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Effects of palytoxins extracted from *Ostreopsis ovata* on the oxidative stress and immune responses in Pacific white shrimp *Litopenaeus vannamei*

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ABSTRACT

Palytoxins (PLTXs) are a group of complex and poisonous marine natural products that are toxic to marine life and even human beings. In the present study, the oxidative stress and immune response in the hepatopancreas and gills of *Litopenaeus vannamei* were assessed for 72 h after injection with PLTX extracts. Chemical and physiological parameters, e.g., the respiratory burst (O_2^-), activities of antioxidant enzymes, oxidative damage to lipids, carbonylation of proteins, and immune gene mRNA expression levels, were analysed. The results showed that the PLTX extract was not fatal to the shrimp but could reduce their mobility. The O_2^- levels in the gills gradually increased after exposure to PLTX extracts and were significantly higher than those in the control from 6 to 72 h. The malondialdehyde content, lipid peroxidation, protein carbonyl levels, and total antioxidant capacity in the gills all peaked at 12 h. At the same time, the gills were loosely connected, there was a clear disintegration of the epithelial tissue, and the stratum corneum disappeared after 12 h. In addition, compared to those in the control group, the PLTX extract treatment increased the O_2^- content, malondialdehyde content, lipid peroxidation, and protein carbonyl levels from 12 to 72 h, 24–48 h, 12–24 h, and 12–72 h after injection in the hepatopancreas of the shrimp, respectively. Both the *Crustin* and *Toll* gene expression levels significantly increased in the hepatopancreas compared to those in the control 6–72 h after injection of the toxin. In parallel, the expression levels of the manganese superoxide dismutase gene gradually decreased from 6 to 48 h and returned to normal levels after 72 h. Interestingly, the total antioxidant capacity also significantly increased compared to that in the control from 6 to 72 h. Our results indicate that although PLTX extracts cause lipid peroxidation and carbonylation of proteins in hepatopancreatic cells, leading to their damage, they did not cause a decrease in the total antioxidant capacity of the hepatopancreas.

1. Introduction

Palytoxins (PLTXs) are a group of complex and toxic non-protein substances and are mainly produced by corals in the genus *Palythoa* and dinoflagellates in the genus *Ostreopsis* [1,2]. To date, twenty analogues of PLTX-like compounds have been discovered, including the structurally related palytoxins, ostreocin, ovatoxins, and isobaric palytoxin [3]. The main biological binding target of PLTXs is the Na^+-/K^+-ATPase pump, a transmembrane enzyme that is involved in the maintenance of transmembrane ionic gradients in animal cells [4,5]. PLTXs can be accumulated in some organisms, e.g., crustaceans, molluscs, animals, fish, and echinoderms, through the food web [6–8]. The ingestion of seafood from areas contaminated with PLTXs may cause physical discomfort and even death of human beings [2]. Due to the high toxicity of PLTXs, according to the European Food Safety Authority, the regulatory limit for PLTX contamination in seafood is 30 μg kg^{-1} in shellfish meat [9].

PLTXs are present in fish, crustaceans, molluscs and echinoderms [6]. The main vectors of PLTXs are crabs (*Demania reynaudii*), parrotfish (*Scarus ovifrons*), goldspot herring (*Herklotsichthys quadrimaculatus*), and serranid fish (*Epinephelus* sp.) [10]. Marine organisms, especially invertebrates, are heavily contaminated by PLTXs. For example, octopi can be found stranded on the beach, sea urchins lose all their spines, and the arms of sea stars fold after exposure to PLTXs [9,11]. In 1995,

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the benthic dinoflagellate *Ostreopsis* was discovered to be the real producers of PLTXs [12–15]. The species *Ostreopsis* is often attached on the surface of seaweed, coral, sand, stones, and invertebrates. When *Ostreopsis* is blooming, they aggregate and formed a film, and the film becomes distributed in the seawater through waves or currents [11,16]. *Ostreopsis* blooms have caused massive deaths of invertebrates in the Mediterranean and in areas of the Tasman Sea around New Zealand in recent years [11,17]. It was proven that there is a strong correlation between *Ostreopsis* blooms and the loss of spines or mortality of sea urchins [11].

Despite the obvious biological damage, little is known about the mechanism of oxidative stress and immune response in marine organisms after their exposure to PLTXs. Some studies have reported that the PLTXs produced by *O. ovata* have an effect on the immunology, histology, and oxidation system of the mussel *Mytilus galloprovincialis* [18–20]. However, it is still unclear about the oxidative stress and immune response in marine organisms after their exposure to PLTXs.

The Pacific white shrimp *Litopenaeus vannamei* inhabits near-shore, sandy bottomed, sea waters and is the most commonly cultured shrimp species worldwide, especially in Southeast Asia. During a large *O. ovata* bloom high concentrations of PLTXs can be found in the water. The effect of PLTXs has great influence on marine animals (including crustaceans). To date, less research has been conducted on the direct immune response and oxidative stress response of shrimp to PLTXs. In this study, the toxicity activities of PLTXs extracted from *O. ovata* on the Pacific white shrimp *L. vannamei*, the most common marine-cultured species, were evaluated. The primary objectives of this study were to preliminarily investigate the effects of PLTXs on oxidative stress and immune responses in the Pacific white shrimp *L. vannamei*.

2. Materials and methods

2.1. Algae culture and toxin extraction

O. ovata cultures were initially established by picking individual vegetative cells from the surface of seaweed (at an approximate 1–3 m depth below chart datum) on the east coast of Weizhou Island, China (20° 02′ 11.2" N, 109° 05′ 04.0" E). *O. ovata* was cultured in L1 medium at a salinity of 30‰ under a 12:12 h light: dark cycle (100 μmol photons $m^{-2}s^{-1}$) at 25 °C. *O. ovata* was kept at the "Marine Algae Culture Collection at the Research Centre of Harmful Algae and Marine Biology of Jinan University".

O. ovata was cultured in several 1000-mL glass conical flasks. Algal cells in the stationary phase were collected by membrane filtering (pore size, 10 μm, and 47 mm in diameter). Cells present in each flask were estimated by counting the cells in three 0.1-mL aliquots per flask. The algal lysates were prepared on ice using an ultrasonic pulse with a wave-strength of 20% for 3 min and an interval of 0.2 s. The lysate samples were observed under a light microscope to ensure complete cell lysis. Lysates were centrifuged (4530 × *g* for 3 min at 4 °C). Next, 15 mL of a methanol/water (1:1, v/v) solution was added to the pellet, and then the mixture was centrifuged at 3000 × *g* for 10 min, after which the pellet was washed twice with 15 mL of methanol/water (1:1, v/v). The extracts were combined and transferred to a 10-kDa ultrafiltration centrifuge tube. The supernatants were dried by aeration with pure $N_2$and stored in a refrigerator at −20 °C for use. The dried samples were reconstituted to provide a toxin extract solution (1 mg ml^{-1} with 1% Tween-60 in saline).

2.2. Preparation and analysis of O. ovata extracts

The toxins were extracted by adding 5 mL of methanol/water (1:1, v/v), transferred to a 10-kDa ultrafiltration centrifuge tube, and then filtered through a 0.22-μm filter. The filtrate was collected and directly analysed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

The liquid chromatography conditions were as follows: an Agilent 1100 (US) high-performance liquid chromatography system equipped with a Phenomenex Gemini C18 column (150 mm × 2.00 mm, 3 μm) was used. Mobile phase A was ultrapure water, and mobile phase B was 100% acetonitrile. The elution method consisted of 0–2 min, 20–100% A; 2–8 min, 100% A; 8–8.1 min, 100–20% A; and 8.1–18 min, 20% A. The flow rate was 0.2 mL min^{-1}, the column temperature was 20 °C, and the injection volume 20 μL.

The mass spectrometry conditions were as follows: an API4000 QTRAP mass spectrometer (AB Sciex, USA) equipped with an electrospray ionisation ion source was used. The detection mode was multiple reaction monitoring. Nitrogen was atomising gas and the drying gas. The quantitative transition ion pair was selected as 1332.1/327.1, the qualitative transition ion pair was selected as 1341.1/327.1, and 1352.1/327.1 was used as the reference.

2.3. Shrimp and culture conditions

Healthy juvenile *L. vannamei* samples, with an average weight of 4.8 ± 0.2 g, were randomly collected from a local hatchery and reared in a semi-intensive culture pond in Shenzhen, China. The shrimp were cultured in filtered, aerated seawater (salinity: 30‰, pH: 8.2, and dissolved oxygen: 6.0 ± 0.5 mg/L) at 30 °C for one week before processing and fed daily at a ratio of 5% body weight with formulated pellet feed (Haida Feed, Jieyang, China).

2.4. Palytoxin exposure and sampling

After adaptation, 180 healthy shrimp at the moulting stage were randomly divided into two groups: a control group and a toxin group, with each group having three replicate tanks (30 shrimp per tank). For the control group, shrimp received 40 μL of sterile 0.86% saline solution in each of their second abdominal segments. For the PLTX-injected group, shrimp were injected individually in their second abdominal segments with 40 μL of the toxin solution. The toxin group and control group shrimps were returned to the PVC tanks containing the aerated seawater and fed at 30 °C, as described above. The hepatopancreas and gills from three shrimp in each treatment group were randomly sampled at 0, 6, 12, 24, 48, and 72 h post-injection, flash-frozen in liquid nitrogen, and stored at −80 °C for biochemical assays and gene expression analysis.

2.5. Histological analysis

The hepatopancreases from shrimp in each treatment group (i.e., the toxin group and the control group) were randomly sampled at 0, 6, 12, 24, 48, and 72 h post-injection and fixed in 4% paraformaldehyde for 2 h. After rinsing with water for 8 h, the tissues were dehydrated in a series of ethanol solutions (70%, 80%, 90%, and 100%), cleared with xylene, embedded in paraffin, and cut with a microtome (Leica, RM2016, Germany) to yield slices of 4-mm thickness. After hematoxylin-eosin (H&E) staining, the stained sections were examined and photographed under an Olympus X61, microscope (Olympus, Tokyo, Japan).

2.6. Biochemical analysis

The hepatopancreas and gills, obtained from shrimp taken at the same time point from each of two different treatment groups, were homogenised with a sterile 0.86% saline solution at a ratio of 10% (w:v). The homogenate was centrifuged at 1500 × *g* for 10 min at 4 °C, and the supernatant was immediately collected for the measurement of biochemical parameters, including the levels of ROS ($\cdot O_2^-$ generation capacity), malondialdehyde (MDA), lipid peroxidation (LPO), and protein carbonyl (PC). The total antioxidant capacity (T-AOC) and total nitric oxide synthase (T-NOS) activity using commercial test kits

(Jiancheng, Ltd., Nanjing, China) following the manufacturer's instructions and read on a microplate reader (Bio-Rad, California, USA). The total protein concentration in the tissue supernatant was measured using a Coomassie Brilliant Blue Assay Kit (Jiancheng, Ltd, Nanjing, China) according to the manufacturer's protocol. All assays were performed using triplicate samples.

The $\cdot O_2^-$ generation capacity was measured spectrophotometrically using xanthine/xanthine oxidase as the $\cdot O_2^-$ generator, and the product absorbance were read spectrophotometrically at 550 nm. The MDA and LPO contents were measured spectrophotometrically with the thiobarbituric acid method, and the product absorbances were read spectrophotometrically at 586 nm and 532 nm, respectively. The PC content was measured spectrophotometrically at 370 nm and expressed as nmol/mg prot. The T-AOC activity, one unit of which was defined as the amount of enzyme per mg tissue protein required at every minute to increase the absorbance by 0.01 at 37 °C, was measured spectrophotometrically at 520 nm. The T-NOS activity was obtained by measuring the absorbance of a catalysation reaction of L-arginine and O_2 at 530 nm. One unit of T-NOS activity was defined as the amount of T-NOS producing 1 nmol NO min $^{-1}$.

2.7. Gene expression analysis

Total RNA was extracted from the hepatopancreas and gills using Trizol Reagent (Invitrogen, Carlsbad, USA). Contaminating DNA was removed from the RNA samples using RQ1 RNase-Free DNase (Promega, Madison, USA). RNA samples were analysed by electrophoresis on 1.0% agarose gels, and the OD_{260} and OD_{280} values were determined. All OD_{260}/OD_{280} ratios were between 1.8 and 2.0. Total RNA (8 μg) was reverse transcribed into first strand cDNA using M-MLV reverse transcriptase (Promega, Madison, USA).

The *Crustin*, *Toll*, and cytoplasmic manganese superoxide dismutase (*cMnSOD*) gene expression levels were assessed by real-time quantitative RT-qPCR using a SYBR PremixEx Taq II kit (TaKaRa, Kyoto, Japan) and ABI PRISM 7500 sequence detection system (Applied Biosystems, California, USA). The β-actin gene from *L. vannamei* was used as an internal control to confirm successful reverse transcription and to provide a means to calibrate each cDNA template. The RT-qPCR reaction was carried out in a total volume of 20 μL, containing 10 μL SYBR® Premix Ex Taq™ II (2 ×) (TaKaRa, Kyoto, Japan), 2 μL of a 1:5 dilution of cDNA, 0.8 μL each of the 10 μmol/L forward and reverse primers (Table 1), 0.4 μL ROX reference dye II (50 ×), and 6 μL of DEPC-treated water. The PCR programme was 95 °C for 30 s, then 40 cycles of 95 °C for 5 s, and 60 °C for 34 s, followed by 1 cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. DEPC-treated water was used as a replacement for the template as a negative control. RT-qPCR data from three replicate samples were analysed with the ABI 7300 system SDS software (Applied Biosystems, California, USA) to estimate the transcript copy numbers for each sample. The relative gene expression levels were calculated by the $2^{-\triangle\triangle CT}$ comparative C_T method [21]. There were three replicates in each tank, and three samples were analysed for each condition.

Table 1
Sequence of primers used in this study.

Primer name	Sequence (5′-3′)	GenBank accession number
Crus-F	CCACGAACCAGAGACACCTG	AY486426
Crus-R	CGAGGCCAGCACACTTGTAG	
Toll-F	TGGTCCTCAGCCTTGGAGAT	DQ923424
Toll-R	CTCCATCACTGGCGCACTTA	
cMnSOD-F	CCGTGCAGATTACGTGAAGG	DQ005531
cMnSOD-R	GTCGCCACGAGAAGTCAATG	
β-actin-F	GCCCTGTTCCAGCCCTCATT	AF300705
β-actin-R	ACGGATGTCCACGTCGCACT	

2.8. Statistical analysis

All data are expressed as the mean ± SE. Statistical analysis was performed using SPSS software (Ver 18.0). Statistical significance was determined using a one-way ANOVA and a post hoc Duncan multiple range tests. Significance was set at $P < 0.05$.

3. Results

3.1. Qualitative and quantitative analysis of PLTXs

A standard solution of the PLTX standard (concentration of 5 mg L^{-1}) was subjected to a full scan in positive ion mode to determine the precursor ion, and then a second mass spectrometry scan was performed to optimise the collision energy and determine the production. The quantitative transition ion pair was determined to be 1332.1/327.1, and the qualitative transition ion pair was 1341.1/327.1. According to the LC-MS/MS analysis, the PLTX standard had a retention time of 6.89 min (Fig. 1). Solutions of gradient concentrations of the PLTX standard were analysed by LC-MS/MS to obtain a standard curve. The regression equation and the coefficient of determination were Y = 105286X-10542 and R^2 = 0.9945, respectively. Y is the peak area, and X is the standard sample concentration (unit: mg·L^{-1}). The signal-to-noise ratio (S/N) was 13.7, the limit of detection of the PLTXs was 0.01 mg L^{-1}, and the linear range was 0.01–5 mg L^{-1}. The average recovery of the experiment was estimated to be approximately 63% (n = 3) by the spiking method (addition of 0.5 mg·L-1 standard), which detected the same amount of toxin, and the results were acceptable. The presence of PLTX was demonstrated in the analysed samples by peaks eluting at the same retention times as that of the PLTX standard (6.89 min). Quantitative analyses indicated a total toxin content of the toxin extract of 0.3588 μg ml^{-1} PLTXs (0.196 pg·$cell^{-1}$). In addition, the presence and biological toxicity of the extract was confirmed to be same as that of PLTXs by mouse bioassay and hemolysis neutralization analysis [22]. The *O. ovata* extract solution is represented as “PLTX extract” in the article.

3.2. Toxicity and behaviour

All the *L. vannamei* were still alive after injection with PLTX extracts at 0.196 pg·$cell^{-1}$ at 72 h; however, their swim ability was significantly affected by the toxin injection. In the first 6 h of treatment, the shrimp did not move and remained stationary at the bottom of the tank; their swim ability improved afterwards, but the ability was still less than that in the control group.

3.3. Structural changes of the gill and hepatopancreas tissues

The tissue structures of the gills and hepatopancreas of *L. vannamei* 72 h after injection of the toxin extracts were checked under a microscope. Pathological lesions were found in the shrimp injected with the PLTX extracts, and the hepatopancreatic lobes collapsed in the treatment group after 48–72 h. Twenty-four hours after injection of the toxin, the number of blasenzellen cells (B-cells) increased, their vacuoles expanded, and the cells then collapsed; the number of embryonic cells (E-cells) decreased in the hepatopancreas tubules during this period. Injection of the toxin extracts showed a clear disruption of gill histology in the shrimp. The gills were loosely connected, there was a clear disintegration of the epithelial tissue, and the stratum corneum disappeared after 12 h but were restored to their normal structure after 24 h (Fig. 2).

3.4. Oxidative stress parameter changes

After injection of the PLTX extracts, the O_2^- generation capacity in the gills gradually increased with time and was significantly higher

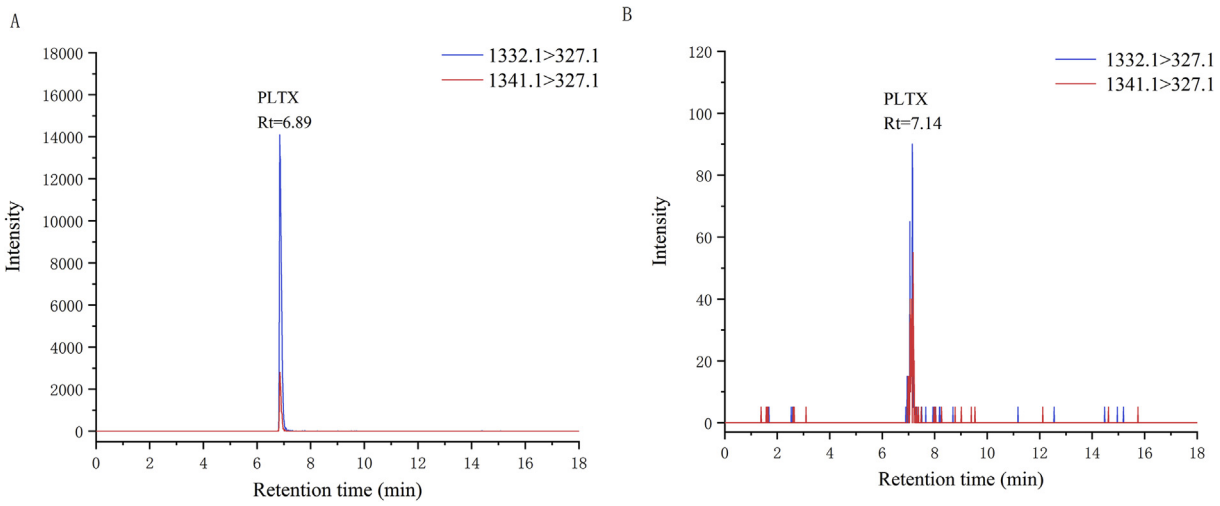

Fig. 1. LC-MS/MS chromatographs of PLTX. A: PLTX standard; B: toxin extract.

than that in the control from 6 to 72 h ($P < 0.05$). The LPO and MDA levels in the gills both peaked at 12 h, after which the MDA levels decreased rapidly, becoming significantly lower than the control from 24 h to 72 h. The PC levels were lower than those in the control from 6 to 72 h, except at 12 h. The changes in oxidative stress in the shrimp hepatopancreas after injection of the toxin extracts were different from the changes observed in the gills. In the hepatopancreas, the O_2^- generation capacity decreased significantly after 6 h but then increased and was higher than that in the control group from 12 to 72 h and peaked at 72 h; the LPO levels were significantly higher than those in

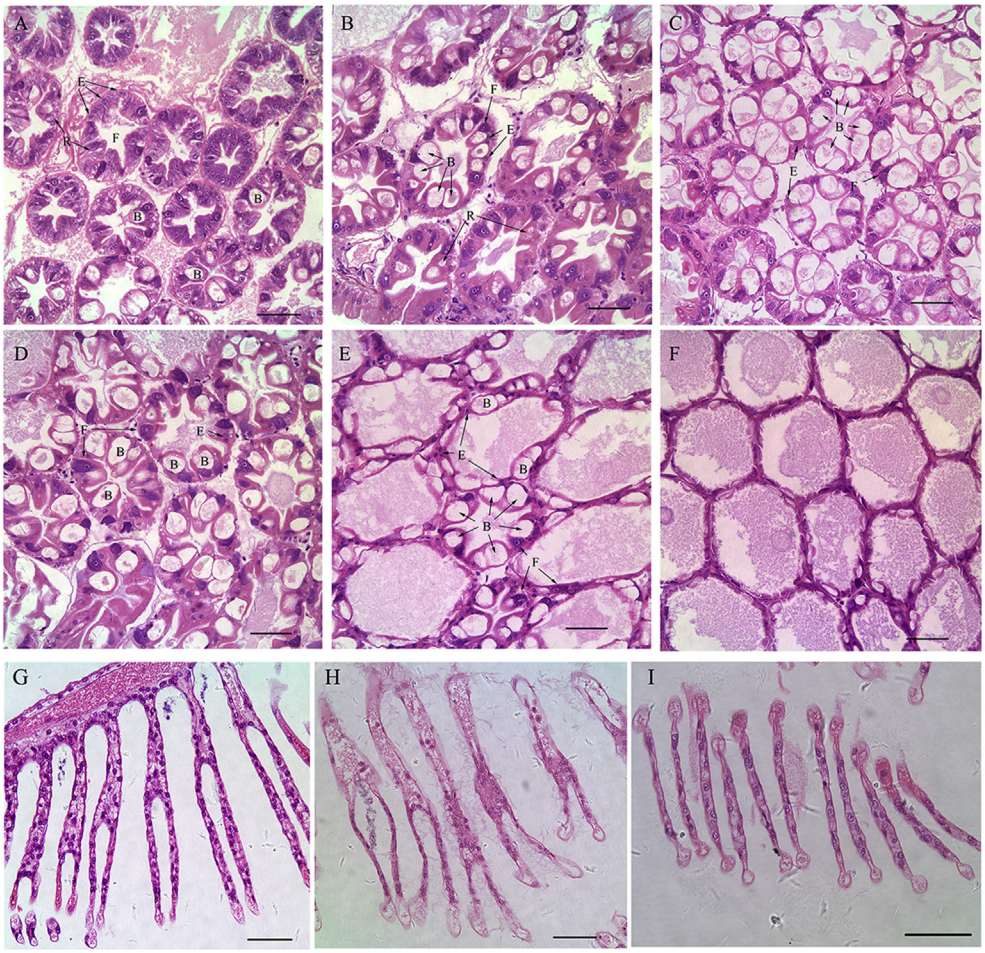

Fig. 2. Tissue structures of the gill and hepatopancreas of *L. vannamei* 72 h after injection of the toxin. **(A)** The hepatopancreas of the control; **(B–F)** changes in hepatopancreas 6 h, 12 h, 24 h, 48 h, and 72 h after injecting the toxin into shrimp; **(G)** gills of the control; **(H)** gills of shrimp 12 h after injection with the toxin; **(I)** The gills of shrimp 24 h after injection with the toxin at 24 h. Scale bar = 50 μm. B = B-cells (Blasenzellen cells) with their large apical secretory vacuoles; E = E-cells (Embryonic cells); R = R-cells (Restzellen cells); F = F-cells (Fibrillenzellen cells).

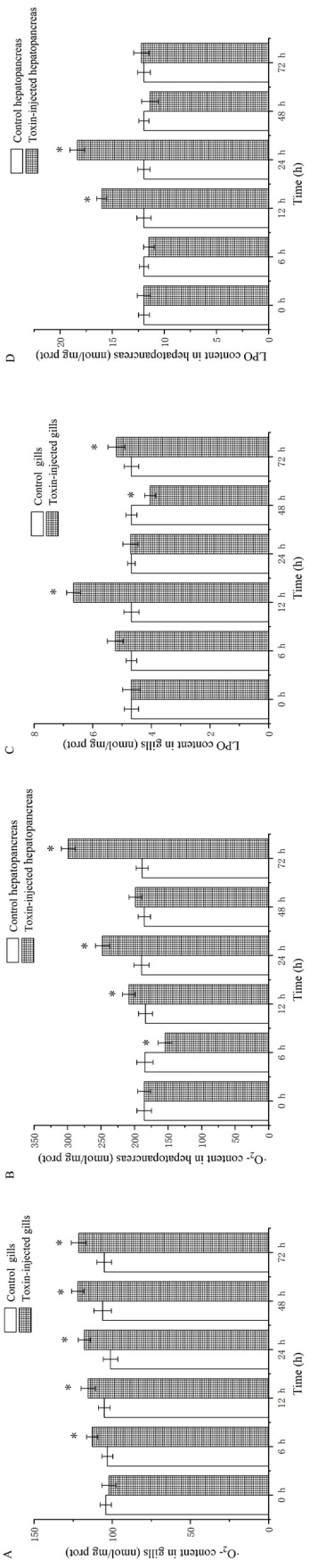

Fig. 3. Oxidative stress responses in *L. vannamei* gills and hepatopancreas at different time intervals after injection of the PLTX extract. (**A-B**) Superoxide anion ($\cdot O_2^-$) generation capacity; (**C-D**) LPO content; (**E-F**) MDA content; (**G-H**) PC content. Vertical bars represent the mean ± SE (N = 3). * indicates a significant difference ($P < 0.05$) compared with the control.

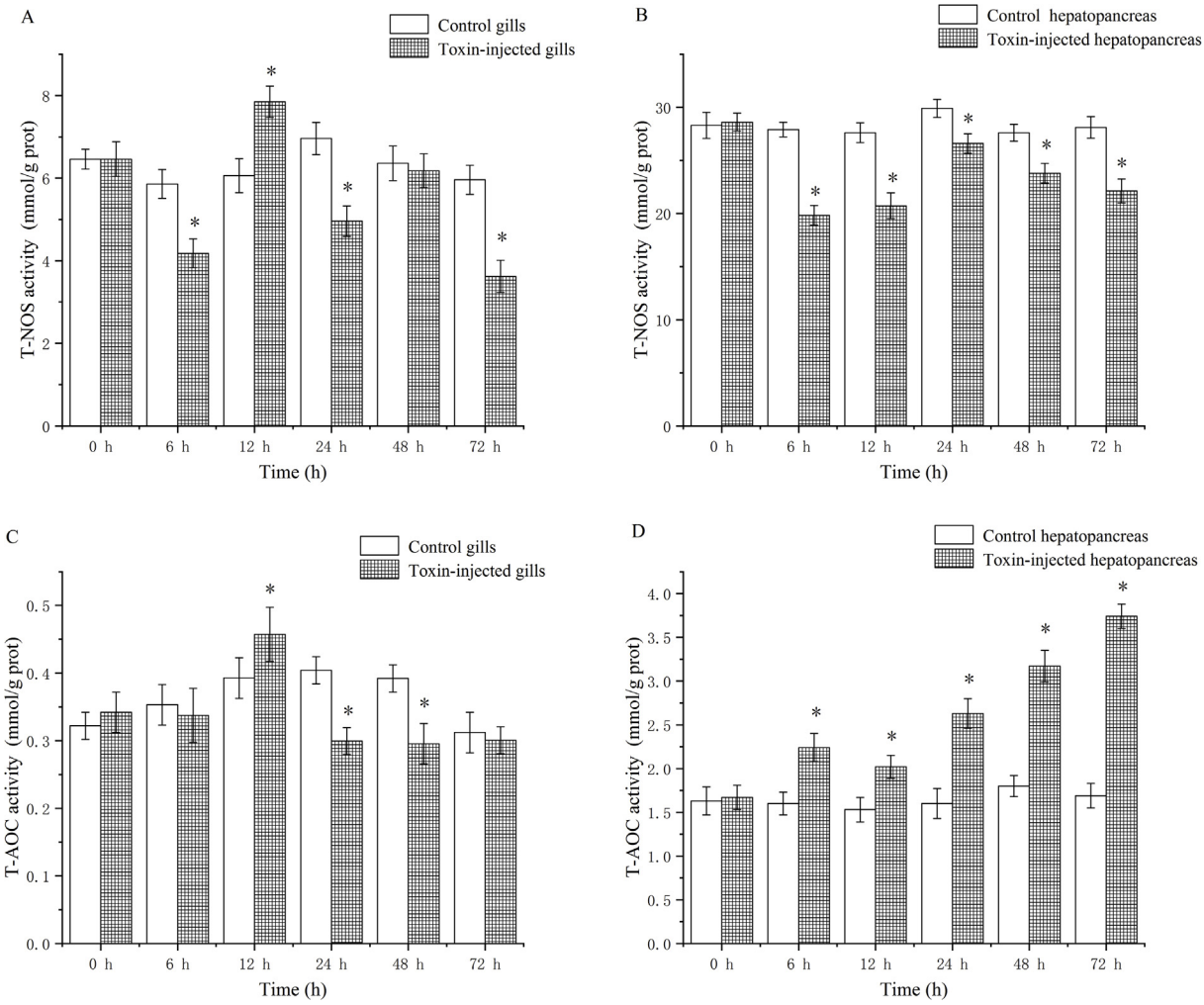

Fig. 4. Immune biochemical parameters in *L. vannamei* gills and hepatopancreas at different time intervals after injection of the PLTX extract. (**A-B**) T-NOS activity; (**C-D**) T-AOC activity. Vertical bars represent the mean ± SE ($N = 3$). * indicates a significant difference ($P < 0.05$) compared with the control.

the control from 12 to 24 h; the MDA levels were significantly higher than those in the control group from 12 h to 48 h; and the PC levels were significantly lower after 6 h but significantly increased from 12 to 72 h (Fig. 3).

3.5. Immune biochemical parameters

After the shrimp were injected with the PLTX extracts, the T-NOS content in the gills showed some degree of fluctuation, while there was a clear and significant reduction in the T-NOS content in the hepatopancreas 6–72 h after toxin injection. The T-AOC levels in the gills in the experimental group increased significantly after 12 h and then decreased from 24 to 48 h. In the hepatopancreas, the T-AOC content increased compared to the control at 6 h–72 h (Fig. 4).

3.6. Changes in immune gene expression level

Compared to those in the control group, after the injection of PLTX extracts, the expression levels of the *Crustin* gene in the gills increased significantly after 6 h and then gradually decreased from 12 to 72 h. In contrast, the *Crustin* gene expression levels increased continuously in the hepatopancreas 6–48 h after toxin injection and then declined after 72 h but were still significantly higher than those in the control group. The *Toll* gene expression levels in the gills significantly decreased from 6 to 48 h after toxin injection but then showed a marked and significant increase 72 h after injection. The *Toll* gene expression levels in the hepatopancreas of the injected shrimp were significantly higher than those in the control group from 6 to 72 h. The *cMnSOD* gene expression levels in the gills were significantly lower than those in the control at 6, 24, and 72 h after toxin injection. The *cMnSOD* gene expression levels in the hepatopancreas significantly decreased 6–48 h after toxin injection but then returned to normal levels 72 h after injection (Fig. 5).

4. Discussion

PLTXs have a long polyhydroxylated and partially unsaturated aliphatic backbone [23]. The plasmalemma Na^+-K^+-ATPase pump is known to be a receptor for PLTXs. Following binding, the pump is essentially converted into an ion channel, resulting in K^+ efflux, Na^+ influx, membrane depolarisation, and osmoregulatory imbalances [21,24]. The Na^+-K^+-ATPase is mainly used to maintain the cell's osmotic balance and is essential for cell function [25]. The changes in ion fluxes are the immediate effects of PLTXs in all mammalian cells and result in cell death. Previous studies have shown that PLTXs induce the lysis of bovine aortic endothelial cells and induce primary necrosis and secondary apoptosis in Caco-2 cells [26,27]. PLTXs have been detected in crustaceans and were especially higher in tissues, such as gills, viscera, and crab eggs [28,29]. However, the interaction between PLTXs and shrimp cells was still unknown. Our results indicated that PLTX extracts caused oxidative stress in shrimp as well as damage to the hepatopancreas with the progressive disintegration of cells and the formation of vacuoles.

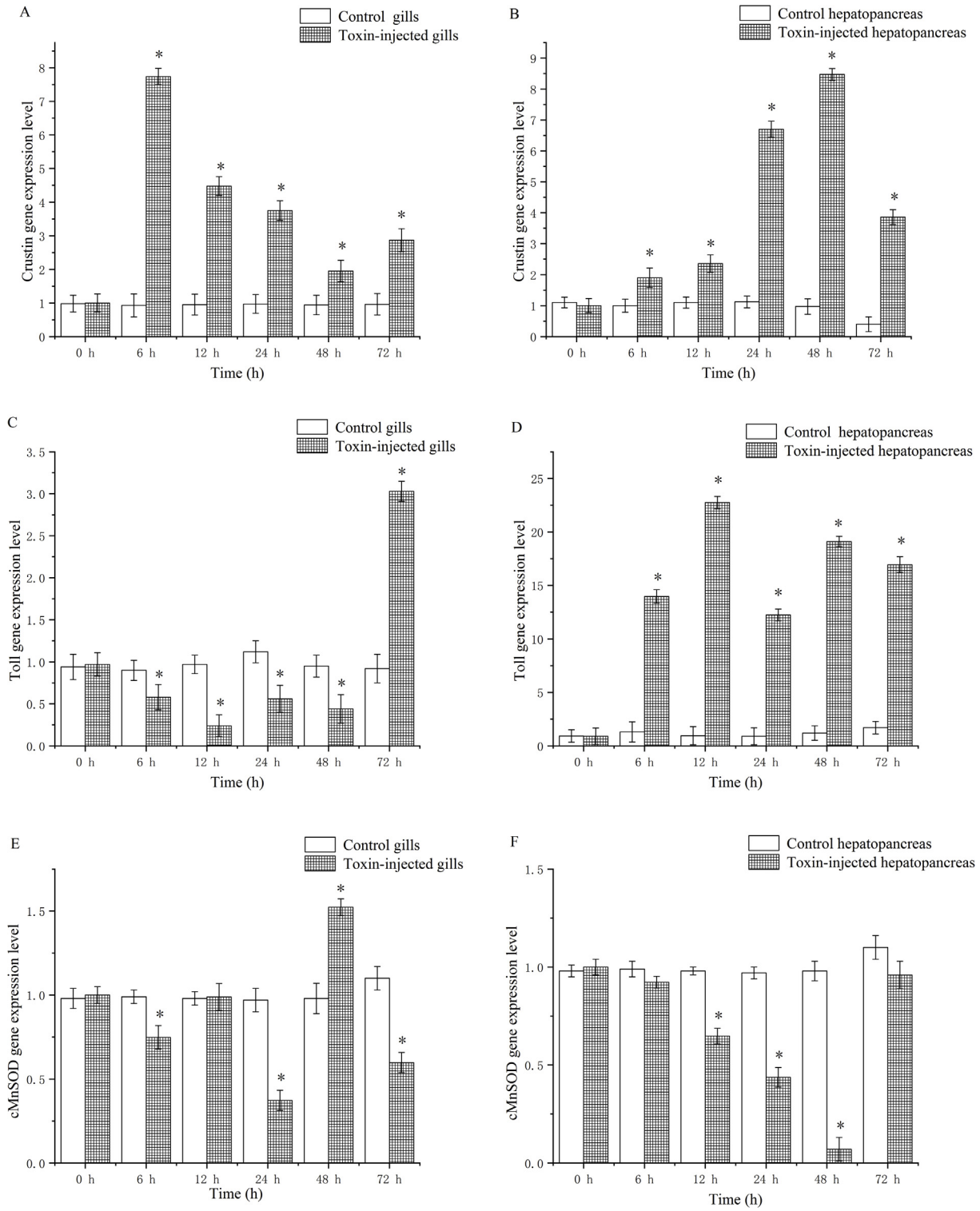

Fig. 5. Changes in the expression of immune-related genes in *L. vannamei* gills and hepatopancreas at different time intervals after injection of the PLTX extract. Vertical bars represent the mean ± SE ($N = 3$). * indicates a significant difference ($P < 0.05$) compared with the control. (**A-B**) *Crustin*; (**C-D**) *Toll*; (**E-F**) *cMnSOD*.

Superoxide anion production (respiratory burst) is a useful indirect indicator for the response capacity of pathogens in evaluating shrimp immune status [30]. The O_2^-, or singlet oxygen radical, is the first oxygen radical generated by organisms after an infection, from which other ROS and reactive nitrogen species are then derived [31,32]. Free radicals attack the lipid membrane to initiate oxidative stress and cause lipid peroxidation [31], and the peroxidation of polyunsaturated fatty acids in cell membranes then leads to cell damage. In this study, the levels of O_2^- in the hepatopancreas were significantly reduced 6 h after the injection of the PLTX extracts, while the in vivo antioxidant mechanism was maintained at its normal physiological level. However, the O_2^- levels in the hepatopancreas increased significantly 12–72 h after toxin injection and caused the induction of oxidative stress. Lipid peroxidation usually increases when the antioxidant defence system for detoxifying excess O_2^- fails [33]. In the present study, the LPO levels increased significantly 12–24 h after injection of the PLTX extracts,

indicating that the unsaturated fatty acids in the lipid membranes of the hepatopancreas were attacked by oxygen-free radicals and that the lipids were oxidised to LPO. The PC showed the same trend as that of MDA and was higher than that in the control group after 24–48 h. Changes in the protein carbonylation process are a biochemical perturbation resulting from oxidative stress and are considered to be a key indicator for oxidative damage to proteins [33,34]. These changes indirectly reflected that the hepatopancreatic cells were damaged. Peroxidation of the membrane will severely affect the structural integrity and cell function and lead to a change in membrane permeability, such as an increase of the water permeability, a decrease of the bilayer thickness or alterations in the lipid membrane order and fluidity [35,36].

The PLTX extracts caused oxidative stress and simultaneously damaged the hepatopancreas in shrimp. First, the lumen of the hepatopancreas became enlarged, B-cells became deformed, and small lipid vacuoles in restzellen cells (R-cells) became larger 6 h after injection of the PLTX extracts into the shrimp. Microvillar on the apical border of the R-cells sloughed and fell into the lumen of the hepatopancreas tubules. Second, vacuoles in both the R-cells and B-cells were enlarged, and the typical microvillar border of the R-cells disintegrated 12 h after injection. Finally, the R-cells and B-cells swelled, the number of vacuoles increased, and the cell lining was severely damaged, with more lesions and collapsed hepatopancreatic lobes. The hepatopancreas is the main detoxification organ in shrimp. The damage caused by PLTX in the hepatopancreas of *L. vannamei* was similar to that caused by the T-2 toxin [37]. Previous studies also showed that histological damage was observed in the liver, lung, kidney, brain, and gastrointestinal tract of mammals after PLTX toxin injection [38]. The results of the present study showed that the hepatopancreas was damaged after injection of PLTX extracts, but the damage did not lead to shrimp death. Therefore, the hepatopancreas was proposed to be the functional site of PLTXs.

Shrimp, the same as other invertebrate species, lack an adapted-specific immune system, and they mainly rely on inborn immune responses to protect them against invaders and environmental stresses [39]. Nitric oxide (NO) and NOS activity plays a pivotal role in the shrimp defence system. NO is the second messenger molecule, and it plays diverse physiologic and pathophysiologic roles in multiple functions, such as neuronal transduction, cardiac disease, and immune responses [40]. The NO produced by NOS was shown to be used as a bactericidal molecule by molluscs and crustaceans in their immune response [41]. In the present study, the activity of T-NOS in the hepatopancreas of shrimp decreased significantly after the injection of PLTX extracts, indicating that PLTX extracts can inhibit the shrimp immune response and reduce immunity.

T-AOC reflects the total intracellular antioxidant defence status of organisms. Usually, the antioxidant capability of an organism under certain unfavourable conditions can reflect its health status [42]. The effective and rapid elimination of ROS is performed by antioxidant defence mechanisms, including SOD, which scavenges the superoxide anion [43]. *cMnSOD* is an important protein and contributes greatly to the total SOD activity in the gill and hepatopancreas [44]. When infected or under adverse environmental conditions (such as hypoxia and temperature changes), the expression levels of *MnSOD* in prawn are upregulated [45–47]. In the present study, however, quantitative real-time RT-PCR analysis showed that the levels of the *cMnSOD* transcript in the *L. vannamei* hepatopancreas gradually decreased from 6 to 48 h after PLTX extract injection. A previous study also showed that the expression levels of the *MnSOD* gene in the gills and hepatopancreas of *L. vannamei* were reduced under hypoxic conditions or cyclic serious/medium hypoxia enhanced conditions [44,48]. It should be noted that the T-AOC levels gradually increased with time, indicating that the antioxidant capacity of the hepatopancreas in the shrimp increased. Within a certain concentration range, some biotoxins or environmental pollutants, e.g., ammonia-N, sulphide, and the T-2 toxin, can cause an increase in T-AOC enzyme activity in the hepatopancreas of shrimp [49]. To date, few studies have investigated the effect of PLTXs on the antioxidant parameters of organisms. One study has conducted the exposure of the mussel *M. galloprovincialis* to *O. ovata*, and the observed antioxidant parameters suggested that the influence of *O. ovata* on the oxidative stress of *M. galloprovincialis* was very limited [19]. The present study demonstrated that PLTX extracts could reduce *cMnSOD* at the transcriptional level but could not damage the antioxidant defence system in the hepatopancreas of shrimp; moreover, the PLTX extracts did not decrease the total antioxidant capacity of shrimp.

This study showed that PLTX extracts caused an apparent oxidative stress and immune response in shrimp. Additionally, 6 h after injection of the PLTX toxin, the levels of O_2^-, MDA, and PC in the hepatopancreas of shrimp were significantly decreased compared with those in the control group ($P < 0.05$). Subsequently, the O_2^- level increased significantly from 12 to 72 h in the hepatopancreas. When the O_2^- level was increased, the level of *cMnSOD* transcription in the hepatopancreas gradually decreased and was significantly lower than that in the control group from 12 to 48 h ($P < 0.05$). O_2^- is the first product released from a respiratory burst initiated by NADPH oxidase [50], and hydrogen peroxide, hydroxyl radical, and single oxygen are produced by the catalysis of SOD [44,51]. Oxidative bursts are one of the most important defence strategies of crustaceans against stress conditions, including chemical, physical, and biological stressors [51]. The steady decrease of *cMnSOD* expression was suspected to generate higher local levels of ROS and to limit the toxicity of PLTX extracts in the hepatopancreas. In the present study, the level of *cMnSOD* transcription markedly decreased in PLTX toxin-treated *L. vannamei*, accompanied by a reduction in antioxidative function and an increase in O_2^- production and accumulation. As antioxidants in cells decreased, the PLTX-induced increase in ROS exceeded the cell cleavage capacity. This, in return, resulted in higher MDA, LPO, and PC levels due to the increased O_2^- concentration. Thus, the results indicate that oxidative stress is the underlying mechanism of cytotoxicity induced by PLTX extracts.

In conclusion, the present study revealed changes in oxidative stress parameters (LPO and MDA), immune enzymes (T-AOC and T-NOS), and the mRNA expression of immune-related genes (*Crustin*, *Toll*, and *cMnSOD*) in the hepatopancreas and gills of *L. vannamei* after PLTX extract injection. Our results indicated that the PLTX extracts can cause lipid peroxidation and carbonylation of proteins in hepatopancreatic cells and then damage the tissues. However, the T-AOC levels indicate that PLTX extracts do not cause a decrease in the total antioxidant capacity of shrimp.

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