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RESEARCH REPORT

The immunosuppressive effects of continuous CpG ODNs stimulation in chinese mitten crab, *Eriocheir sinensis*

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Abstract

CpG oligodeoxynucleotides (CpG ODNs) have been widely used as a novel vaccine adjuvant in mammals due to its immune protection, long effectiveness and safety. In the present study, the long-term immune effects as well as immunosuppression of CpG ODNs was evaluated by comparing the immune parameters of Chinese mitten crab, *Eriocheir sinensis* after continuous or interval feeding with CpG ODNs-supplement diet for 21 days (designated as CC and CI group, respectively). In the CI group, the mRNA transcripts of *Es*Tolls (*Es*Toll1 and *Es*Toll2) and *Es*MyD88 (adaptor molecule) in hepatopancreas maintained at a significantly higher level ($p < 0.05$) compared with the CC group after 7 days and 14 days feeding, while there was no significant difference between them at the 21st day. Moreover, after a significant increase at 7th day, the expression level of *Es*LITAF mRNA [Lipopolysaccharide-induced tumor necrosis factor (TNF)-α] in CC group decreased at 14th-21st day, while that in CI group kept increasing at 14th day, followed by a decrease at 21st day. The TNF-α in plasma of CC group was abnormally increased at the 21st day ($p < 0.05$) in CC group compared with the significant raise at 7th-14th in CI group. Moreover, the phagocytic activity and reactive oxygens (ROS) level of hemocytes in continuous CpG ODNs feeding crabs were significantly lower than those in interval feeding crabs. These results indicated that long-term and continuous CpG ODNs stimulation could reduce the activation of pro-inflammatory cytokine production, hemocyte phagocytosis and ROS generation, displaying immunosuppressive effects on the immune system of crabs.

Key Words: *Eriocheir sinensis*; CpG ODNs feeding; immunosuppressive effects; Toll-like receptor pathway; tumor necrosis factor-α; phagocytosis

Introduction

DNA from bacteria has been demonstrated to induce the immune response of hosts by a specific structure termed CpG motifs (Yamamoto *et al.,* 1992; Tassakka and Sakai, 2002). The CpG motifs possess typical palindromic sequences with unmethylated cytosine and guanine triphosphate deoxynucleotides (CpG ODNs), and they are important immunomodulators to induce or enhance various immune responses (Klinman *et al*., 1996). In vertebrate, CpG ODNs have been used as a kind of therapeutic agents to prevent host from various pathogens. After internalized by target cells, CpG ODNs could be recognized by Toll-like receptors, and trigger various immune responses in mammals, such as phagocytic activities, the generation of reactive oxygen species (ROS), and the production of pro-inflammatory cytokines (Stacey *et al.*, 1996; Klinman, 2004; Aguilar and Rodriguez, 2007). For instance, after interacting with human TLR9, CpG ODNs activate the MyD88 dependent TLR pathways, resulting in the production of immune factors, such as IFN-α and TNF-α.

CpG ODNs is not only widely applied as effective vaccine adjuvants for mammalian models, its immunoenhancement effect have also been observed in some aquatic animals (Stacey *et al.,* 1996; Zhang *et al.,* 2010). Accumulating evidences have demonstrated that CpG ODNs can effectively

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activate the innate immune response in aquatic animals. For instance, CpG ODNs could induce phagocytic activity and respiratory burst activities of kidney cells and the lysozyme activity in serum of *Cyprinus carpio* (Tassakka and Sakai, 2002). CpG ODNs have also been reported to activate macrophages and increase levels of superoxide anion, hydrogen peroxide, acid phosphatase (ACP) and the bactericidal activity in *Ctenopharyngodon idellus*, which was consistent with the results from *Paralichthys olivaceus* (Lee *et al.,* 2003; Meng *et al*., 2003). In addition, CpG ODNs could activate the prophenoloxidase (proPO) system in *Macrobrachium rosenbergii* (Chuo *et al*., 2005). In our previous studies, CpG ODNs were found to promote regeneration of circulating hemocytes, and enhance the phagocytosis and ROS production in *Litopenaeus vannamei* (Sun *et al*., 2013a).

Although immunostimulants can improve the resistance of hosts against pathogens, the immunosuppression has also been observed in some aquatic animal because of the abnormal administration of immunostimulants. For instance, long-term oral administration of peptidoglycan could decrease immune response of rainbow trout as well as catfish against the infection of *Vibrio anguillarum* (Matsuo and Miyazono, 1993). β-glucan or glycyrrhizin could cause the immunity fatigue in shrimp after continuous applying into the diet (Bai *et al.,* 2010). CpG ODNs has been considered as one of prospective immunostimulants in aquatic animals. To our knowledge, recently studies are mainly focused on its immune stimulation mechanism as well as its short-term effects, while the dysimmunity of its long-term stimulation is still not well known.

Chinese mitten crab, *Eriocheir sinensis*, is an important economic crustacean in China (Chen *et al.,* 2013). In recent years, massive mortalities of crabs have caused decline of productions and huge economic losses by infections of various pathogens (Wang and Gu, 2002; Bricknell and Dalmo, 2005; Wang, 2011). Immunostimulants can directly initiate the innate immune system of hosts, which would be benefit for invertebrates lacking the adaptive immunity (Bricknell, 2005; Ringø, 2011). The use of biological immunostimulants such as CpG ODNs is an innovative approach to substitute antibiotics for disease control in aquaculture (Zhang *et al.,* 2010). In our previous research, the enhancements of immuno-protection induced by CpG ODNs were observed in crab (Sun *et al*., 2013b). In the present study, CpG ODNs was added into the dietary and fed crabs with two feeding strategies (interval and continuous) for 3 weeks. The mRNA expression levels of TLR pathway related genes (*Es*Tolls, *Es*MyD88, TNF-α transcription factor *Es*LITAF), and the release of TNF-α in plasma as well as the ability of phagocytosis and generation of reactive oxygen species (ROS) were examined to explore the effects of long-term CpG ODNs administration in crabs, hopefully providing theoretical guidance for the application of CpG ODNs in aquaculture as an immunostimulant.

Materials and Methods

Crabs and CpG ODNs

Chinese mitten crabs, *Eriocheir sinensis*, weighing approximately 20 g, were collected from a commercial farm in Lianyungang, China, and cultured in flat-bottomed circular tanks (80×90 cm) at 18-23 ℃, pH 7.3-7.5, for a week before processing. CpG ODNs used in the present study were synthesized by Sangon Biotech Co. (China) containing five CpG rich fragments (Fig. 1), which were previously found effective to mammalian and aquatic animals (Sun *et al*., 2013b, 2014). The CpG ODNs were sub-cloned into the plasmid pUC57, and transformed into *Escherichia coli*. The recombinant plasmids were extracted and purified by using alkaline lysis, then heated at 100 ℃ for 15 min and cooled down rapidly for fragmentation (Sun *et al*., 2015). The linear and fragmented DNA was then freeze-dried and stored at -80 ℃ for the further experiment.

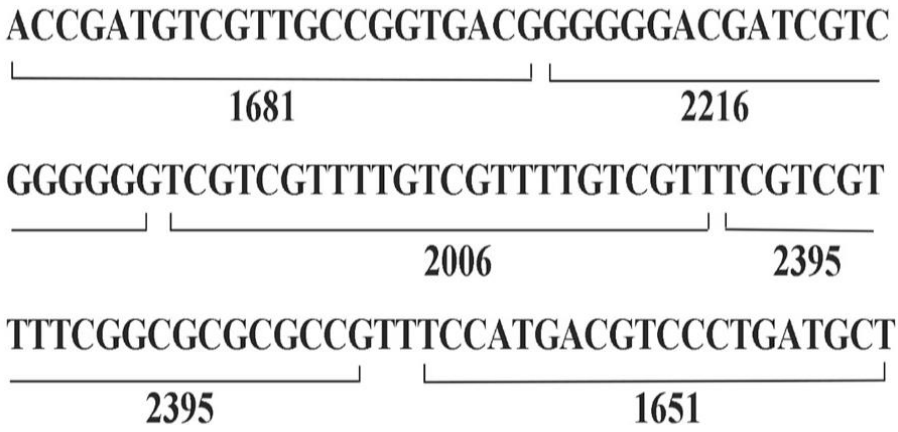

Fig. 1 Description of tandem ODNs sequence. Five CpG ODNs in this figure previously used effectively in mammalian and aquatic animals were designed to connect one another in series to form a CpG-rich fragment in the present study.

Table 1 The composition of the basic diets for crab

Ingredients	Composition (g/kg)	Ingredients	Composition (g/kg)
Soybean meal	250	Phosphatidylcholine	5
Peanut meal	50	Cholesterol	5
Shrimp meal	100	Choline chloride	5
Wheat flour	80	Vegetable oil	20
Corn flour	140	$NaH_2PO_4 \cdot 2H_2O$	2
Fish meal	300	$Na_2HPO_4 \cdot 12H_2O$	3
Multi-vitamin	10	$Ca(H_2PO_4)_2 \cdot 2H_2O$	3
Vitamin C	2	Sodium Alginate	10
Glycine	15		

Diet preparation

The ingredients for the basic diet of crabs were shown in Table 1, and they were mixed evenly following the reported proportion (Sun *et al.,* 2013b). The linear and fragmented CpG ODNs were dissolved in the double distilled water and added into the basic diet at a final proportion of 40 mg kg^{-1}. The ingredients of the diet were well mixed and extruded by a pellet extruder. The pellets were dried by the microwave oven and stored at 4 ℃.

CpG ODNs-supplement diet feeding experiments, hemolymph and tissue collection

A total of 135 crabs were randomly selected from the flat-bottomed circular tanks and equally divided into 3 groups with same conditions as described above. In the first group, crabs were continuously fed with the basic diet during the whole experimental period (21 days) as a control (named B group). The crabs in the second group were alternately fed with the CpG ODNs-supplement and basic diet according to a modified interval strategy (Sun *et al.,* 2013b), 4 days feeding of CpG ODNs containing diet following 3 days feeding of basic diet (named CI group). In the third group, crabs were continuously fed with the CpG ODNs-supplement diet every day (named CC group) (Fig. 2). All the crabs were fed twice a day to ensure the repletion at 8:30 a.m. and 6:30 p.m., and the water was changed every two days. Six crabs were randomly sampled from three groups at the 0th, 7th, 14th and 21st day after feeding. The hemolymph (about 2 mL each crab) was collected from the last walking legs using a syringe (5 mL) with 2 mL pre-cooled anticoaglulant solution (1L: 29.835 g NaCl, 18 g glucose, 38.426 g sodium citrate, 8.823 g trisodium citrate, 3.7224 g EDTA·2Na, pH 7.3) and immediately centrifuged at 800*g*, 4 ℃ for 10 min to harvest hemocytes for determination of phagocytosis and ROS. The supernatants (plasma) were stored at -80 ℃ for subsequent enzyme activity examination. Hemolymph from two crabs was mixed as a single sample and there were three biological replicates for each group. The hepatopancreas was collected from crabs and added into 1.5 mL microcentrifuge tube with 600 μL Trizol reagent (TaKaRa, Japan), stored at -80 ℃ for RNA extraction.

Quantification of Es*Tolls, EsMyD88 and* Es*LITAF expression by quantitative real-time PCR*

Total RNA was extracted from hepatopancreas samples using Trizol reagent (TaKaRa, Japan) according to the manufacturer's protocol. The fist-strand cDNA synthesis was carried out based on M-MLV RT usage information using the RQ1 Dnase (Promega, USA) treated total RNA as a template and oligo (dT)-adaptor as a primer (Table 2) (Invitrogen, USA). The synthesis reaction was performed at 42 ℃ for 1 h, terminated by heating at 95 ℃ for 5 min. The cDNA mix was diluted to 1:30 and stored at -80 ℃ and quantitative real-time PCR

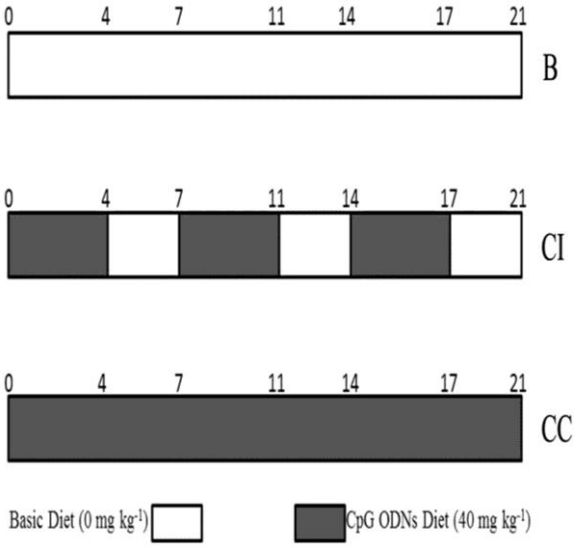

Fig. 2 The feeding strategy diagram. The crabs of basic group were continuously fed basic diet for 21 days (B group). The one of experiment groups was fed using the interval feeding method, which was 4 days feeding with CpG ODNs-supplement diet following 3 days feeding with basic diet for 3 weeks (CI group). Another experiment group was continuously fed CpG ODNs-supplement diet until end of the experiment (CC group).

(qRT-PCR). The mRNA expression levels of *Es*Toll1 (JX295852), *Es*Toll2 (AGT21374.1), *Es*MyD88 (AIM45535.1) and *Es*LITAF (Lipopolysaccharide-induced TNF-α factor, KF892539) in hepatopancreas were detected by qRT-PCR in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA). The specific primers of *Es*Toll1 (P1 and P2), *Es*Toll2 (P3 and P4), *Es*MyD88 (P5 and P6), and *Es*LITAF (P7 and P8) were used to amplify the corresponding products. The β-actin gene fragment from *E*. *sinensis*, amplified with primers P8 and P9, was chosen as a reference gene for internal standardization (Table 2). The assay was carried out in a total volume of 10 μL, containing 5.2 μL of SYBR Green Mix (TaKaRa, Japan), 0.2 μL of each primer (10 μmol L^{-1}), 1 μL of the 30 times diluted cDNA, and 3.4 μL of DEPC-water. The thermal procedure for the qRT-PCR program was 50 ℃ for 2 min, 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 60 s. Dissociation curve analysis of amplification products was performed to confirm that only one PCR product was amplified and detected. After the PCR program, the data were analyzed using ABI 7500 SDS software V2.0 (Applied Biosystems, USA). The relative expression of *Es*MyD88, *Es*Toll1, *Es*Toll2 and *Es*LITAF gene was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All the data were given in terms of relative mRNA expressed as mean ± S.E. (n = 3).

Measurement of TNF-α level in plasma

TNF-α activity in plasma was determined by using the commercial fish TNF-α ELISA kit (Baolai, China) following the manufacturer's instructions. Briefly, the 96 wells plates coated by the purified fish TNF-α antibody, were incubated with 10 μL plasma (diluted 5-fold) from different groups at 37 ℃ for 30 min. After 5 times of washing, the plate was incubated with 50 μL Horseradish Peroxidase (HRP)-fish TNF-α antibody at 37 ℃ for 30 min. After the final washing of 5 times, chromogenic agent and stop buffer were added, and the absorbance was determined at 450 nm by a precision microplate reader (BioTek, USA).

Analysis of phagocytic rate of crab hemocytes

The phagocytic activity of hemocytes was measured according to the method modified from Wu's description (Wu *et al*., 2007). Briefly, hemocytes (10^6 cells ml^{-1}) from different groups were washed and resuspended with 1×Leibovitz L-15 medium. Then they were incubated with the Latexs Beads (Sigma, USA) marked fluorescent yellow-green in advance for 60 min with dark. After the incubation, hemocytes were centrifuged at 900*g*, 4 ℃ for 10 min and resuspended with 400 μl 1×Leibovitz L-15 medium. The relative intensity of fluorescence was analyzed by using Becton Dickinson FACC scan (USA).

Measurement of intracellular ROS generation

The levels of intracellular ROS were detected by using the peroxide-sensitive fluorescent probe DCFH-DA (Beyotime, China) as a substrate according to the method from Sun's description (Sun *et al.,* 2015). Briefly, hemocytes from different groups were collected and incubated with 10 μmol L^{-1} DCFH-DA in Leibovitz L-15 medium (Gibco, USA)

Table 2 Names and sequences of the primers used in this study

Primer	Sequence
Oligo(dT) -adaptor	5'-GGCCACGCGTCGACTAGTAC(T)17-3'
***Es*Toll1**	
P1	CTCCTTCACCTGCCCTAACTGCT
P2	CTCCAGTTTGTATTGCTGTGCGAAA
***Es*Toll2**	
P3	CATTGATTGCTCTTACCTGAACCTA
P4	CTGCAAGCTATCTAGGCTCGTTAAG
***Es*MyD88**	
P5	GCAACAGGTGGACTTTGAGGAGTG
P6	CACGGACAAACCACGACTAAACC
***Es*LITAF**	
P7	GATTCCCTGCTGTATGGATGACT
P8	CTTTCTGGATGCGTTGTTTAACC
β-*Es*Actin	
P8	GCATCCACGAGACCACTTACA
P9	CTCCTGCTTGCTGATCCACATC

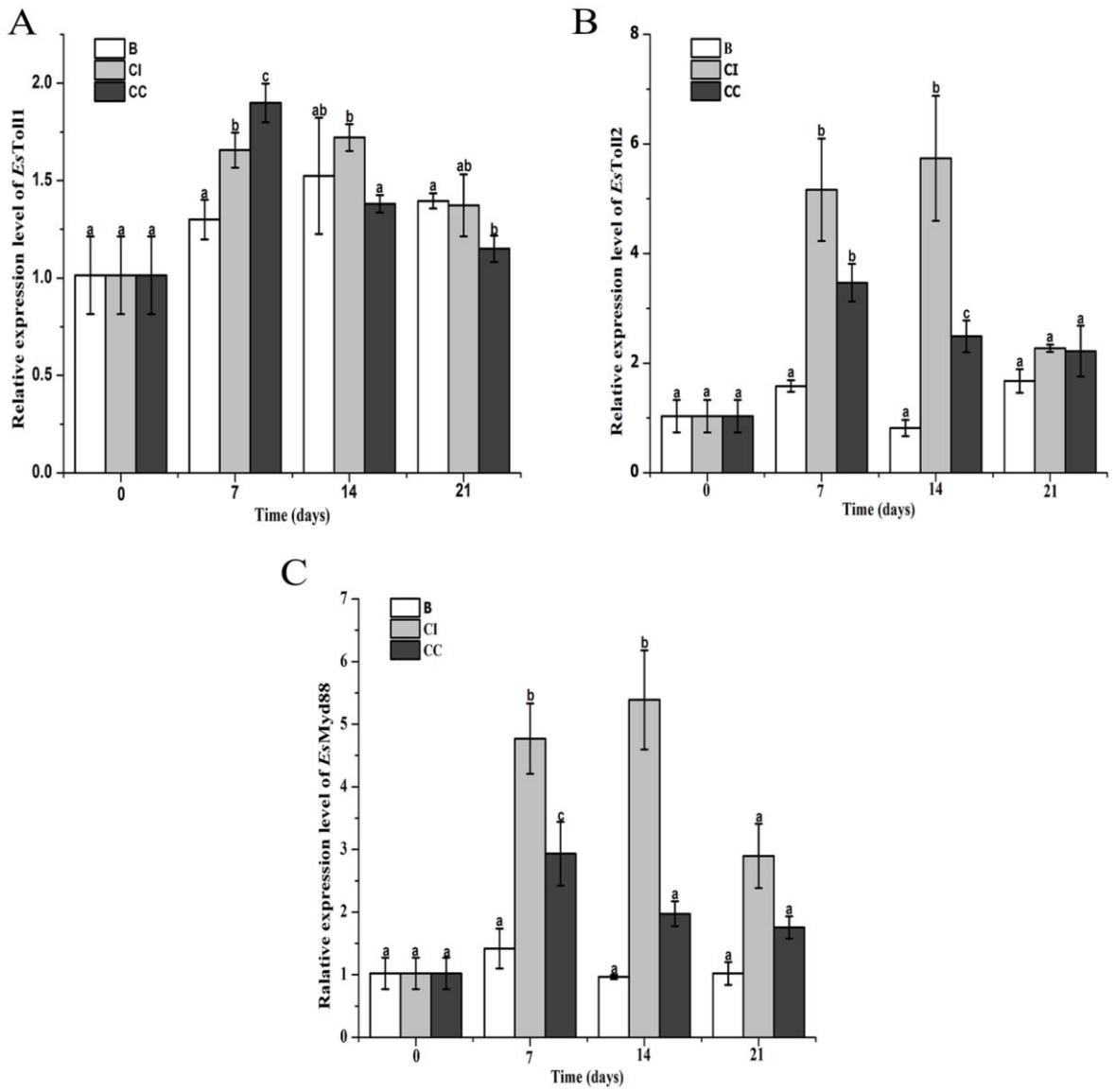

Fig. 3 Real-time PCR analysis of *Es*Toll1 (A), *Es*Toll2 (B) and *Es*MyD88 (C) mRNA expression in hepatopancreas of crabs after feeding CpG ODNs-supplement diet. A comparison of the level of mRNA (relative to actin mRNA) among different time points was performed using Student's t-test. Each bar represents the mean with the standard error (SD), n = 3. Different letters indicate significant difference from each other. The significant differences among the control and treated groups were subjected to one-way analysis of variance (one-way ANOVA) ($p < 0.05$).

(containing 0.27 g L^{-1} KCl, 10.1 g L^{-1} NaCl, 0.3 g L^{-1} $CaCl_2$, 2.0 g L^{-1} $MgCl_2$, 0.5 g L^{-1} $MgSO_4$) at room temperature for 20 min. The supernatant was discarded after washing for three times and the pellet was suspended with modified Leibovitz L-15 medium. The relative fluorescence intensity was analyzed using Becton Dickinson FACS scan (USA) and Lysis II software.

Statistical analysis

Statistical analysis was performed using SPSS 16.0. Data from three samples of each time-point were subjected to a one-way ANOVA. When overall differences were significant at less than 5 % level ($p < 0.05$), Duncan's test was used to compare the mean values between individual treatments.

Results

The mRNA expression of Es*Tolls (*Es*Toll1 and* Es*Toll2) and* Es*MyD88 in hepatopancreas after interval or continuous feeding with CpG ODNs*

*Es*Tolls and *Es*MyD88 were selected to detect the interval or continuous activation of CpG ODNs to the immune system of crabs. For *Es*Toll1, the mRNA expression in CI group was significantly upregulated ($p < 0.05$, 0.27-fold) at 7th day, and no difference was observed at 14th and 21st day than that in control group. In CC group, the expression level of *Es*Toll1 mRNA upregulated significantly at 7th day ($p < 0.05$) and deceased at 21st day ($p < 0.05$), which was 1.47-fold and 0.88-fold of that in control group, respectively (Fig. 3A).

The mRNA expression level of *Es*Toll2 in CI group was significantly higher than that in control group except the samples at the 21st day, which was 3.26-fold ($p < 0.05$) and 7.04-fold ($p < 0.05$) of that in control group at the 7 and 14th day, respectively. In CC group, the expression level of *Es*Toll2 mRNA increased significantly at the 7th day (1.19-fold, $p < 0.05$) and 14th day (2.05-fold, $p < 0.05$) in comparison to that in control group, while it was 0.57-fold ($p < 0.05$) lower than that in CI group at 14th day. There was no significant difference in the mRNA expression level of *Es*Toll2 among the three groups at 21st day (Fig. 3B).

*Es*MyD88 is considered as an important adapter protein in the TLR signaling pathway. After interval feeding of CpG ODNs, its mRNA expression level increased significantly at the 7th and 14th day, which was 2.36-fold and 4.58-fold higher ($p < 0.05$) than that in control group. After continuous feeding of CpG ODNs, the expression of *Es*MyD88 mRNA increased significantly ($p < 0.05$, 1.07-fold) at the 7th day and no significant difference was observed at 14th and 21st day comparing to the control group. Compared with the CC group, the expression level of mRNA was significantly higher at 7th day (0.63-fold, $p < 0.05$) and 14th day (1.73-fold, $p < 0.05$) in CI group (Fig. 3C).

*Alteration of Es*LITAF *mRNA after feeding with CpG ODNs diets*

The expression level of *Es*LITAF mRNA in hepatopancreas of crabs after the interval and continuous feeding of CpG ODNs was investigated. The expression of *Es*LITAF mRNA was significantly up-regulated from 7th to 21st day after intermittently feeding CpG ODNs diet, which was 2.57-fold ($p < 0.05$), 1.42-fold ($p < 0.05$) and 0.79-fold ($p < 0.05$) higher than that in control group, respectively. In CC group, its expression reached the maximum level (2.53-fold, $p < 0.05$) at the 7th day, while no significant difference was observed at the 14th and 21st day compared with the control group (Fig. 4).

The comparison of TNF-α protein level in plasma after feeding of CpG ODNs diets

The TNF-α protein level in plasma was determined using the fish TNF-α ELISA kit after the feeding with CpG ODNs-supplement diet for three weeks. Compared to the control group, TNF-α concentration in CI group was significantly up-regulated (0.60-fold, $p < 0.05$) at the 7th day, and then reached a peak (0.99-fold, $p < 0.05$) at the 14th day, while no significant difference was determined at 21st day. After continuous feeding, the concentration of TNF-α in CC group was up-regulated (0.22-fold, $p < 0.05$) at the 7th day, followed by a decrease at 14th day, and then abruptly increased (1.52-fold, $p < 0.05$) at the 21st day, which was 2.52-fold ($p < 0.05$) and 1.90-fold ($p < 0.05$) of that in control and CI group, respectively (Fig. 5).

The phagocytic rate of crab hemocytes after the feeding of CpG ODNs diets

The phagocytic rate of hemocytes was determined after the feeding of CpG ODNs diets. The phagocytosis rate of crab hemocytes kept in high level after interval feeding with of CpG ODNs diets, which was 1.77-fold ($p < 0.05$), 1.69-fold ($p < 0.05$) and 1.14-fold ($p < 0.05$) of that in control group

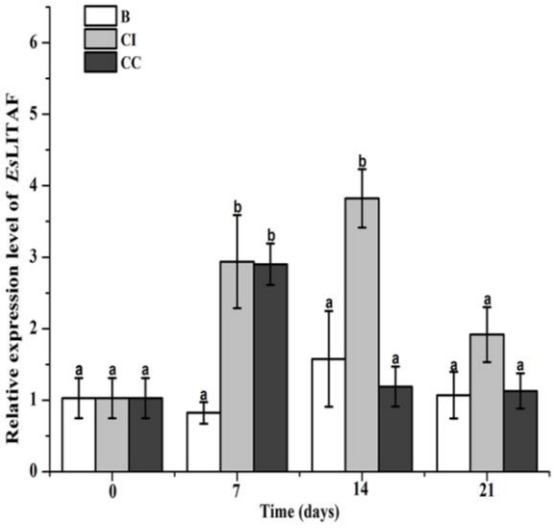

Fig. 4 Real-time PCR analysis of LPS-induced TNF-α factor *(Es*LITAF) mRNA expression of hepatopancreas after feeding the CpG ODNs at the 7th, 14th and 21st day in the three groups. Data presented as mean ± S.D. (n = 4). Different letters indicate significant differences from each other. The significant differences among the control and treated groups were subjected to one-way analysis of variance (one-way ANOVA) ($p < 0.05$).

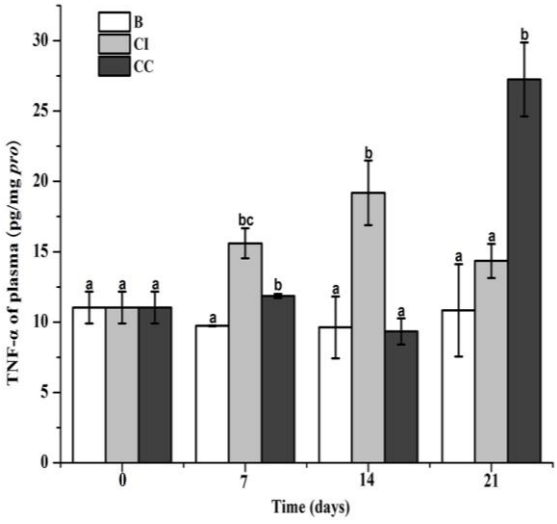

Fig. 5 Determination of plasma TNF-α in three groups after feeding CpG ODNs for 21 day using the commercially fish TNF-α ELISA kit. Each bar represents the mean with the standard error (SD), n = 4. Different letters indicate significant differences from each other. The significant differences among the control and treated groups were subjected to one-way analysis of variance (one-way ANOVA) ($p < 0.05$).

at 7th, 14th and 21st day, respectively. While in CC group, the phagocytosis rate was significantly upregulated at 7th ($p < 0.05$, 0.47-fold) and 14th ($p < 0.05$, 0.27-fold) day, and no difference was observed at 21st day compared with the control group. Meanwhile, the phagocytosis rate in CC group was 0.17-fold ($p < 0.05$) and 0.25-fold ($p < 0.05$) lower than that in CI group at 7th and 14th day, and there was no significant difference determined at 21st day between CI and CC groups (Fig. 6).

The production of reactive oxygen species (ROS) after continuous or interval CpG ODNs stimulation

The ROS level in hemocytes after continuous or interval CpG ODNs stimulation was recorded by the relative fluorescence intensity. ROS level in CI group was significantly up-regulated at the 14th day ($p < 0.05$, 1.69-fold), and decreased at the 21st day ($p < 0.05$, 0.52-fold) compared with that in the control group. And after continuous CpG ODNs stimulation, the ROS kept high level at the 7th and 14th day, and then significantly decreased at 21st day, which was 1.91-fold ($p < 0.05$), 2.30-fold ($p < 0.05$) and 0.41-fold ($p < 0.05$) of that in control group, respectively (Fig. 7).

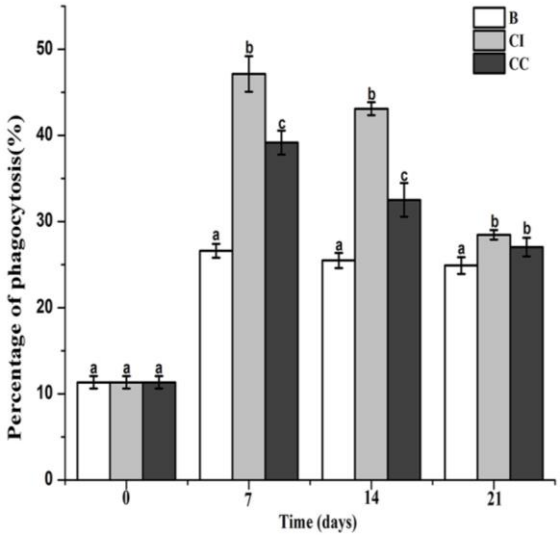

Fig. 6 Hemocytes phagocytotic activity were determination after feeding CpG ODNs for three weeks. The phagocytotic activity was represented by the percentage of Latexs Beads (marked with fluorescent yellow-green) phagocytosed by hemocytes measured by flow cytometer. Each bar represents the mean with the standard error (SD), n = 4. Different letters indicate significant differences from each other. The significant differences among the control and treated groups were subjected to one-way analysis of variance (one-way ANOVA) ($p < 0.05$).

Discussion

Immunostimulants are well known to improve the resistance of hosts to pathogens, but they are not absolutely safe and even seriously damage physiological processes of organisms after the long-term and continuous administration. It was found that CpG ODNs could lead to the disruption of normal lymphoid tissue and multifocal liver necrosis after administered daily to mice for three weeks (Heikenwalder *et al.,* 2004). CpG ODNs could cause severe mortality of murine when they were co-administered with sub-pathological does of LPS and D-galactosamine (Sparwasser, 1997). Nowadays, the short-term immunostimulatory effects of CpG ODNs have been investigated in aquaculture animals (Ian Bricknell, 2005; Ringø et al., 2011). However, little was known about its long-term immune effects in aquaculture animals. In the present study, the immune parameters were investigated after continuous or interval feeding with CpG ODNs-supplement diet to evaluate its long-term immune effects in Chinese mitten crabs.

As a classical type of PAMPs, CpG ODNs are recognized by conserved PRRs, such as TLR9 in mammals and TLR21 in chicken which were identified in the previous studies (Bauer et al., 2001; Keestra et al., 2010). In our previous studies, *Lv*Toll1 and *Lv*Toll3 were confirmed as the recognition receptors of CpG ODNs in shrimp *L*. *vannamei* (Sun *et al.,* 2014). Recently, *Es*Toll1 and *Es*Toll2 have also been cloned from *E*. *sinensis*, and *Es*Toll2 was found to induce a strong immune response than *Es*Toll1 after interval challenge with lipopolysaccharide, peptidoglycan and zymosan (Yu *et al*., 2013). In the present study, the mRNA expression level of *Es*Toll2 and *Es*Toll1 was significantly up-regulated after the feeding of CpG ODNs-supplement diet, suggesting that *Es*Toll2 and *Es*Toll1 might be potential receptor of CpG ODNs to activate the downstream signaling pathways. Similarly, *Es*Toll2 showed a relative higher expression level than *Es*Toll1, indicating that *Es*Toll2 might induce a strong response against CpG ODNs stimulation than *Es*Toll1. Moreover, the relative lower expression level of *Es*Toll2 mRNA in CC group was observed for the first two weeks than those in CI group. This was consistent with the previous observation, in which the immune tolerance of bacterial lipoprotein could be induced through downregulation of TLR2 expression (Wang *et al.,* 2002). The results suggested that successive CpG ODNs stimulation could reduce the recognition sensitivity of the immune cells by decreasing the expression levels of CpG ODNs receptors, which would further affect the activation of downstream immunostimulatory cascade induced by CpG ODN.

After interacting with human TLR9, CpG ODNs could activate the MyD88-dependent TLR signaling pathway (Kurlander and Pisetsky, 1996; Häcker *et al*., 2000; Mogensen, 2009). It has also been reported that the recognition of CpG DNA requires MyD88 in *Drosophila* (Schnare *et al*., 2000). Accumulating evidences have proved the existence and the activation of MyD88-dependent TLR pathway in crustacean. For instance, the MyD88-dependent signaling pathway in *Fenneropenaeus chinensis* and *Penaeus monodo*n was reported to involve in defense against WSSV infection (Wen *et al.,* 2013; Deepika *et al*., 2014). *Es*MyD88 was found to have a key function in innate

immune defense of *E. sinensis* (Li *et al*., 2013; Huang *et al*., 2014). In the present study, the mRNA transcripts of *Es*MyD88 were significantly increased after the feeding of CpG ODNs. It was also found in *L. vannamei* that the injection of CpG ODNs could enhance the expression of *Lv*MyD88, suggesting that the TLR pathways in crustacean could be activated by CpG ODNs (Zhang *et al*., 2012). Moreover, the expression level of *Es*MyD88 mRNA in CC group was significantly lower than that in CI group after two weeks' feeding, which was consistent with the tendency of *Es*Toll2 mRNA. Considering the existence of conserved TLR pathways in crustacean, it could be inferred that the continuous CpG ODNs-supplement diet feeding could weaken the activation of the downstream signal transduction by adaptor molecule MyD88 in crabs.

It is reported that after the binding of CpG ODNs, TLR pathways are activated to produce various pro-inflammatory cytokines and other immune factors (Stacey *et al*., 1996; Klinman, 2004; Aguilar and Rodriguez, 2007). LITAF is an important transcription factor in downstream of TLR signaling pathway, which is closely associated with transcriptional regulation of TNF-α and other cytokines dependent on MyD88 (Tang *et al.,* 2006; Li *et al.,* 2014). In the present study, the mRNA expression of *Es*LITAF in CI group kept a higher level compared with that in control group during the whole experiment, while it was dramatically decreased in CC group from 14th to 21st day. Moreover, the level of pro-inflammatory factor TNF-α in plasma was consistent with the change tendency of *Es*LITAF mRNA in both CI and CC feeding groups at 7th -14th day. These results indicated that continuous feeding of CpG ODNs might induce a suppression regulation to the downstream of TLR signaling pathway. Interestingly, the concentration of TNF-α in CC group was found significantly increased at the 21st day after CpG ODNs feeding. The serious tissue injury (multifocal liver necrosis and hemorrhagic ascites) was observed in mice after 20 days' daily injection of 60 μg CpG ODNs, which is closely related to the abundant production of pro-inflammatory cytokines TNF-α (Heikenwalder *et al*., 2004). The dramatic increasing of TNF-α at the 21st day in the present study might be related to the serious collapse and vacuolization observed in hepatopancreas of crabs after continuous CpG ODNs feeding (data not shown). These results further indicated that the repeated CpG ODNs stimulation could not only reduce the activation of TLR signaling pathway, but also suppress downstream transcription factors expression and pro-inflammatory cytokine production, and even lead to disorder of cytokine production after administration of three weeks.

Lots of evidences have proved that CpG ODNs could induce phagocytic activity and respiratory burst activities in various aquatic animals (Tassakka and Sakai, 2002; Meng *et al*., 2003; Sun *et al.,* 2013a). Phagocytosis is considered to be one of the powerful cellular responses and plays significant roles for the invertebrate lacking adaptive immune system (Allen and Aderem, 1996). In the present study, the phagocytic activity of crab hemocytes was

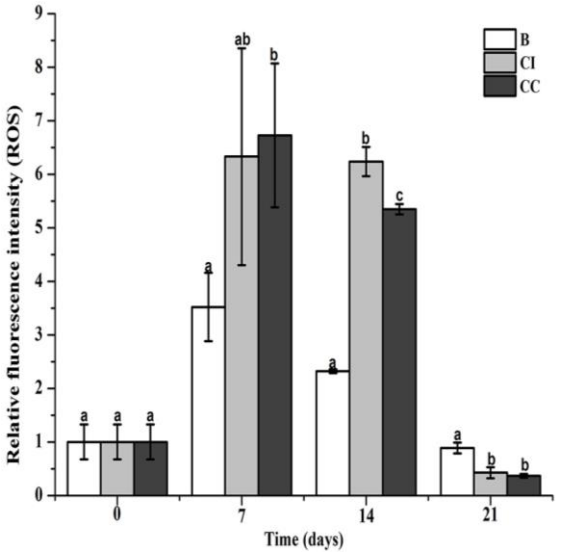

Fig. 7 Changes of hemocytes reactive oxygen (ROS) after three CpG ODNs feeding methods. The ROS values were represented by the mean fluorescence intensity of hemoncytes measured by flow cytometer. Each bar represents the mean with the standard error (SD), n = 4. Different letters indicate significant differences from each other. The significant differences among the control and treated groups were subjected to one-way analysis of variance (one-way ANOVA) ($p < 0.05$).

dramatically enhanced after 7 - 14 days feeding of CpG ODNs, similar to the observation in shrimps (Sun *et al*., 2013). Moreover, the phagocytic ability of hemocytes in CC group was significantly lower than that in CI group. The neutrophils of CpG ODN-treated mice showed the elevated phagocytic activities against bacteria and the increased production of reactive oxygen species (ROS) through the up-regulation of phagocytic receptors (Weighardt *et al.,* 2000). Moreover, various TLRs differentially promote phagocytosis through induction of a phagocytic gene program and TLR9 being the strongest mediator of this process by MyD88 in mice (Doyle *et al.,* 2004). These results indicated that the declined phagocytic activity of hemocyte in continuous CpG ODN-fed crabs in the present study may be caused by the suppression of *Es*Tolls as well as phagocytic receptors. ROS is widely considered as an indicator of phagocytic activity, and it contributes to the increased capability to kill bacteria (Meng *et al*., 2003; Waris *et al.,* 2005). In the present study, the ROS level of both CI and CC group reached the lowest level at 21st day after significant increase at 7th and 14th day, and the ROS level in interval feeding crabs was significantly higher than that in continuous feeding crabs at 14th day. These results suggested that continuous CpG ODNs stimulation could suppress the efficacy of the hemocyte phagocytosis as well as the ROS generation in comparison with interval CpG ODNs stimulation.

In conclusion, continuous CpG ODNs-supplement diet feeding could reduce the activation of *Es*Tolls and adaptor protein *Es*MyD88 as well as its downstream production of pro-inflammatory cytokine and hemocyte phagocytosis and ROS generation. Compared with the continuous feeding strategy, the interval CpG ODNs feeding could effectively activate immune response at the first two weeks although the suppression was also observed at the last week, indicating that the interval feeding strategies needs further optimization. We hope that this information can provide theoretical guidance for the application of CpG ODNs in aquaculture as an immunostimulant.

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