

Article

# Effects of Marine Toxin Domoic Acid on Innate Immune Responses in Bay Scallop *Argopecten irradians*

Cheng Chi <sup>\*,†</sup>, Caiyan Zhang <sup>†</sup>, Jiadai Liu and Xiaochuan Zheng

Key Laboratory of Aquatic Nutrition and Feed Science of Jiangsu Province, College of Animal Science and Technology, Nanjing Agricultural University, No. 1 Weigang Road, Nanjing 210095, Jiangsu Province, China; 2018105050@njau.edu.cn (C.Z.); 2017105048@njau.edu.cn (J.L.); 15951977892@163.com (X.Z.)

\* Correspondence: chicheng0421@126.com; Tel.: +86-25-84395270; Fax: +86-25-84395382

† These authors contributed equally to this work.

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**Abstract:** Domoic acid (DA) is an amnesic shellfish poisoning toxin produced by some species of the genera *Pseudo-nitzschia* and *Nitzschia*. This toxin has harmful effects on various species, especially scallops. This study aimed to investigate the effects of DA exposure on the immune and physical responses of bay scallop, *Argopecten irradians*. Various immunological and physical parameters were assessed (acid phosphatase (ACP), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), lipid peroxide (LPO), nitric oxide (NO), and the total protein content) in the haemolymph of scallops at 3, 6, 12, 24, and 48 h post-exposure to DA at different concentrations (10, 50, and 100 ng/mL). Moreover, the expression of immune-related genes (*CLT-6*, *FREP*, *HSP90*, *MT*, *PGRP*, and *PrxV*) was assessed. The activities of ACP, ALP, and LDH and the total protein content and LPO increased upon exposure to DA at different concentrations, while NO levels were decreased. Furthermore, immune-related genes were assessed upon DA exposure. Our results showed that exposure to DA negatively impacts immune function and disrupts physiological activities in bay scallops.

**Keywords:** Domoic acid; bay scallop; *Argopecten irradians*; harmful algal blooms; immune response

## 1. Introduction

Harmful algal blooms, caused by environmental changes and water pollution, have harmful effects on various organisms including humans, not only affecting the coastal ecology, but also causing serious economic losses [1]. One prominent underlying reason is that harmful algal blooms produce marine phycotoxins and other metabolites [2]. Domoic acid (DA) is an amnesic shellfish poisoning (ASP) toxin, produced naturally by some species of the genera *Pseudo-nitzschia* and *Nitzschia* [3]. DA is an amino acid with three carboxyl groups and one amino group, and is structurally similar to glutamic acid and kainic acid [4]. Glutamate is a putative excitatory neurotransmitter in the vertebrate central nervous system [5]. Because of this structural similarity, DA binds to the glutamate receptor [GluRs] family to induce neuroexcitatory and neurotoxic effects [6]. DA exerts neurotoxic effects on the central nervous system of mammals and other vertebrates [4,7]. These investigations focused on mice, humans, birds and others, revealing the neurovirulence of DA, which included brain injury, acute retina injury and age and gender susceptibility [1,2], exposure routes for vertebrates, and vertebrate susceptibility [3]. Although few researchers studied marine organisms including fish and marine mammals, they only revealed the susceptibility (ED<sub>50</sub>/LD<sub>50</sub>) to DA, aggressive behavior, swimming behavior, and metabolic activity in brain of marine mammals and fish [4]. Moreover, many other studies have focused on the morphology, distribution, and toxicity of DA producing algae species including

*Pseudo-nitzschia* spp., *Nitzschia bizertensis* and *Nitzschia navis-raringica* [5]. Although there are some investigations related to invertebrates, most of them reported that the DA content accumulated in the various tissues as the important DA vectors to top predators, whereas fewer have documented toxicological effects on themselves resulting from exposure [5]. Generally, the immunotoxic potential of DA, a well-characterized neurotoxin, has not been fully investigated. The previous results in mice suggest that DA may exert its immunotoxic effects both directly and indirectly by affecting monocyte phagocytosis [6]. Bivalves, including scallop *Argopecten irradians*, are among the important marine fishery resources in China, South Korea, and Japan. Bivalves are filter-feeding organisms, therefore, biotoxins produced during harmful algal blooms can accumulate in their body tissues [8]. However, there are great physiological differences between vertebrates and invertebrates, in case of the immune system.

Scallops, as an invertebrate, lack adaptive immunity and rely primarily on the innate immune system for their defense [9], and humoral components mediate innate immunity primarily via the haemolymph. Acid phosphatase (ACP) and alkaline phosphatase (ALP) are important lysosomal enzymes which are involved in humoral immune responses and participate in the degradation of foreign carbohydrates, proteins, and lipids [10,11]. They are primarily present in lysosomes of granulosa cells and contribute to phagocytosis and secretion into the serum through degranulation [12]. Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme that potentially reflects damage to cells, tissues, and organs [13]. Lipid peroxide (LPO) is the product of the reaction between oxygen free radicals and polyunsaturated fatty acids, and increased LPO levels potentially indicate structural or functional damage to cells or cell membranes [14]. Nitric oxide (NO) is widely distributed in various organs and participates in many important physiological and pathological activities such as immune defense [15]. It is synthesized by inducible (i) NO synthase (iNOS), and usually considered to be related to the immune response in mammals. In bivalves, NO has been demonstrated that it is involved in innate immunity, iNOS-like activity has been detected in hemocytes of the zhikong scallop *Chlamys farreri* after LPS stimulation [9,16], and NO has been shown to be synthesized in scallop hemocytes [17], directly or indirectly contributing to pathogen elimination [11]. It is also been demonstrated that NO promotes apoptosis, phagocytic and anti-bacterial ability by developing oxidative toxicity in order to avoid excessive auto-toxicity during the last phase of immunity, playing a crucial role in the modulation of the immune response [9,11]. Therefore, the innate immune-related responses of scallops exposed to DA can be understood through the assessment of these immune-related parameters.

Generally, this study aimed to investigate the effects of DA exposure on the immune and physical responses of bay scallop by measuring the activities of ACP, ALP, LDH, and contents of NO, LPO as well as the expression of immune-related genes in bay scallop after different concentrations of DA exposure. The results of current study will provide a novel insight into the immunotoxicity of DA in scallops, and the present results also are of particular interest and use to toxicologists and researchers involved in studies on cellular and molecular toxicity of xenobiotics [18].

## 2. Materials and Methods

### 2.1. Domoic Acid

Domoic acid (DA), 90–100% on HPLC, was purchased from Sigma-Aldrich Co. LLC (Sigma, St. Louis, MO, USA) and stored at 4°C in a refrigerator until use.

### 2.2. Animals

Three hundred and sixty bay scallops were collected by a fisheries wholesale market in Jiangsu Province, China. The average shell length of the scallops was 59–70 mm; average weight, 45.32±2.43 g. Before the experiments, the scallops were stored in lantern nets suspended in an 800-litre container supplied with filtered and aerated seawater for two weeks to adapt to laboratory conditions (temperature: 10 ± 1 °C; salinity: 30‰ ± 0.1‰). Instant Algae® Shellfish Diet (Reed Mariculture

Inc., Campbell, CA, USA) was fed to scallops with  $1.2 \times 10^{10}$  algal cells per scallop. Half of seawater was changed daily.

The bay scallops were distributed as four groups: control group and three treatment groups (DA exposure). Each group comprised 30 scallops with 3 replicates. The DA stock solution was prepared by dissolving DA in millipore water, and the equal volume of millipore water was added in the control. The experimental groups were exposed to DA at four concentrations (0, 10, 50, and 100 ng/mL), which were selected on the basis of previous studies reporting the toxicity of DA accumulation and its effects on juvenile scallops [19]. To maintain the DA concentrations, no water exchange or tank cleaning was performed during this experiment. One millilitre of haemolymph was sampled from each bay scallop at 3, 6, 12, 24, and 48 h upon exposure to DA. Three scallops per group were used as triplicates. The haemolymph sampled from each group was divided into two parts: one part (100  $\mu$ L) was used to extract RNA, and the other part was centrifuged for 3 min at 750  $\times g$  and stored at  $-80^{\circ}\text{C}$  to measure the humoral immune parameters. This present study was approved by the Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) (permit number: SYXK (Su) 2017-0007).

### 2.3. Measurement of Non-Specific Immune Responses

ACP and ALP activity in the haemolymph were quantified using assay kits (Nanjing Jiancheng Bioengineering Institute, China). The unit definition of ACP enzymatic activity corresponded to 1 mg nitrophenol within 30 min at  $37^{\circ}\text{C}$  according to the instruction of the commercial kit. One microgram of phenol was released per 100 mL haemolymph, corresponding to one unit of ALP activity according to the instruction of the ALP assay kit.

Lactate dehydrogenase (LDH) in haemolymph was quantified using an assay kit (Nanjing Jiancheng Bioengineering Institute, China). LDH catalyzes the synthesis of pyruvic acid from lactic acid, and pyruvic acid reacts with 2,4-dinitrophenylhydrazine to produce pyruvic dinitrophenylhydrazine. The product is reddish brown in an alkaline solution. The activity of the enzyme was determined by measuring the absorbance at 450 nm.

NO levels were determined using the nitrate reductase assay using an assay kit (Nanjing Jiancheng Bioengineering Institute, China). NO is oxidized to nitrate and nitrite by oxygen and then reacts with the chromogenic agent to generate azo dyes, which can be quantified using a spectrophotometer.

LPO and the total protein content in haemolymph were estimated using assay kits (Nanjing Jiancheng Bioengineering Institute) in accordance with the manufacturer's instructions.

### 2.4. Real-Time Quantitative PCR Analyses of Gene Expression

Total RNA was extracted using TRIzol Reagent from haemolymph samples [11]. The concentration and purity of RNA were determined using a spectrophotometer, based on the OD 260/280 values ( $1.8 < \text{ratio} < 2.0$ ). cDNA was synthesized using a PrimeScript<sup>TM</sup> RT Reagent Kit (TaKaRa Bio, Otsu, Japan) in accordance with the manufacturer's instructions and stored at  $-80^{\circ}\text{C}$  for the real-time quantitative PCR (qPCR).

qPCR was performed using SYBR Premix Ex Taq<sup>TM</sup> Kits (TaKaRa Bio) on a RT-PCR Detection System (Qiagen, Hilden, Germany). Gene expression was standardized by  $\beta$ -actin. The sequences of primers for the target and reference genes (*CLT6*, *FREP*, *HSP90*, *MT*, *PGRP*, *PrxV* and  $\beta$ -actin) are enlisted in Table 1. The reactions proceeded at a final volume of 12  $\mu$ L, containing 6.25  $\mu$ L SYBR Premix Ex Taq<sup>TM</sup>, 1  $\mu$ L forward primer, 1  $\mu$ L reverse primer, and 1  $\mu$ L cDNA and ultra-pure water. The cycling conditions were as follows: a pre-run at  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles at  $95^{\circ}\text{C}$  for 45 s,  $56^{\circ}\text{C}$  for 45 s, and  $72^{\circ}\text{C}$  for 30 s. After the amplification stage, the melting curve was analysed. The relative mRNA levels were determined using the  $2^{-\Delta\Delta\text{CT}}$  method [20]. In all cases, PCR was performed in triplicate.

**Table 1.** Primers used for the analysis of mRNA expression via qRT-PCR analysis.

Genes	Primer Sequence	Accession No.
<i>β-actin</i>	F:5'CAAACAGCAGCCTCCTCGTCA 3' R: 5'CTGGGCACCTGAACCTTTCGTT 3'	AY335441
<i>CTL6</i>	F: 5'CAGTTGCTACAGGGTTCG 3' R: 5'GGGCGTTATCTGGCTCAT 3'	GQ202279
<i>FREP</i>	F: 5'CGTCGCAAATGCTGAAGATG 3' R: 5'TAAGTTGTGGTCGGTCCTGAGA 3'	EU399719
<i>HSP90</i>	F: 5'TCAGTATGGTTGGTCCGCTAA 3' R: 5'CGGTTGCCTTTTCCTTCAGA 3'	EF532406
<i>MT</i>	F: 5'AACTTGCTGTAGTGGGAATG 3' R: 5'AGGCTGGAACTGCTGTGGT 3'	EU734181
<i>PGRP</i>	F: 5'GGGCAAGTGTATGAGGGAAGAG 3' R: 5'TCCGATGAAGGAGACAGCGTAG 3'	AY437875
<i>PrxV</i>	F: 5'AATCAAGGAGCGGCTGGCA 3' R: 5'TCAACTTCTCAATCTTCCCGTCAT 3'	HM461987

### 2.5. Statistical Analyses

The data were tested for normality and homogeneity of variance, using the Kolmogorov-Smirnov and Cochran's tests. Data were analyzed using one-way analysis of variance (ANOVA). The data were presented as mean  $\pm$  standard deviation (SD) values. The differences were determined using the LSD test in the SPSS 19.0 software (IBM Corp., Armonk, NY, USA) with  $p < 0.05$  indicating statistical significance.

## 3. Results

### 3.1. Non-Specific Immune Responses

#### 3.1.1. ACP Activity

ACP activity (Figure 1A) was significantly enhanced in 10 ng/mL of DA treated group at 3–24 hours post exposure (hpe) comparing with control, and the highest level of ACP activity was observed at 3 hpe. In 50 ng/mL of DA treated group, an obvious enhancement in ACP activity was observed at 3–12 hpe. ACP activity was significantly increased in 100 ng/mL of DA treated group at 3–48 hpe except for 24 hpe.

#### 3.1.2. ALP Activity

ALP activity (Figure 1B) was significantly enhanced in 10 ng/mL of DA treated group at 3–48 hpe. ALP activity was significantly raised in the 50 ng/mL DA-treated group at all time intervals. In the group treated with 100 ng/mL of DA, ALP activity was increased at 3–24 hpe and reverted to baseline levels up to 48 hpe.

#### 3.1.3. LDH Activity

LDH activity (Figure 1C) was significantly increased in the group treated with 10 ng/mL of DA at 3–12 hpe and then reverted to baseline levels at 24–48 hpe. LDH activity was significantly increased in the 50 and 100 ng/mL DA-treated groups at 3–24 hpe and then reverted to baseline levels at 48 hpe.

#### 3.1.4. LPO Level

LPO levels (Figure 1D) were significantly increased in all DA-treated groups at all time intervals.

3.1.5. NO Level

NO levels (Figure 1E) were significantly decreased at all time intervals upon exposure to 10, 50, and 100 ng/mL of DA.

3.1.6. Total Protein Concentration

The total protein concentration (Figure 1F) was significantly increased in the 50 and 100 ng/mL DA-treated groups at all time intervals. In the group treated with 10 ng/mL of DA, the total protein concentration was significantly increased at 3–12 hpe and reverted to baseline levels at 24–48 hpe.

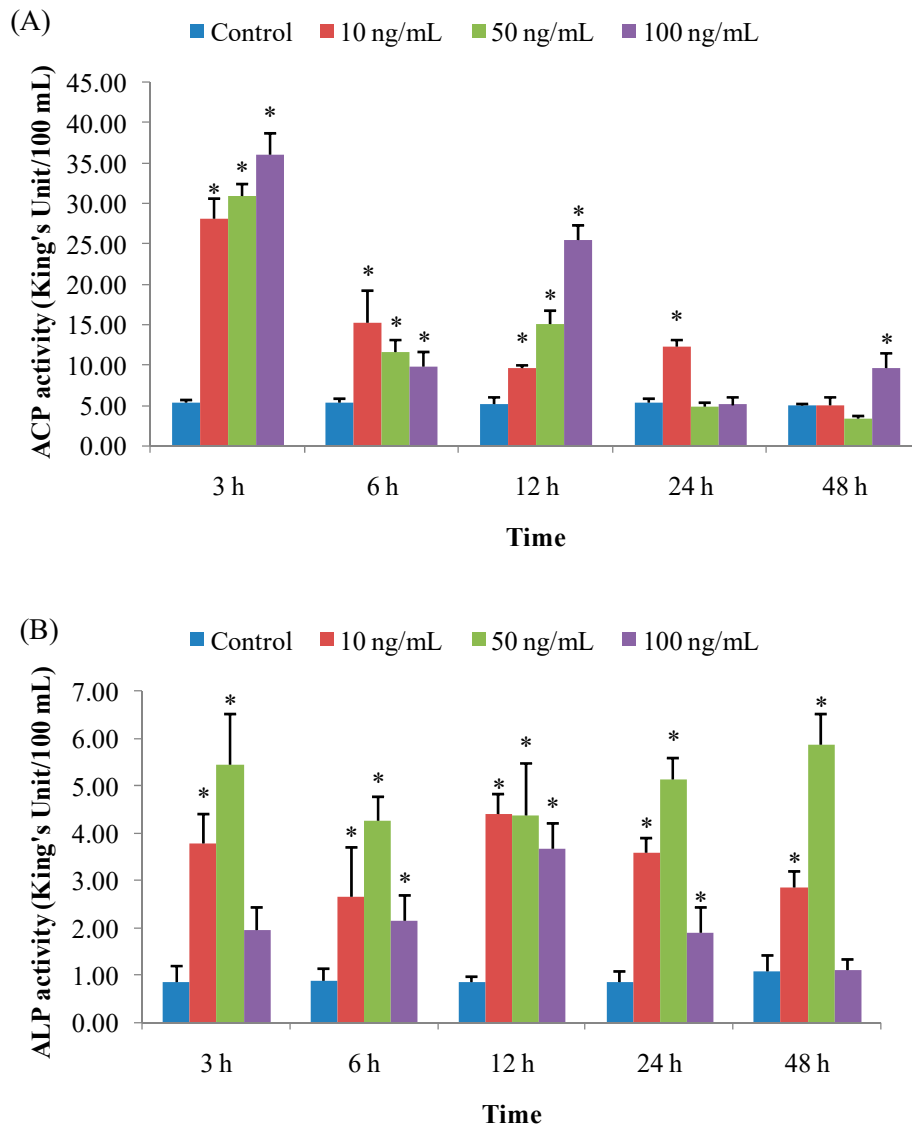


Figure 1. Cont.

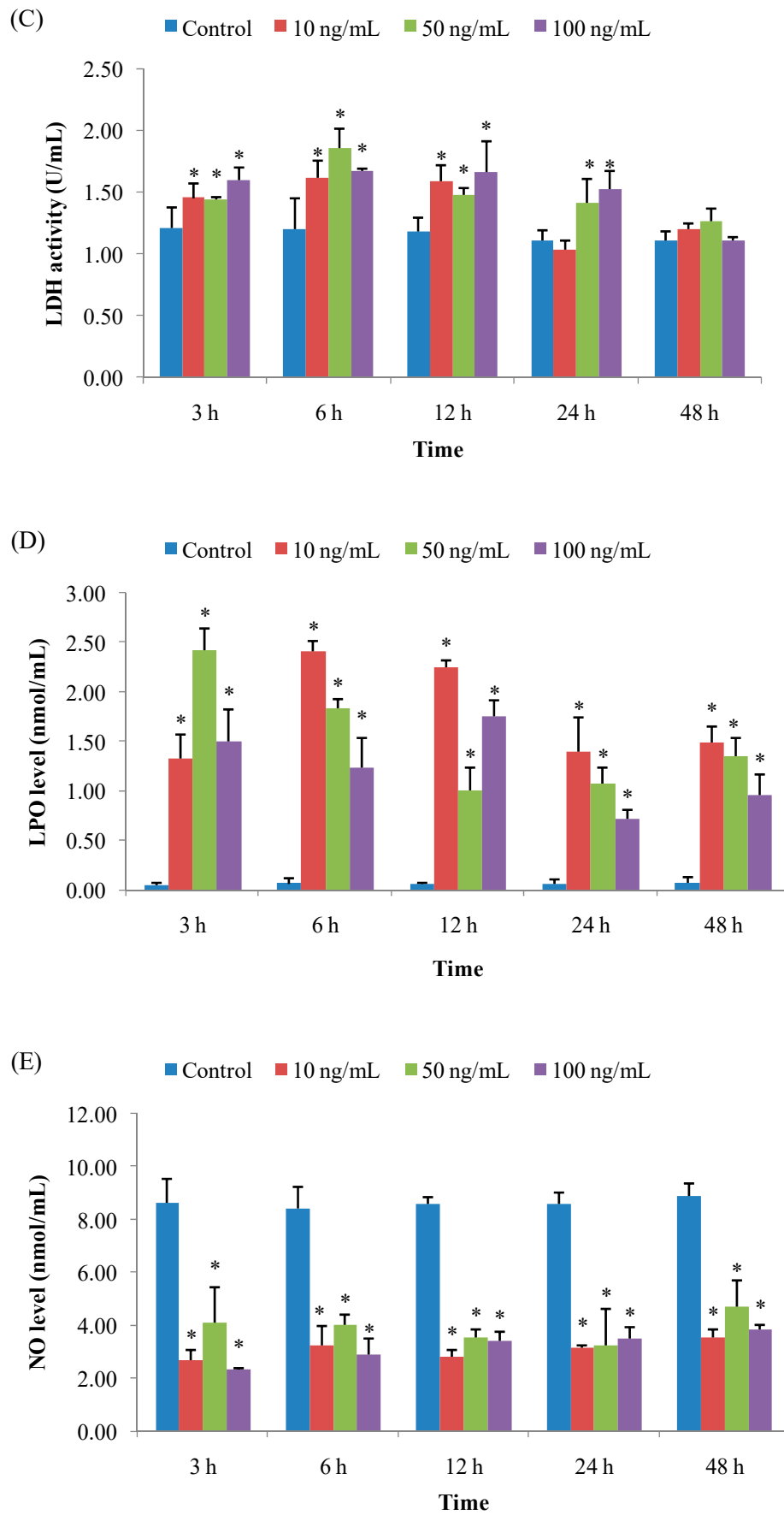
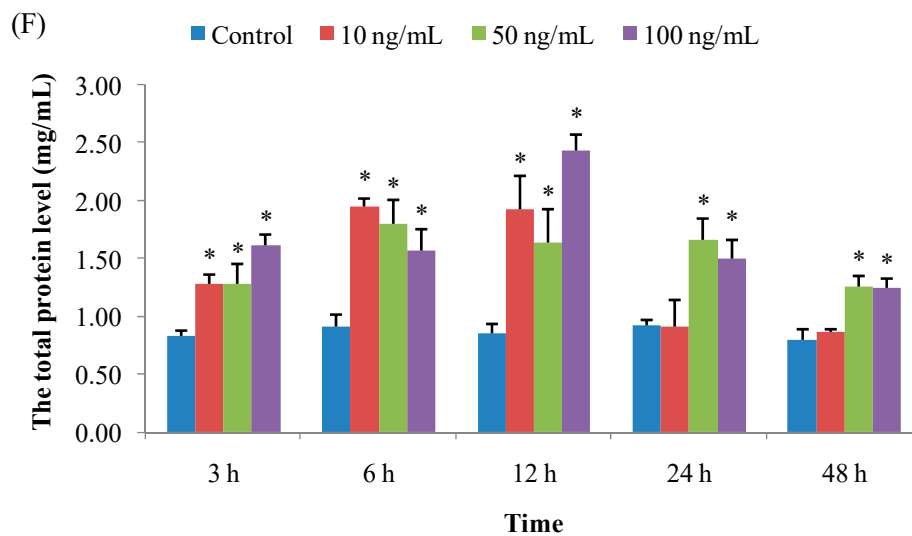


Figure 1. Cont.



**Figure 1.** Effects of domoic acid (DA) on non-specific immune responses of the bay scallop *Argopecten irradians* at different time intervals after exposure to DA at three concentrations (10, 50, and 100 ng/mL). (A) acid phosphatase (ACP); (B) alkaline phosphatase (ALP); (C) lactate dehydrogenase (LDH); (D) lipid peroxide (LPO); (E) nitric oxide (NO) level; (F) total protein concentration. Data represent mean  $\pm$  SD values ( $n = 3$ ) during sampling with “\*” denoting significant differences between the treatments of each exposure time ( $p < 0.05$ ).

### 3.2. Expression of Immune-Related Genes

#### 3.2.1. Expression of CTL6

*CTL6* mRNA levels (Figure 2A) remained almost unchanged in the group treated with 10 ng/mL of DA at 3–12 hpe; however, *CTL6* was significantly upregulated at 24–48 hpe. Accordingly, *CTL6* mRNA levels in the 50 ng/mL DA-treated group were significantly higher than those in the control group at 3–48 hpe, except at 12 hpe, which reverted to baseline levels. *CTL6* mRNA expression levels remained almost unchanged on treatment with 100 ng/mL of DA at 3–6 hpe; however, they were significantly decreased at 12 hpe and sharply increased ( $p < 0.05$ ) at 24–48 hpe.

#### 3.2.2. Expression of FREP

FREP mRNA levels (Figure 2B) in the 10 ng/mL DA-treated group were significantly increased at 6–24 hpe rather than in the control group and sharply decreased ( $p < 0.05$ ) at 48 hpe. FREP mRNA was strongly induced ( $p < 0.05$ ) at 12 hpe in the 50 ng/mL of DA group and then decreased markedly at 48 hpe. FREP mRNA expression levels in the 100 ng/mL DA-treated group were significantly increased at 3 hpe and reverted to baseline levels at 6–24 hpe; thereafter, they sharply decreased ( $p < 0.05$ ) at 48 hpe.

#### 3.2.3. Expression of HSP90

*HSP90* mRNA expression levels (Figure 2C), upon treatment with 10 ng/mL DA, increased significantly at 3–24 hpe and gradually decreased with an increase in the exposure time, followed by a significant reduction in comparison with the control group at 48 hpe. *HSP90* mRNA expression upon treatment with 50 ng/mL DA was significantly increased at 3 hpe, followed by a marked reduction at 24–48 hpe. *HSP90* mRNA expression levels upon treatment with 100 ng/mL DA decreased at all time intervals, decreasing significantly at 6–48 hpe.

### 3.2.4. Expression of MT

MT mRNA levels (Figure 2D) did not differ significantly on treatment with 10 ng/mL DA at 3–12 hpe; however, they were significantly increased at 24–48 hpe. MT mRNA was significantly upregulated at 3–6 hpe on treatment with 50 ng/mL DA and then reverted to baseline levels at 12–48 hpe. On treatment with 100 ng/mL DA, MT mRNA was strongly upregulated ( $p < 0.05$ ) at 3–24 hpe.

### 3.2.5. Expression of PGRP

PGRP mRNA levels (Figure 2E) were significantly increased on treatment with 10 ng/mL DA at all time intervals. PGRP mRNA expression levels were significantly increased on treatment with 50 ng/mL DA at 3–12 hpe and then significantly decreased at 24–48 hpe. On treatment with 100 ng/mL of DA, MT mRNA was significantly downregulated at 3 hpe and upregulated at 12–48 hpe.

### 3.2.6. Expression of PrxV

PrxV mRNA levels (Figure 2F) were significantly increase on treatment with 10 ng/mL DA at 3–24 hpe. On treatment with 50 ng/mL DA, PrxV mRNA was significantly upregulated at 3–12 hpe and then significantly downregulated at 48 hpe. PrxV mRNA expression levels did not differ significantly on treatment with 100 ng/mL DA at 3–6 hpe; however, they were significantly increased at 12–24 hpe and reverted to baseline levels at 48 hpe.

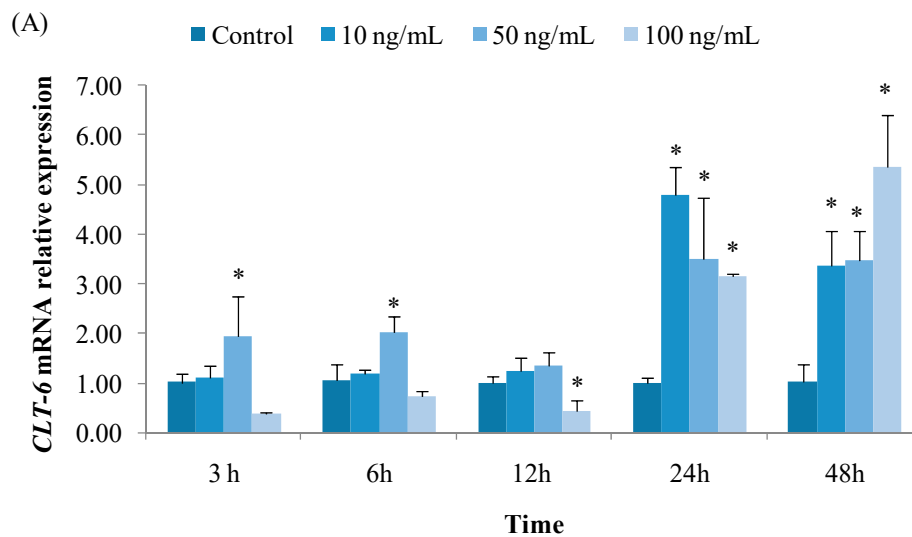


Figure 2. Cont.



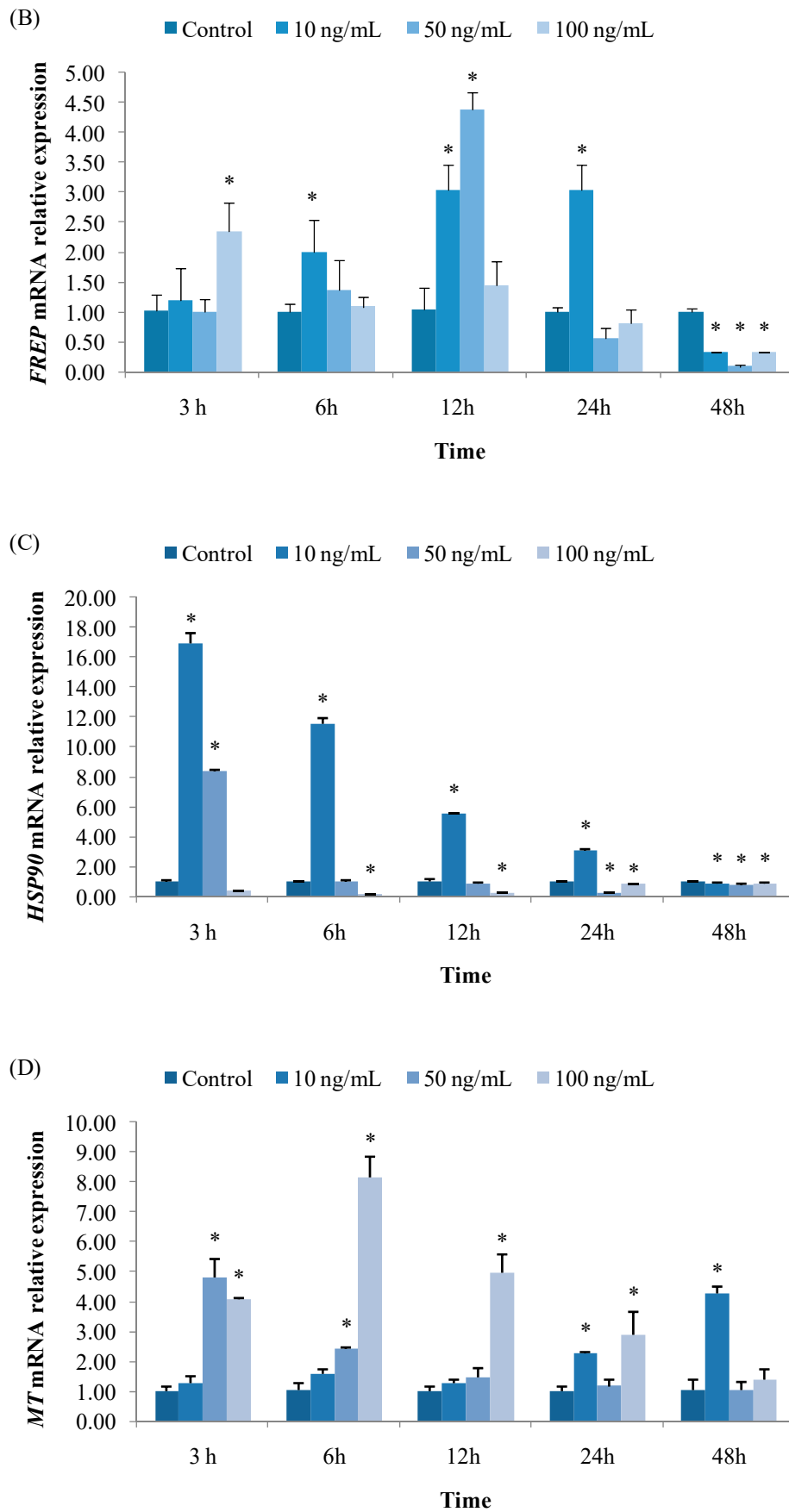
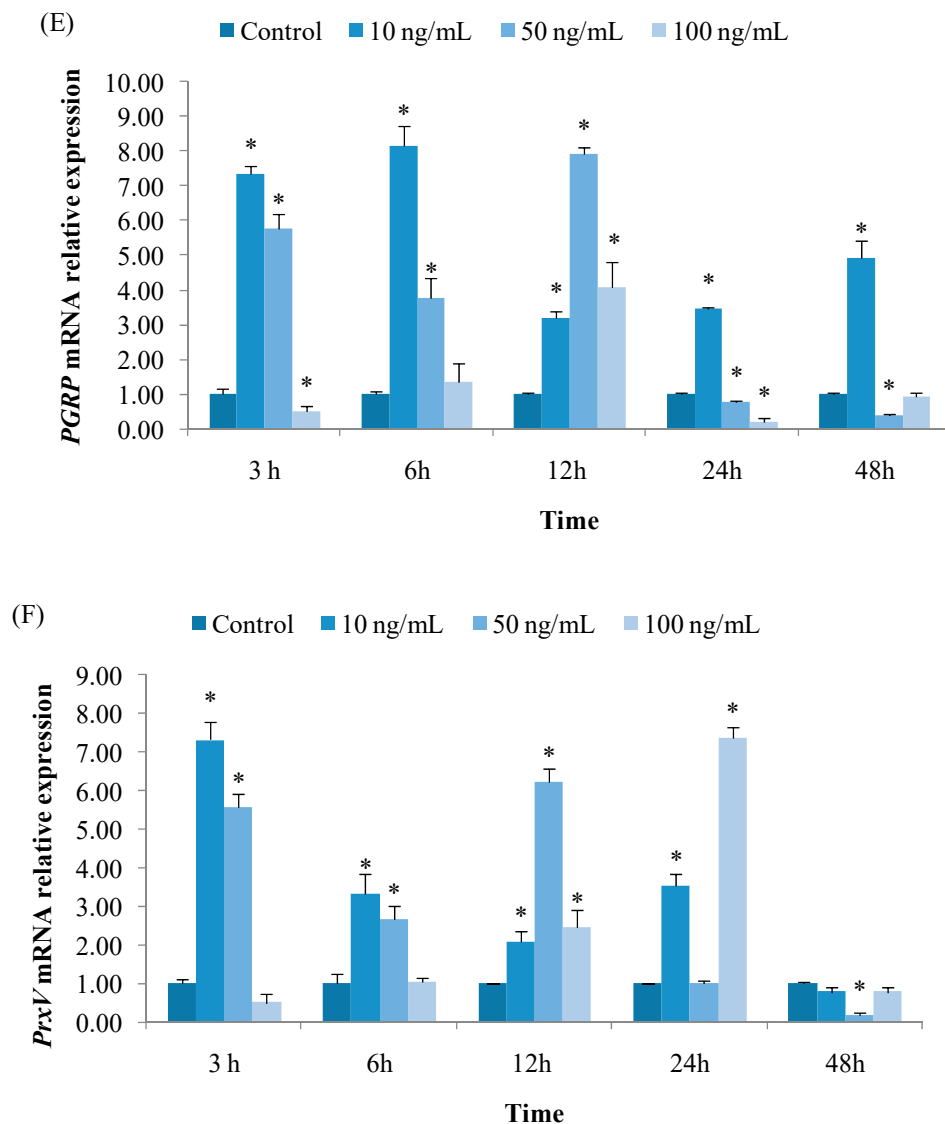


Figure 2. Cont.



**Figure 2.** Effects of domoic acid (DA) on non-specific immune responses of the bay scallop *Argopecten irradians* at different time intervals after exposure to DA at three concentrations (10, 50, and 100 ng/mL). (A) *CLT6*; (B) *FREP*; (C) *HSP90*; (D) *MT*; (E) *PGRP*; (F) *PrxV*. Data represent mean  $\pm$  SD values ( $n = 3$ ) at the same sampling time with “\*” denoting significant differences the treatments of each exposure time ( $p < 0.05$ ).

#### 4. Discussion

DA is an amnesic shellfish poisoning (ASP) toxin, which can be accumulated in the bodies of fish and shellfish [21]. To analyse the effects of DA on scallop physiology and immune-related responses, we assessed some non-specific immune responses and the expression of the immune-related genes in the haemolymph of scallops exposed to different concentrations of DA (10, 50, and 100 ng/mL). Although continuous high levels of DA exposure, up to 100 ng/mL, is unlikely for scallops in their natural habitat, due to the floating nature of an algal bloom, the relatively high DA concentration used in the present study could help to understand its negative effects as scallops in the natural habitat may be exposed to dissolved DA or toxic algae repeatedly. Scallop may be naturally exposed to 10 and 50 ng/mL of DA used in this study, as DA at concentrations up to 100 nmol/L (=331 ng/mL) was occasionally found during natural blooms of *Pseudo-nitzschia* spp. [22]. The concentrations of dissolved DA (10, 50 and 100 ng/mL of DA) were used to study DA effects on juvenile king scallop *P. maximus* [19]. Although few previous investigations studied the DA produced by *Pseudo-nitzschia* spp., they only

revealed the accumulation of DA by the sea scallop (*Placopecten magellanicus*)-fed cultured cells of toxic *Pseudo-nitzschia multiseriis* [23], the effects on immune responses were neglected.

ACP and ALP are two important hydrolases, which are involved in humoral immune responses and participate in the degradation of xenobiotics by hydrolysis [10,11]. In this study, ACP activity was increased after exposure to 10, 50, and 100 ng/mL of DA at 3–12 hpe in comparison with the control group, concurrent with Jing et al. [24], who reported an increase in ACP activity in *Pinctada fucata* after exposure to copper. It indicated that external stimulus including DA could induce stronger ACP activity to make phagocytes more effective for opposing and cleaning the DA invasion. However, we observed that ACP activity after exposure to 10 ng/mL of DA after 6 h is higher than the activity after exposure to 50 and 100 ng/mL after the same time; ACP activity after exposure to 100 ng/mL of DA increases after 3 h then decrease at 6 h and increase again after 12 h. It may indicate that higher concentrations or longer time exposure might cause fluctuant ACP activities with the increase of exposure concentration and extension of time. The induced ACP activity may be attributed to increasing number of lysosomes as a lysosomal ACP enzyme. Whereas, the ACP declining as a response to the increase of exposure concentration and the extension of time, in the current investigation, may be due to decreasing in number of lysosomes and in turn decreased levels of ACP. However, the regulatory mechanism of the fluctuation of ACP activity based on different concentrations and exposure times needs to be further confirmed. Moreover, in the present investigation, the ALP activity was increased in scallop *Argopecten irradians* after exposure to DA, concurrent with Chi et al. [25], reporting that 500 nM of okadaic acid (OA) significantly induced ALP activity in bay scallops from 12 to 24 hpe. These results indicate that scallops treated with DA may resist DA exposure through the increased ACP and ALP activities.

NO is an important immune signaling molecule in all vertebrates and invertebrates [26] and it is synthesized by inducible (i) NO synthase (iNOS), and usually considered to be related to the immune response in mammals. In bivalves, NO has been demonstrated that it is involved in innate immunity, and be synthesized in scallop hemocytes [17], contributing to pathogen elimination [11]. It is also been demonstrated that NO promotes apoptosis, phagocytic and anti-bacterial ability by developing oxidative toxicity in order to avoid excessive auto-toxicity during the last phase of immunity, playing a crucial role in the modulation of the immune responses. Our results show that NO levels were decreased at all time intervals upon exposure to 10, 50, and 100 ng/mL DA. Similarly, Barbosa et al. [27] reported that  $\text{NO}_2^-$  levels were reduced after the ascidian *Styela plicata* was exposed to the marine water-soluble fraction of diesel oil (WFDO), indicating a reduction in NO production. Manju et al. [28] demonstrated the protective properties by using a mouse model of DA-induced temporal lobe epilepsy. Therefore, it is speculated that NO may act against DA and the decreasing of NO level may probably due to eliminating DA by combining with it.

LDH is expressed in most living cells, wherein it catalyzes the conversion of pyruvate to lactic acid during carbohydrate metabolism [29]. Therefore, LDH levels reflect the damage to cells, tissues, and organs. Our results show that LDH activity increased after exposure to DA at different concentrations, indicating that DA potentially causes cellular membrane damage in scallops. Ravindran et al. [30] also reported that the LDH activity in U-937 cells was increased significantly after 4 h of OA exposure and peaked at 16 h. Similarly, Chi et al. [31] reported that scallops treated with higher concentrations of palmitoleic acid (PA) displayed higher LDH activity, potentially resulting in cellular membrane damage. These results are consistent with our present results that LDH activities were increased after DA exposure, suggesting that DA exposure may induce cellular membrane damage of hemocytes of bay scallop. Moreover, the previous study reported that the account of hemocytes of the pacific oyster (*Crassostrea gigas*) increased caused by exposure to DA in the diatom *Pseudonitzschia pungens* [32]. Therefore, the increasing of activity of LDH in the haemolymph of bay scallop may be also due to the increasing of hemocytes after DA exposure. Moreover, the LDH activities in low concentration of DA (10 ng/mL) returned to basal level at 24 hpe, while in higher concentrations of DA (50 and 100 ng/mL) at 48 hpe, which is indicating its dose-dependent. LPO is the product of the reaction between oxygen free radicals and polyunsaturated fatty acids. Increased LPO content indicates an excessive amount of

reactive oxygen species (ROS), which can cause serious damage to lipids and proteins [33]. In this study, LPO levels were increased in all DA-treated groups at all time intervals. Furthermore, Hannam et al. [34] reported a significant increase in LPO after acute oil exposure in Arctic scallop *Chlamys islandica*. Similarly, these results indicate that DA exposure may induce structural damage to cell membranes, resulting in physiological functional disorders, subsequently decreasing the immunity of bay scallop. Moreover, total protein levels in haemolymph can also reflect the health status of bivalves [35]. Simoes et al. [36] reported a significant increase in the total protein level during the blooms of the toxic alga *Dinophysis acuminata* in the mussel *Perna perna*. Consistent with these results, the total protein level in the haemolymph of bay scallop increased upon DA exposure, indicating that DA exposure caused histolysis in the scallop.

C-type lectins potentially recognize and bind to terminal sugars on glycoproteins and glycolipids, and play important roles in non-self-recognition and the elimination of foreign particles [37]. In this study, scallops stimulated with 10 and 50 ng/mL of DA displayed *CLT6* upregulation, while exposure to 100 ng/mL DA downregulated *CLT6* mRNA at 3–12 hpe and upregulated *CLT6* at 24–48 hpe, suggesting that low concentrations of DA effectively induce *CTL6* expression, and the high *CTL6* expression levels result in resistance towards DA toxicity in scallops. However, at higher concentrations, DA potentially inhibits *CLT6* expression in a short period, thus negatively impacting immune responses in scallops. On prolonging the exposure duration up to 24–48 h, the *CLT6* expression upregulation in the haemolymph of bay scallop was a response to DA invasion. Fibrinogen-related protein (*FREP*), which is another pattern recognition receptor in innate immune system responses, potentially scavenges xenobiotic substances [38]. In our study, *FREP* mRNA was expressed at 3–24 hpe after DA exposure; however, it was inhibited in all treatment groups at 48 hpe. These results indicate that prolonged exposure to DA inhibits the ability of scallops to recognize and clear xenobiotic particles. Peptidoglycan recognition protein (*PGRP*) can recognize peptidoglycan and peptidoglycan-containing bacteria and play a major role in recognition and regulation of natural and acquired immune responses [39]. In this study, 10 ng/mL of DA induced the expression of *PGRP* mRNA; however, 50 ng/mL of DA suppressed *PGRP* expression at 24–48 hpe, indicating that DA at lower concentrations can induce immune responses of scallops to eliminate non-autoimmune substances; however, with an increase in the exposure duration, DA suppressed the immune response in scallops. Moreover, it was speculated that high DA concentrations could suppress the *PGRP* expression in short period, and then induced to counter DA damage to scallops momentarily, whereas it could inhibit *PGRP* expression at 48 hpe due to the immune fatigue.

Peroxiredoxins (*Prxs*) are very important in the antioxidant defense system and can alleviate oxidative damage caused by ROS and regulate oxidative stress levels through  $H_2O_2^-$  mediated signaling [40]. In this study, *Prxs* was upregulated at 3–24 hpe after exposure to low concentrations of DA. However, on prolonging the exposure duration, *Prxs* was downregulated in all treatment groups at 48 hpe, suggesting that DA exposure at low concentrations and for short durations potentially induces resistance to oxidative stress induced by DA. However, on prolonged DA exposure, *Prxs* expression was inhibited, thus weakening the ability of scallops to resist oxidative stresses induced by DA exposure. In addition, heat shock proteins (*HSPs*) are crucial to prevent irreversible protein denaturation, promoting either repair or destruction of damaged proteins [41]. Our results show that DA at lower concentrations induced *HSPs* in a short period; however, on prolonged exposure and an increase in DA concentration, *HSP* expression was significantly inhibited. These results indicate that DA might reduce antioxidant buffering capacity, subsequently protein damage in scallops in a dose- and time-dependent manner. Metallothionein (*MT*) plays an important role in responses to oxidative stress, tissue injury, infection, and inflammation [26,42,43]. In this study, *MT* mRNA was upregulated in scallops after exposure to higher concentrations of DA up to 24 hpe. Similarly, Chi et al. [44] reported that scallops treated with PA displayed higher *MT* expression. This phenomenon may be attributed to more rapid induction of *MT* mRNA expression by higher concentrations of DA exposure to counter tissue injury or oxidative stress.

In conclusion, this study showed that exposure to DA at different concentrations markedly influenced physiological and immune responses of the bay scallop, as reflected through ACP, ALP, and LDH activities and LPO, NO, and TP content, along with immune-related gene expression, showing that DA disrupts immune signaling and physiological phenomena in the bay scallop. The present results potentially provide preliminary evidence regarding the early responses or adaptation of bay scallops with novel insights into the immunotoxicity of DA in scallops and provides a theoretical basis to develop countermeasures against DA to subsequently improve the production efficiency during scallop culture. However, the effects of DA exposure on oxidative stresses or apoptosis as well as DA accumulation in haemolymph of bay scallop after different concentrations of DA exposure, as well as the regulatory mechanism of these physiological and immune processes based on different concentration and exposure time needs to be further confirmed, should be investigated further.

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