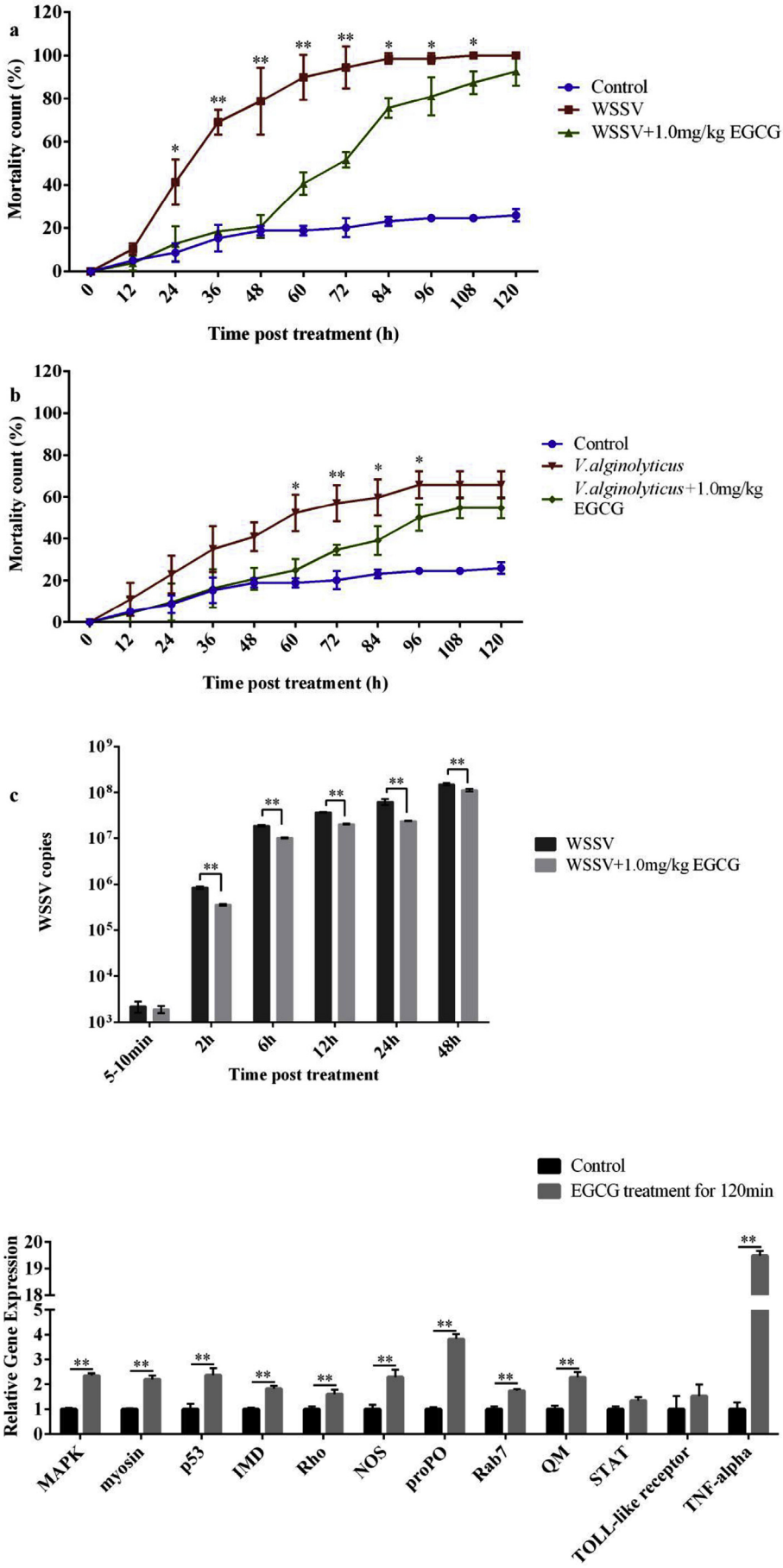

Fig. 1. Cumulative mortality of pathogen-challenged shrimps treated with/without EGCG and WSSV copies detection. Shrimps were treated with PBS, WSSV, WSSV +1.0 mg/kg EGCG, *V. alginolyticus*, *V. alginolyticus* + 1.0 mg/kg EGCG, separately. PBS only groups was defined as control. Each group contained at least 20 individuals. Shrimp mortality of each group was recorded continuously twice a day for 5 days. To represent mortality variation more clearly, EGCG only group was not showed in the figure, while WSSV group (a) and *V. alginolyticus* (b) group were demonstrated separately. WSSV copies (c) were detected at 5–10 min, 2 h, 6 h, 12 h, 24 h, 48 h after WSSV challenge. These experiments were repeated three times as biological replications, figures showed the mean value with standard deviation error bar. Asterisk marked the significance of difference between pathogen group and pathogen + EGCG group (* = $P < 0.05$, ** = $P < 0.01$), analyzed by two-way ANOVA and Multiple *t*-test method.

Fig. 2. Real-time quantitative PCR analysis of immune gene expression. The expression of twelve immune genes (*MAPK*, *myosin*, *p53*, *IMD*, *Rho*, *NOS*, *pro*PO, *Rab7*, *QM*, *STAT*, *Toll-like receptor* and *TNF-alpha*) in the hemocytes of *M. japonicus* was detected 2 h post 1 mg/kg EGCG treatment. The amount of gene mRNA was normalized to the *GAPDH* transcript level. Data are shown as means ± standard deviation of three separate individuals. The asterisks indicate a significant difference ($P < 0.01$) between 1.0 mg/kg EGCG group and control groups, analyzed by Multiple *t*-test method.

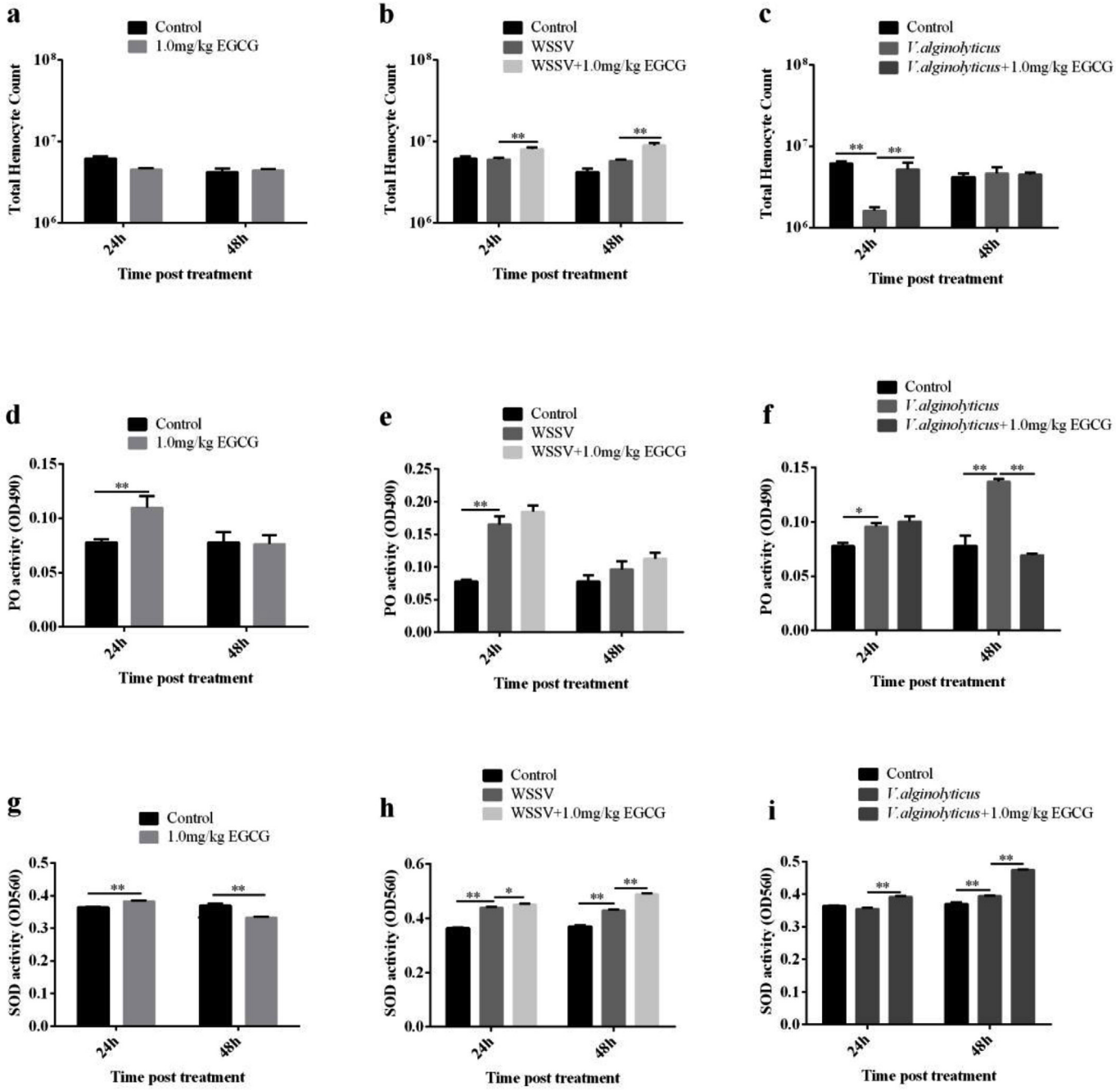

Fig. 3. The immune parameters measured in challenge experiment. Shrimps were treated with PBS (control), 1.0 mg/kg EGCG, pathogens (WSSV or *V. alginolyticus*), combination of 1.0 mg/kg EGCG and pathogens, separately, to compare the differences of three immune parameters, THC (total hemocyte count), PO (phenoloxidase) activity, and SOD (superoxide dismutase) activity before and after pathogen challenging, and the influence of EGCG treatment on shrimp immunity. For the co-treatment of pathogens and EGCG, shrimps were challenged with pathogens 10min after 1.0 mg/kg EGCG treatment. The variation of 1.0 mg/kg EGCG on THC (a), proPO activity (d), and SOD activity (g) showed the influence of EGCG treatment on healthy shrimps. (b) (e) (h) showed the influence of EGCG on WSSV infection process. (c) (f) (i) showed the influence of EGCG on *V. alginolyticus* infection process. Each treatment at each time point contained at least three shrimp individuals, mean values were showed in column with error bar representing standard deviation. The significant differences were represented with asterisk signal (* = $P < 0.05$, ** = $P < 0.01$), analyzed by Multiple *t*-test method.

combination of pathogen and EGCG, respectively. Shrimp THC was unaffected by 1.0 mg/kg EGCG treatment after 24 h and 48 h ($p = 0.01$ at 24 h; $p = 0.625$ at 48 h) (Fig. 3a), and WSSV infection did not affect THC at 24 h or 48 h (Fig. 3b). When EGCG was applied, shrimp THC increased to 8.09×10^6/mL at 24 h and 8.9×10^6/mL at 48 h, which were both higher than levels observed in the WSSV-infected groups ($p = 0.006$ at 24 h; $p = 0.004$ at 48 h) (Fig. 3b). *V. alginolyticus* infection led to an opposite result, with significantly decreased THC from 6.1×10^6/mL to 1.6×10^6/mL at 24 h ($p < 0.0001$). Following EGCG treatment, THC increased significantly relative to levels in the *V. alginolyticus*-infected group (5.19×10^6/mL vs. 1.6×10^6/mL; $p = 0.0005$), whereas no decrease was observed as compared with the PBS group (5.19×10^6/mL vs. 6.1×10^6/mL; $p = 0.201$) (Fig. 3c). No differences in THC among groups were observed at 48-h post-*V. alginolyticus* infection.

PO activity significantly increased ($p = 0.0076$) following EGCG treatment (0.109 U) as compared with that in the PBS group (0.077 U) (Fig. 3d) at 24 h, and at 48 h, PO activity in the EGCG-treatment group

decreased (0.076 U) to similar levels with those in the PBS group (0.078 U) ($p = 0.863$), indicating that EGCG treatment temporarily enhanced hemolymph PO activity. In virus-challenge experiments, PO activity increased ($p = 0.00035$) nearly 2-fold (Fig. 3e) at 24 h, whereas at 48 h, no difference ($p = 0.112$) was observed relative to the control. When EGCG was applied in virus-challenge experiments, PO activity showed no difference as compared with that observed in the WSSV-only group ($p = 0.103$ at 24 h; $p = 0.15$ at 48 h). At 24 h post *V. alginolyticus* infection, PO activity was higher than that in the PBS group (0.096 U vs. 0.077 U; $p = 0.002$), with EGCG treatment not altering this change ($p = 0.242$). At 48 h, the PO activity of infected shrimp significantly increased to 0.137 U ($p = 0.0005$), whereas that in *V. alginolyticus* + EGCG-treated shrimp significantly decreased to 0.069 U ($p < 0.0001$) (Fig. 3f).

SOD activity in healthy shrimp increased slightly from 0.364 U to 0.383 U at 24 h ($p = 0.00036$), followed by a decrease from 0.440 U to 0.333 U at 48 h ($p = 0.00036$) (Fig. 3g). In WSSV-challenge experiments, SOD activity significantly ($p < 0.0001$) increased to 0.439 U at 24-h and 0.429 U at 48 h post WSSV infection. When EGCG was applied in the challenged groups, at 24 h, SOD activity increased slightly to 0.439 U ($p = 0.024$), whereas at 48 h, there was a significant increase to 0.488 U ($p < 0.0001$) (Fig. 3h). In *V. alginolyticus*-challenge experiments, SOD activity in *V. alginolyticus*-infected shrimp showed no variation at 24 h ($p = 0.029$) as compared with the control, but at 48 h, it increased from 0.394 U to 0.440 U ($p = 0.00015$). When EGCG was applied to the challenged groups, SOD activity significantly increased to 0.391 U ($p = 0.00019$) at 24 h and 0.474 U ($p < 0.0001$) at 48 h (Fig. 3i).

3.4. Effects of EGCG on the apoptosis of hemocytes

The hemocyte apoptosis rates in the presence or absence of 1 mg/kg EGCG treatment were detected using flow cytometry. The apoptosis rate in the PBS control group was approximately 34% (Fig. 4a), which was relatively higher than that observed in mammalian apoptosis experiments (usually < 10%). This might be explained by the Annexin V used in our experiments being designed for regular mammalian cells. Despite dilution of the dyes using high-saline PBS and adjustment of the working concentrations of propidium iodide (PI) and fluorescein isothiocyanate (FITC)-Annexin V to the minimum standards for flow cytometry, it is possible that the PI concentration might still cause cell damage. Furthermore, the shrimp hemocytes used in these experiments were highly vulnerable, given that experimental operations included centrifugation, fluid impact associated with pipetting, and subsequent resuspension. We attempted to minimize damage from external sources to the best of our ability.

Treatment with 1.0 mg/kg EGCG did not alter the hemocyte apoptosis rate (Fig. 4a), suggesting the absence of EGCG-related cytotoxicity to shrimp hemocytes. In the WSSV-infection groups, the apoptosis rate increased to an average of 54.2%; however, upon EGCG treatment, this rate decreased to 53.7% and showed no variation from the WSSV group ($p = 0.786$). In the *V. alginolyticus* groups, the apoptosis rate reached an average of 50.1%, and following EGCG treatment, decreased significantly to 29.6% ($p = 0.00098$).

4. Discussion

The mortality rates in the pathogen-challenge experiments directly indicated the role of EGCG in the shrimp innate immune system. In the WSSV-challenge experiment, 1.0 mg/kg EGCG treatment significantly delayed shrimp death caused by WSSV for approximately 48 h. This result agreed with results of a WSSV-challenge experiment in another invertebrate (mud crab, *Scylla paramamosain*), in which mortality was delayed and decreased by about30% [7]. WSSV copy number was detected at 5–10 min, 2 h, 6 h, 12 h, 24 h, and 48 h post WSSV injection, respectively, showing rapid increases in WSSV copy number in the shrimp hemolymph and confirming the high degree of pathogenicity of WSSV and its ability to double its replication in only 2 h and duplicate over 1×10^7/mL in just 12 h. Over 24 h, over50% mortality was observed; however, when shrimp were pretreated with 1.0 mg/kg EGCG, the mortality rate and copy number decreased. EGCG also reportedly inhibits grass carp reovirus (GCRV) infection in *Ctenopharyngodon idellus* kidney cells by blocking GCRV attachment [18]. In the present study, *V. alginolyticus* was also applied as a pathogen to challenge shrimp, with similar results to those obtained for WSSV infection. EGCG treatment delayed and reduced *V. alginolyticus*-related mortality, confirming that EGCG was capable of protecting hosts from both viral and bacterial infection.

To determine which parts of the shrimp innate immune system were influenced by EGCG, the expression of multiple immune-related genes was detected. Alterations in gene expression following EGCG treatment showed that, except for *STAT* and *TLR* genes, most innate immune-related genes were significantly up-regulated. The IMD pathway was up-regulated by EGCG. The IMD pathway, which responds to Gram-negative bacteria and WSSV infections, regulates the expression of antibacterial-peptide gene expression [5,19]. Following EGCG treatment, the expression of the *proPO* gene was up-regulated, whereas pathogen-challenge experiments showed that PO activity in shrimp treated with EGCG increased, indicating the proPO non-self-recognition system [20] was promoted by EGCG treatment. PO is the terminal enzyme of the proPO pathway and converts tyrosine to dihydroxyphenylalanine (DOPA), which is subsequently converted to DOPA-quinone and melanin synthesis [20]. Melanization is among the most important immune responses in crustaceans [4]. In *M. japonicus*, Xu et al. [21] found that the QM protein interacts with hemocyanin and myosin to regulate the PO enzyme activity. Our findings showed that EGCG treatment promoted PO activity and up-regulated *proPO*, *QM*, and *myosin* expression, indicating that EGCG enhanced enzyme synthesis, as well as activity.

WSSV infection with EGCG co-treatment resulted in significantly delayed mortality, decreased WSSV copy number, increased THC, and enhanced SOD activity. THC represents the hemocyte population in the shrimp hemolymph, and hemocytes contribute to innate immunity in both cellular and humoral defense responses, as they can directly recognize and engulf foreign targets and act as the source [6] and storage sites [22] of many humoral molecules. Higher THC represents greater immune defense and allows increased numbers of immune cells to act in a protective role. SOD activity is crucial to shrimp homeostasis and health. The reactive oxygenic species (ROS) released during the innate immune response can damage bacteria or viruses; however, large amount of ROS can also non-specifically attack host proteins, lipids, and DNA, causing cell damage [23]. To avoid ROS self-damage, SOD is activated as part of the antioxidant defense for scavenging superoxide anions and maintaining homeostasis during the immune response [17]. The results of the present study indicated that EGCG induced hemocyte proliferation following viral infection, thereby strengthening host defense and enhancing SOD activity to better maintain homeostasis during pathogen response. Compare the mortality count and corresponding WSSV copies, the difference of mortality count is much more significant than the reduction of WSSV copies, it seems that EGCG protects shrimps from WSSV infection mainly by enhancing the host innate immunity, not directly working on viron although EGCG did have influence on virus replication. In our previous research on mud crab *Scylla paramamosain*, the inhibition of EGCG on WSSV copies and mRNA expression level of WSSV replication related genes include endonuclease, DNA polymerase, and VP26 were much more significant and efficient [7]. This discrepancy might be explained by the differences of shrimp and mud crab since shrimps, not the crabs, are the nature host of WSSV virus, makes it easier for WSSV to take advantage of shrimp protein to replicate than the crab protein. This hypothesis needs further research.

The *p53* tumor-suppressor gene and *TNF-α* are apoptosis-related genes [24]. TNF-α is a proinflammatory cytokine and functions mainly

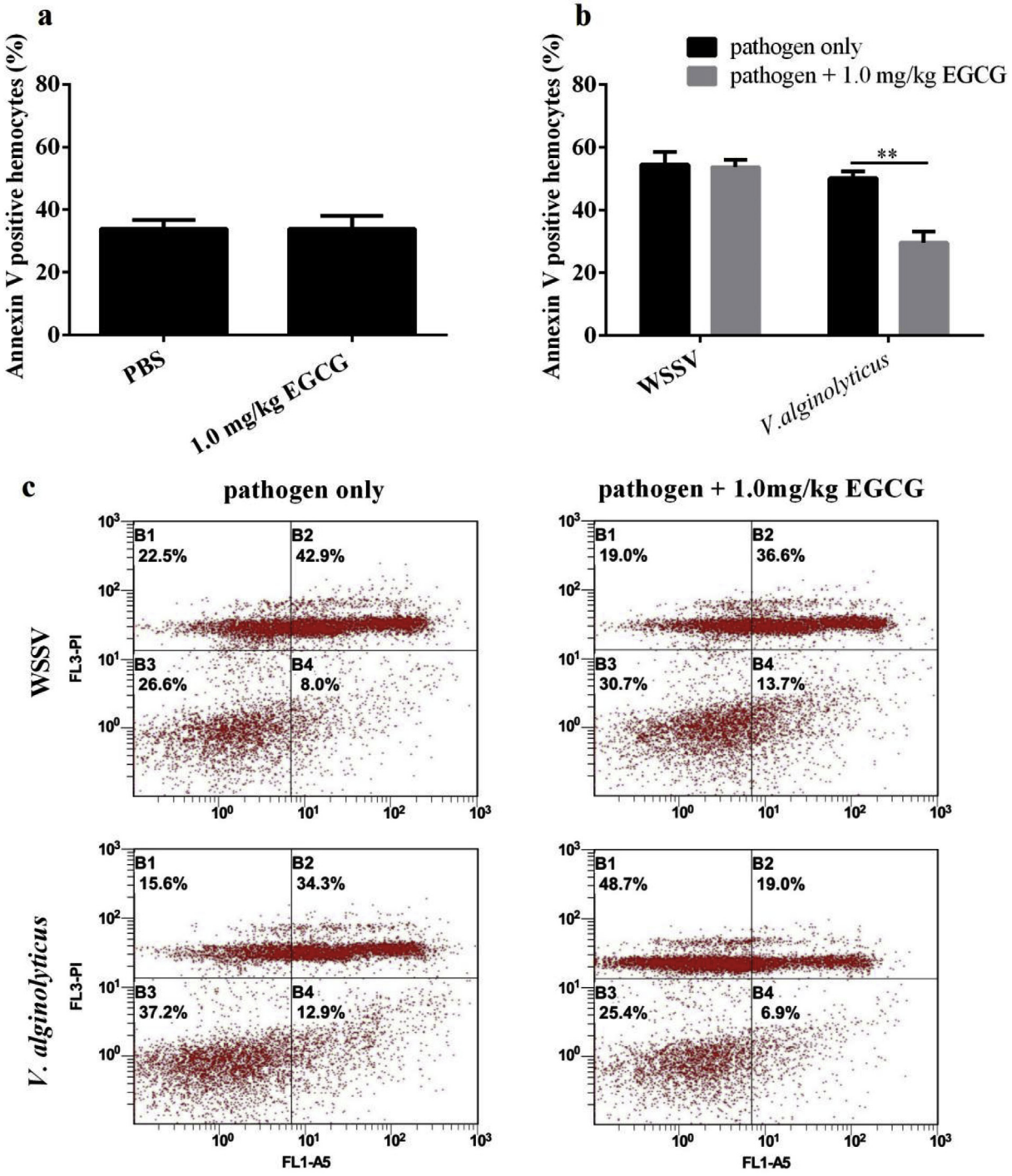

Fig. 4. Apoptosis analyze of EGCG treatment by flow cytometry. Shrimps were treated with PBS/1.0 mg/kg EGCG, followed by a second treatment of PBS/WSSV/*V.alginolyticus* as pathogen challenge. 24 h post pathogen challenge, the hemocyte samples of different treatment samples were collected and stained with Annexin-V FITC and PI for flow cytometry detection. Fluorescent 1-Annexin V (FL1-A5) represents apoptosis cells. Fluorescent 3-PI (FL3-PI) represents died or damaged cells. The threshold was determined based on empty control, negative control and positive control. The percentage of Annexin V positive cells represents the apoptosis rate of hemocyte sample. The apoptosis rate of control and EGCG control were showed in (a), each column represents the mean value of three isolated repeats with error bar. (b) showed the apoptosis rate of challenged groups. The scatter plots of one of the challenged groups were showed in (c), B1 represents cell fragments caused by centrifuge and re-suspend operation, B2 represents the late-stage apoptosis, B3 represents normal cells, B4 represents early-stage apoptosis. The apoptosis rate of sample was determined by the total fluorescent intensity of B2 and B4.

in the central nervous system as a signaling and an effector molecule [25]. Apoptosis represents a programed cell-death process controlled by a complex and conserved molecular network and is involved in all fundamental immune responses [26]. Apoptosis regulates the course of immune response by removing surplus, damaged, or diseased cells [27]. TNF-α signaling gives rise to either apoptosis or activation of transcription factor NF-κB, indicating a potential influence of EGCG treatment on the upstream signaling of apoptosis [24]. P53 protein in a normal cell is kept at a relatively low concentration, upstream events or signals like DNA damage or environmental stimuli could activate p53 protein transcriptionally and leading to the activation of downstream signals including p21, MDM2, GADD45, Bax, IGF-BP and cyclin G to

either stop cell cycle, repair damaged DNA or leading directly to program cell death [28]. Direct signaling like sensing oncegene also leads to apoptosis. In our experiment, EGCG treatment elevated the expression level of p53, indicates the shrimp hemolymph was stimulated by this external material. Meanwhile in the apoptosis experiments, the accurate apoptosis rate of shrimp hemolymph showed no change in EGCG treatment compared with PBS treatment shrimps, indicates that EGCG stimulation could not further activate p53 protein transcription or could not activate the downstream genes which would trigger apoptosis. Thus, our results indicated that 1.0 mg/kg EGCG treatment caused no cell damage to normal hemocytes. The high apoptosis rate induced by *V. algiolyticus* infection was reduced by 1.0 mg/kg EGCG, while the apoptosis rate of WSSV-infected shrimp was not affected by EGCG treatment; however, our previous study in crabs (*Scylla paramamosain*) showed that apoptosis caused by WSSV was reduced by EGCG treatment [7], although this response was not as efficient as that observed in relation to *V. alginolyticus* infection. In HT-29 colon cancer cells, EGCG treatment suppresses cancer-cell proliferation by inducing apoptosis [29]. Consider the enhancement of EGCG treatment on PO activity and SOD activity, we supposed the decrease of apoptosis rate was the result of an enhanced humoral immunity. The EGCG could enhance the gene expression of apoptosis related gene, but not able to trigger transcription activity of apoptosis gene or the downstream signals. Further experiments were needed to discover the influence of EGCG on signal pathway of apoptosis. Apoptosis is considered a critical aspect of cellular immunity in invertebrates; however, apoptosis in penaeid shrimps was found to be susceptible to WSSV, given evolutionary changes enabling the manipulation of this cell activity in this species [27]. It is possible that WSSV might intentionally be captured and engulfed by host defense cells, thereby allowing the viral proteins to interact with host proteins to inhibit the immune response [30–34]. The underlying mechanism associated with viral-invertebrate host interactions requires further study. Our findings suggested differences in apoptosis between WSSV and *V. alginolyticus* infection, which might provide insight into WSSV exhibiting a different invasion strategy to take advantage of host immunity. These results also revealed that EGCG treatment efficiently enhanced the humoral immunity of shrimp, while its influence on cellular immunity was not as efficient.

Rho-family proteins establish polarity through dynamic regulation of the actin cytoskeleton [35]. Myosin participates in formation and maintenance of the cytoskeleton and regulates several biological functions, including cell motility, membrane-structure regulation, and interactions with viral proteins to positively regulate shrimp phagocytosis activity [36]. Shrimp Rab7 is a WSSV-binding protein that belongs to the small GTP-binding protein family. In *Penaeus monodon*, *pm*Rab7 is a receptor for the WSSV VP-28 protein and reduces and delays mortality upon WSSV challenge through autophagy [37] and phagocytosis [38]. In the present study, up-regulation of *Rab7* indicated that cell motility and phagocytosis might have been promoted following EGCG treatment. Although we did not perform phagocytosis or autophagy assays, a previous report showed that EGCG induced protein aggregation, inhibited the release of high-mobility group box 1 protein, and induced autophagy in murine macrophage-like RAW 264.7 cells [39].

In conclusion, our results showed that EGCG treatment promoted the activity of several innate immune pathways especially the humoral immunity following viral and/or bacterial infection. Additionally, EGCG treatment inhibited pathogen replication and delayed and reduced mortality upon viral and bacterial infection. These findings suggested that EGCG could be applied in shrimp cultures as an immune promotor and a potential antiviral therapeutic for further research.

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References

[1] 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.
[2] Y.T. Wang, W. Liu, J.N. Seah, C.S. Lam, J.H. Xiang, V. Korzh, J. Kwang, White spot syndrome virus (WSSV) infects specific hemocytes of the shrimp *Penaeus merguiensis*, Dis. Aquat. Org. 52 (2002) 249–259.
[3] M.C. Balebona, M.J. Andreu, M.A. Bordas, Z. Irene, M.A. Morinigo, J.J. Borrego, Pathogenicity of *Vibrio alginolyticus* for cultured gilt-head sea bream (*Sparus aurata* L.), Appl. Environ. Microbiol. 64 (1998) 4269–4275.
[4] A. Tassanakajon, K. Somboonwiwat, P. Supungul, S. Tang, Discovery of immune molecules and their crucial functions in shrimp immunity, Fish Shellfish Immunol. 34 (2013) 954–967.
[5] F.H. Li, J. Xiang, Signaling pathways regulating innate immune responses in shrimp, Fish Shellfish Immunol. 34 (2013) 973–980.
[6] M.D. Lavine, M.R. Strand, Insect hemocytes and their role in immunity, Insect Biochem. Mol. 32 (2002) 1295–1309.
[7] Z. Wang, B.Z. Sun, F. Zhu, Epigallocatechin-3-gallate inhibit replication of white spot syndrome virus in *Scylla paramamosain*, Fish Shellfish Immunol. 67 (2017) 612–619.
[8] G.N. Dale, D. Ferreira, Y. Zhou, Epigallocatechin-3-gallate (EGCG)-chemical and biomedical perspectives, Phytochemistry 67 (2006) 1849–1855.
[9] N.S. Brahma, S. Shankar, R.K. Srivastava, Green tea catechin, epigallocatechin-3-gallate (EGCG) mechanisms, perspectives and clinical applications, Biochem. Pharmacol. 82 (2011) 1807–1821.
[10] S. Masahito, Y. Shirakami, H. Sakai, H. Tatebe, et al., EGCG inhibits activation of the insulin-like growth factor (IGF)/IGF-1 receptor axis in human hepatocellular carcinoma cells, Canc. Lett. 262 (2008) 10–18.
[11] G.R. Daniel, M.F. Schlachter, E. Volker, P. Weber, Effect of EGCG on lipid absorption and plasma lipid levels in rats, J. Nutr. Biochem. 14 (2003) 326–332.
[12] H.X. Zhao, P. Xie, X.L. Li, W.Q. Zhu, et al., A prospective phase II trial of EGCG in treatment of acute radiation-induced esophagitis for stage III lung cancer, Radiother. Oncol. 114 (2015) 351–356.
[13] F. Zhu, X.B. Zhang, The Wnt signaling pathway is involved in the regulation of phagocytosis of virus in *Drosophila*, Sci. Rep. 3 (2013) 2069–2078.
[14] Z. Wang, F. Zhu, Minichromosome maintenance protein 7 regulates phagocytosis in kuruma shrimp *Marsupenaeus japonicus* against white spot syndrome virus, Fish Shellfish Immunol. 55 (2016) 293–303.
[15] B.Z. Sun, Z. Wang, F. Zhu, The crustin-like peptide plays opposite role in shrimp immune response to *Vibrio alginolyticus* and white spot syndrome virus (WSSV) infection, Fish Shellfish Immunol. 66 (2017) 487–496.
[16] Z. Wang, F. Zhu, MicroRNA-100 is involved in shrimp immune response to white spot syndrome virus (WSSV) and *Vibrio alginolyticus* infection, Sci. Rep. 7 (2017) 42334–42345.
[17] A.I. Campa-Cordova, N.Y. Hernandez-Saavedra1, R. De Philippis, and F. Ascencio, Generation of superoxide anion and SOD activity in haemocytes and muscle of American white shrimp (*Litopenaeus vannamei*) as a response to β-glucan and sulphated polysaccharide, Fish Shellfish Immunol. 12 (2002) 353–366.
[18] H. Wang, W. Liu, F. Yu, Identification of (-)-epigallocatechin-3-gallate as a potential agent for blocking infection by grass carp reovirus, Arch. Virol. 161 (2016) 1053–1059.
[19] E. DE Gregorio, P.T. Spellman, P. Tzou, G.M. Rubin, B. Lemaitre, The Toll and IMD pathways are the major regulators of the immune response in Drosophila, EMBO J. 21 (2002) 2568–2579.
[20] K. Sritunyalucksana, K. Soderhall, The proPO and clotting system in crustacean, Aquaculture 191 (2000) 53–69.
[21] J. Xu, S. Wu, X.B. Zhang, Novel function of QM protein of shrimp (*Penaeus japonicus*) in regulation of phenol oxidase activity by interaction with hemocyanin, Cell. Physiol. Biochem. 21 (2008) 473–480.
[22] C.H. Chiu, Y.K. Guu, C.H. Liu, T.M. Pan, W. Cheng, Immune responses and gene expression in white shrimp, *Litopenaeus vannamei*, infected by *Lactobacillus plantarum*, Fish Shellfish Immunol. 23 (2007) 364–377.
[23] P.T. Schumacker, Reactive oxygen species in cancer: a dance with the devil, Canc. Cell 27 (2015) 156–157.
[24] D.J. Van Antwerp, S.J. Martin, T. Kalri, D.R. Green, L.M. Verma, Suppression of TNF-α-induced apoptosis by NF-kB, Science 274 (1996) 787.
[25] S. Alvarez, A. Blanco, M. Fresno, M.A. Munoz-Fernandez, TNF-α contributes to Caspase-3 independent apoptosis in neuroblastoma cells: role of NFAT, PLoS One 6 (2011) 16100–16113.
[26] L. Wang, B. Zhi, W. Wu, X.B. Zhang, Requirement for shrimp caspase in apoptosis against virus infection, Dev. Comp. Immunol. 32 (2008) 706–715.
[27] J.H. Leu, S.J. Lin, J.Y. Huang, T.C. Chen, C.F. Lo, A model for apoptotic interaction between white spot syndrome virus and shrimp, Fish Shellfish Immunol. 34 (2013) 1011–1017.
[28] A.J. Levine, p53, the cellular gatekeeper for growth and division, Cell 88 (1997) 323–331.
[29] J.T. Hwang, J.H. Ha, I.J. Park, S.K. Lee, H.W. Baik, Y.M. Kim, O.J. Park, Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway, Canc. Lett. 247 (2007) 115–121.
[30] S.H. Abeer, M.D. Hassan, M. Shariff, DNA fragmentation, an indicator of apoptosis, in cultured black tiger shrimp *Penaeus monodon* infected with white spot syndrome virus (WSSV), Dis. Aquat. Org. 44 (2001) 155–159.
[31] J.H. Leu, C.H. Lo, A Review of the Strategies Evolved by WSSV to Thwart Host Responses to Infection and Ensure Successful Virus Replication in Cells-apoptosis

and Anti-apoptosis Strategies, DAA VII, (2011).
[32] Y. Zhang, M.E. Hinojosa, N. Yoo, M.J. Holtzman, Viral and host strategies to take advantage of the innate immune response, Am. J. Res. Cell Mol. 43 (2010) 507–510.
[33] T. Ye, R.R. Zong, X.B. Zhang, The role of white spot syndrome virus (WSSV) VP466 protein in shrimp antiviral phagocytosis, Fish Shellfish Immunol. 33 (2012) 350–358.
[34] Y.E. Guo, J.A. Steitz, Virus meets host microRNA: the destroyer, the booster, the hijacker, Mol. Cell Biol. 20 (2014) 3780–3787.
[35] M. Wherlock, H. Mellor, The Rho GTPase family: a racs to wrchs story, J. Cell Sci. 115 (2002) 239–240.
[36] S. Taengchaiyaphum, P. Havanapan, S. Roytrakul, C.F. Lo, K. Sritunyalucksana, C. Krittanai, Phosphorylation is required for myosin regulatory light chain (PmMRLC) to control yellow head virus infection in shrimp hemocytes, Fish Shellfish Immunol. 34 (2013) 1042–1049.
[37] J. Stefanie, C. Bucci, T. Isei, T. Ueno, E. Kominami, P. Saftig, E.L. Eskelinen, Role for Rab7 in maturation of late autophagic vacuoles, J. Cell Sci. 117 (2004) 4837–4848.
[38] S. Kallaya, W. Wannapapho, C.F. Lo, T.W. Flegel, PmRab7 is a VP28-binding protein involved in white spot syndrome virus infection in shrimp, J. Virol. 80 (2006) 10734–10742.
[39] W. Li, S. Zhu, J.H. Li, A. Assa, A. Jundoria, J.Y. Xu, et al., EGCG stimulates autophagy and reduces cytoplasmic HMGB1 levels in endotoxin-stimulated macrophages, Biochem. Pharmacol. 81 (2011) 1152–1163.