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Up-regulation of Nrf2-dependent antioxidant defenses in *Perna viridis* after exposed to *Prorocentrum lima*

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ABSTRACT

It is well documented that diarrhetic shellfish poisoning (DSP) toxins have strong genetic toxicity, cytotoxicity and oxidative damage to bivalve species. However, these toxic effects seem to decrease with the extension of exposure time and the increment of the toxin concentration, the mechanism involved remained unclear, though. In this paper, we found that expression of the genes related to cytoskeleton and Nrf2 signaling pathway displayed different changes over time in the gill of *Perna viridis* after exposure to DSP toxins-producing microalga *Prorocentrum lima*. During the short-term exposure (3 h and 6 h), KEAP1 gene expression was significantly up-regulated, coupled with up-regulation of MRP, ABCB1 and CAT transcriptions and down-regulation of GPx1 and NQO1 mRNA. After longer exposure to high density of *P. lima*, Nrf2 was significantly up-regulated, accompanied with up-regulation of Nrf2 pathway related genes such as NQO1, SOD, GST-ω and ABCB1, whereas KEAP1 was down-regulated. TUBA1C and TUBB1 transcripts were significantly down-regulated after short-term exposure of *P. lima*, but both of them were up-regulated at 96 h after exposure to high density of *P. lima*. Paraffin section demonstrated that *P. lima* had a strong damage on the gill of mussels during the short-term exposure. However, the negative effect to the gill decreased, and the gill restored after longer exposure (96 h). Taking together, we proposed that *P. lima* had a negative impact on cytoskeleton of mussel gill tissue, could cause oxidative damage to the gills. However, longer exposure of *P. lima* in high density could activate Nrf2 signaling pathway, thereby reducing the influence of toxin on mussel. Our study might provide a novel clue for the resistance mechanism of shellfish to DSP toxins.

1. Introduction

Diarrhetic shellfish poisoning (DSP) toxins are one of the most common lipid soluble phycotoxins, including okadaic acid (OA), dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2) and acyl derivatives dinophysistoxin-3 (DTX3), etc. [1,2]. It has been found that DSP toxins had cytotoxicity, genetic toxicity and embryonic toxicity, and could cause DNA damage [3–6]. However, the toxicological effects on bivalves are not always dose-dependent, and in most cases, are descent over exposure time [7]. It seems that the bivalves have some resistance to the DSP toxins [5–9]. Oxidative damage and apoptosis induced by DSP toxins or DSP toxins-producing algae have been well-characterized in bivalves [5, 7, 10–12]. Romero-Geraldo and Hernández-Saavedra [13] observed the repression of superoxide dismutase (SOD) expression in the *Crassostrea gigas* exposed to *P. lima*. Chi et al. [14] reported the decreases in SOD and acid phosphatase (ACP) activities after 12 h and 48 h exposure of OA. As for the apoptosis induced by DSP toxins, a great body of studies have reported through *in vivo* and *in vitro* experiments. Interestingly, Prado-Alvarez et al. observed significant haemocyte apoptosis in *Ruditapes decussatus* after incubation with OA or feeding with DSP toxins-producing dinoflagellate *Prorocentrum lima* for 4 h [10]. However, they failed to find statistical difference in apoptosis rate after 48 h of *P. lima* exposure, though the clams were proved to accumulate a higher level of OA in tissues. Similarly, Prego-Faraldo et al. [7] observed a significant decrease in oxidative damage in the mussel *Mytilus galloprovincialis* after 48 h exposure of *P. lima*. Vidal et al. [15] reported a similar reduction in damage after longer exposure of *P. lima* in the digestive gland of *M. galloprovincialis*. McCarthy et al. [6] showed an initial increase in percentage tail DNA (%tDNA) after 24 h exposure with a decrease after 7 days. However, the mechanism involved remains ambiguous, though it is crucial to reveal the resistant mechanism to the DSP toxins.

To data, a body of studies have dealt with the metabolism of DSP toxins in bivalves. It has been shown that accumulated toxins

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experienced biotransformation in bivalves including acylation and hydrolysis [16,17]. Transcription factor NFE2-related factor (Nrf2) is an important transcription factor that regulates the expression of antioxidant proteins to protect against oxidative damage induced by exogenous substances [18]. Its induction needs a common DNA sequence named antioxidant response element (ARE) that is similar to the NFE2-binding motif. Activation of the Nrf2/ARE pathway can induce a series of antioxidant enzymes and metabolic detoxification enzymes such as hemeoxygenase 1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST), and glutathione peroxidase (GPx), thus increasing detoxification and elimination of numerous chemicals [19]. Several Nrf2/ARE signaling pathway target genes such as GST, ABC transporters, SOD and catalase (CAT) have been suggested to be involved in the metabolic detoxification of DSP toxins in bivalves [9,12,15,20,21]. Taking together oxidative damages induced by DSP toxins [13,14,22], it is reasonable to speculate that longer exposure of DSP toxins might activate Nrf2/ARE signaling pathway, which in turn alleviate their toxic effects on bivalves.

Cytoskeleton is a dynamic structure, which plays an important role in maintaining cell shape, protecting cells, and cell signaling, etc. DSP toxins-induced changes in cytoskeletal architecture have been reported in bivalves [22–25]. Using a mussel cDNA microarray, Manfrin et al. [23] found that 9% of up-regulated transcripts induced by OA exposure were potentially involved in cytoskeleton organization. Hanana et al. [24] demonstrated that the exposure of OA at 1 μM for 24 h could induce disorganization of actin cytoskeleton, rounding and detachment of the fibroblastic cells in clam heart. Huang et al. [22] found that an annexin-like protein and two cytoskeleton related proteins, actin-related protein (Arp) 2 and Arp 3 were down-regulated in mussel gills after exposure to *P. lima* for 6 h. Unfortunately, changes of cytoskeleton induced by DSP toxins with exposure time have been largely ignored and remained unclear.

To explore the roles of Nfr2/ARE signaling pathway in the adaptation or resistance of bivalves to longer exposure of DSP toxins, here, we observed morphological changes in gill of the mussel *Perna viridis* at 3 h, 6 h and 96 h after exposure to DSP toxins through paraffin section. Changes in genes related to cytoskeleton and Nrf2/ARE signaling pathways were also analyzed by reverse transcription-quantitative PCR (RT-qPCR). In addition, antioxidant enzyme activities were detected.

2. Materials and methods

2.1. Animal

The mussel *P. viridis* (8–10 cm) was purchased from Huangsha Seafood Market in Guangzhou, China. After removing sediment and epibionts, the animals were cultivated in re-circulated aerated aquariums (5 L) with filtered natural seawater at 18 °C. Natural seawater was replaced once a day, and the mussels were fed with 1×10^7 cell/L *Tetraselmis subcordiformis*. After cultured for 8 days, the mussel individuals, whose foot filaments were strong and powerful and the shells opened and closed quickly, were chosen for exposure experiment.

2.2. Exposure experiment

Prorocentrum lima (CCMP 2579) was kindly provided by National Center for Marine Algae and Microbiota (NCMA), which has been proved to produce DSP toxins in previous studies [22]. The strain was grown as batch cultures in sterile Erlenmeyer flasks containing f/2 medium. The mussel individuals were divided into three groups. Individuals in one group were fed with 1×10^7 cells/L *T. subcordiformis* and 2×10^5 cells/L *P. lima*, while animals in another group were fed with 1×10^7 cells/L *T. subcordiformis* and 2×10^6 cells/L *P. lima.* As for the control, only 1×10^7 cells/L *T. subcordiformis* was provided. After exposure for 3 h, 6 h and 96 h, gill tissues were collected from all treatments. In the experiment, total 162 ($18 \times 3 \times 3$) mussel individuals were employed, and each treatment at every time point contained 18 mussels. Tissues from six mussel individuals at each time point within the same treatments were pooled together as one sample for RNA extraction and enzyme activity detection, etc.

2.3. RNA extraction and RT-qPCR

Total RNA from gill tissues was extracted by using a total RNA kit (R6934-01, Omega, USA) according to the manufacturer's instructions. cDNAs were generated from 1 μg total RNA by using the miScript Reverse Transcription kit (Vazyme, China) as described by manufacturer's instructions. qPCR was performed in a Bio-Rad CFX96 Real Time PCR System (Bio-Rad, USA) with AceQ® qPCR SYBR® Green Master Mix (Vazyme, China). The cycling conditions were as follow: 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s. The reaction mixture (20 μl) was composed of 10 μl of AceQ® qPCR SYBR® Green Master Mix, 7.6 μl of H_2O, 0.2 μl of each forward and reverse primers (10 μM) and 2 μl of first-strand cDNA. Specificity of the PCR products was evaluated by melting curve analysis.

Three softwares including NormFinder, geVanNorm and BestKepper were employed to check the expression of six genes such as eukaryotic elongation factor1α (EF1α), phosphatase of regenerating liver-3 (PRL3), phosphatase of regenerating liver-13 (PRL13), ribosomal protein L37a (RPL37a), ubiquitination 52 (UBA52) and tubulin for screening reference genes. UBA52 and RPL3 were found to display the most stable expression, and then employed as reference genes to normalize expression of target genes. All the primers used in RT-qPCR were designed by Primer 5.0, and their characters are summarized in Table 1.

Relative expression of target genes were analyzed by formula NRQ as described by Hellemans et al. [26], in which multi-internal reference genes and inter-run calibration algorithms were considered. Standard curves were generated to check the efficiency of PCR amplification [27]. Amplification efficiencies for each reaction were all over 90%, and correlation coefficients were more than 0.99.

2.4. Detection of oxidative stress parameters

The *P. lima*-induced oxidative stress was evaluated by detecting the level of malondialdehyde (MDA), and activities of GPx and SOD. MDA was detected by using a lipid peroxidation MDA assay kit (Beyotime, China). SOD activity was measured with a total superoxide dismutase assay kit (Beyotime, China). GPx activity was determined with a glutathione peroxidase (GPx) assay kit (Beyotime, China). The protein content was determined by using a BCA protein assay kit (Beyotime, China). All the detections were performed in term of the related manufacturer's instructions. Multi-Mode microplate readers (Tecan Sunrise, Switzerland) were employed to detect absorbance in the experiments.

2.5. Paraffin section

Paraffin section was conducted as described by previous reports with some modifications [28,29]. Briefly, gill tissue was firstly excised and fixed in Bovin's solution for 24 h. After carefully shaping, the tissue was dehydrated in an ascending ethanol series (from 75 to 100%), cleared in xylene, and then embedded in paraffin. Subsequently, the embedded tissue was sectioned at 4 μm by a freezing microtome (Leica CM1900, Germany). The paraffin-embedded section obtained was deparaffinized in xylene, rehydrated in ethanol and water, then stained by hematoxylin and eosin. After cleared in ethanol and xylene, the stained slice was sealed with neutral balsam. The sliced tissues were pictured by using a Nikon Eclipse E100 light microscope, and the obtained pictures were analyzed by using a NIKON DS-U3 imaging system software.

Table 1
Primers for RT-qPCR.

Gene name	Primer sequence 5′-3′	Amplicon size (bp)	Accession number
KEAP1	F: TATCGCTCCAATGAACACGG R: AAGCACTTCTGGGGCTACGC	138	XM_011433558.2
Nrf2	F: TCAACCTGGACAGGAACCCA R: TATCGCGACAGTGTGGACCT	90	XM_020064493.1
ABCB1	F: ACCATCTCCCCTGGTTTTACTG R: ATGTCTGCTGTGGGTGCCTC	147	HF912273.1
MRP	F: AGCAAAGCTCTGGCAAGACA R: GCATGCCCACTTGAAAACTT	104	KT819586.1
GST-ω	F: GTTGGCTCGAAATTAAGTATGGC R: AAACTCCTCCAGTATTTTCTGGTCT	171	KC480256.1
CAT	F: AGTGGAGGACACTTTCGGCA R: AAACCACACTGCGAGGCAAT	104	KX957929.1
NQO1	F: GCAACGAGGAACGATGTAAGG R: GGAAACTGGAAAATGACTAGATCAG	154	XM_021510094.1
GPx1	F: CAACGACCCCCAGATTCAGA R: TCTAGAGTCGGTAGGAGCCAT	108	HQ891311.1
SOD	F: GCAACATTCCTTCAGCACCT R: CCTTGTTCCAAAAGCCTAATTG	154	AJ496219.1
TUBA1C	F: GACGGGTTTCAAAGTGGGTA R: GCTATGGCGGTTGTATTGCT	189	XM_021514976.1
TUBB1	F: ATGTGGTGAGGAAGGAAGCA R: TCCCTGATCTTGCCAACAAT	137	KV589486

KEAP1, Kelch-like ECH-associated protein-1; Nrf2, Nuclear factor erythroid 2-related factor 2; MRP, Multidrug resistance-associated protein; GST-ω, Glutathione S-transferase Omega; CAT, Catalase; NQO1, NAD(P)H:quinone oxidoreductase 1; GPx1, Glutathione peroxidase 1; SOD, Superoxide dismutase; TUBA1C, Tubulin alpha 1 chain; TUBB1, Tubulin beta 1 chain.

2.6. Statistical analyses

All data were given as mean ± standard deviation. Differences between the *P. lima*-exposed groups and control were checked by using Student's-test after testing for homogeneity of variance.

3. Results

3.1. Expression of genes related to cytoskeleton and Nrf2/ARE signaling pathways

As shown in Fig. 1A, tubulin alpha 1 chain (TUBA1C) and tubulin beta 1 chain (TUBB1) genes presented similar expression profile of early inhibition and later up-regulation after exposure to *P. lima*. At 3 h, TUBA1C expression was significantly down-regulated both in the low and high density groups ($p < 0.05$). At 96 h, TUBA1C transcript was significantly up-regulated in the high density group ($p < 0.05$). Similar to TUBA1C, the expression of TUBB1 was significantly decreased at 6 h, but increased at 96 after exposure to 2×10^5 cells/L and 2×10^6 cells/L *P. lima* ($p < 0.05$ or 0.01).

Expression of genes related to Nrf2/ARE signaling pathways after exposure to *P. lima* are given in Fig. 1B. KEAP1 gene expression was significantly up-regulated at 3 h after exposure to *P. lima*. However, it was significantly down-regulated at 96 h after exposure to high density of *P. lima*, while Nrf2 was significantly up-regulated at that time. Correspondingly, expression of Nrf2 downstream genes such as NQO1, SOD, GST-ω and ABCB1 were all up-regulated at 96 h after exposure to high density of *P. lima*. However, these genes related to Nrf2/ARE signaling pathways displayed various expression changes after short-time exposure of *P. lima*. After 6 h exposure of *P. lima*, NQO1 transcript was decreased under 2×10^6 cells/L of *P. lima*, whereas ABCB1 was increased under 2×10^5 cells/L of *P. lima*. In addition, multidrug resistance associated protein (MRP), GPx1 and CAT genes also underwent some alteration after exposure of *P. lima*. The expression of MRP was significantly up-regulated in the low density group at 6 h ($p < 0.05$), but down-regulated at 96 h both in the low and high density group ($p < 0.05$). GPx1 transcript was significantly decreased at 3 h and 6 h in the high density group ($p < 0.05$). CAT gene expression was significantly up-regulated in the high density group at 6 h ($p < 0.05$).

3.2. Antioxidant enzyme activity and MDA content

The level of MDA was increased significantly in gill tissues of the mussel at 6 h and 96 h after exposed to high density of *P. lima* (Fig. 2). Corresponding, the activity of SOD also increased at that time after exposure of high density *P. lima* ($p < 0.05$). However, there was no distinct change in GPx activity after *P. lima* exposure ($p > 0.05$).

3.3. Histological alterations of gill tissue

Paraffin section demonstrated that the gill tissue of *P. viridis* experienced some structural changes after exposure to *P. lima*. In control, the gill filaments were complete, with clear clearance and dense microvilli on the top of the cells. However, after 3 h or 6 h exposure of high density of *P. lima*, the gill tissue structure presented distinct changes (Fig. 3). As shown in Fig. 3, at 3 h after exposure of high density *P. lima*, the branchial filament structure has begun to deform, with extremely irregular arrangement. At 6 h, the branchial filament structure was completely deformed, and the cells atrophied into strips with irregular arrangement. However, the gill filament structure has gradually restored at 96 h after exposure. The clearance regained clear and the cell morphology was restored, and only a few cells have not fully restored to their normal state. Similar morphological changes in gill tissues occurred at 6 h and 96 h in the low density *P. lima*-exposed mussels with unclear and obvious cell gaps and irregular arrangement.

4. Discussion

Cytoskeleton is present in all cells, playing an important role in maintaining cell shape, protecting cells, and cell signaling, etc. It is proposed that the proteins associated with cytoskeleton structure is one of the first targets of oxidative stress mediated by unbalanced calcium homeostasis [30,31]. Interestingly, cytoskeleton is the important target of DSP toxins, and DSP toxins-induced oxidative damage have been well-documented in bivalves. Annexins are usually regarded as Ca^{2+} sensors or effectors in cytosol Ca^{2+}-driven processes [32]. Importantly, an annexin-like protein has been found to be down-regulated in gill of the mussel after exposure of *P. lima* for 6 h [22]. Gill as a robust detoxification system, have rapid amplification of key proteins and genes

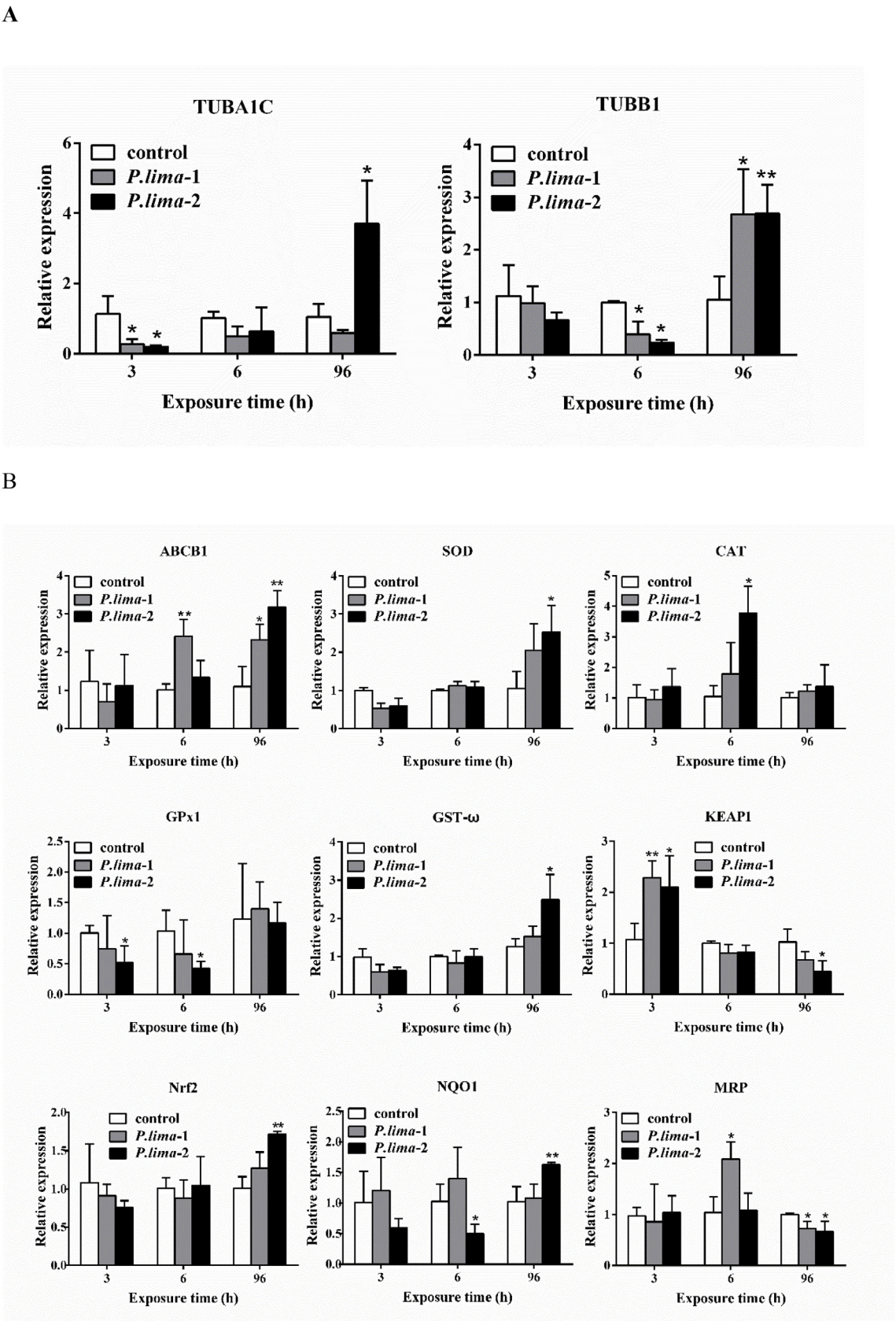

Fig. 1. Changes in expression of genes related to cytoskeleton (A) and Nrf2/ARE signaling pathway (B) after exposure to *Prorocentrum lima*. *P. lima*-1, 2×10^5 cells/L *P. lima*; *P. lima*-2, 2×10^6 cells/L *P. lima*. Data are expressed as mean ± SD (n = 3). Significant differences compared to control are represented by asterisks (*t*-test, $^*p < 0.05$; $^{**}p < 0.01$).

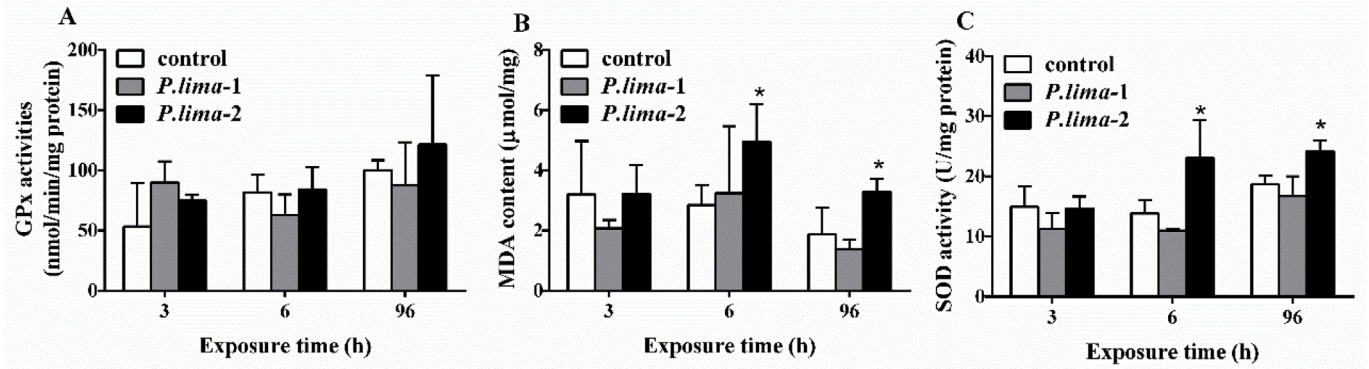

Fig. 2. Changes in SOD activity, GPx activity and MDA content after exposure to *Prorocentrum lima*. *P. lima*-1, 2×10^5 cells/L *P. lima*; *P. lima*-2, 2×10^6 cells/L *P. lima*. Data are expressed as mean ± SD (n = 3). Significant differences compared to control are represented by asterisks (*t*-test, $*p < 0.05$; $**p < 0.01$).

concerning with detoxicification, for instance GST [33]. It has been considered as a major site for antioxidant protection under acute exposure in bivalves [34]. So we chose cytoskeleton of the gill in mussel to investigate the changes of DSP toxins-induced toxicity with exposure time. We found that the branchial filament structure was completely deformed, and the cells atrophied into strips with irregular arrangement after short-time exposure of *P. lima.* Meanwhile, expressions of TUBA1C and TUBB1 in gill of the mussel were significantly down-regulated, and MDA content and SOD activity were increased significantly. Microtubules as important component of cytoskeleton, are very important in a number of cellular processes, concerning maintaining of the cell structure, movement of secretory vesicles, organelles and intracellular macromolecular assemblies, and cell division, etc. Microtubules are assembled from dimers of α- and β-tubulin. Decrease of TUBA1C and TUBB1 transcripts and morphological alternation of gill in the mussel gave direct evidence for that short-time exposure of DSP toxins induced damage of gills. However, after exposure for 96 h, the gill filament structure has gradually restored. Correspondingly, TUBA1C and TUBB1 transcripts were significantly increased. It is obvious that some resistance mechanism against DSP toxin has been triggered, and thus reduced the negative effect of toxin on bivalve.

The Nrf2/ARE signaling pathway is a key mediator in oxidative stress, playing an important protective role for the body to resist oxidative damage [18]. Alterations in Nrf2 and Keap1 expression and dysregulation of the Nrf2/ARE signaling pathway can contribute to reduction of phase II metabolic enzyme genes such as GST and NQO1, which in turn results in increased sensitivity to various oxidative stress sources, and aggravates the toxic effect of oxidative stress on cells [35]. To explore the reason for the reduction in damage after longer exposure of DSP toxins, we analyzed expression of genes related to Nrf2/ARE signaling pathway. It was found that KEAP1 expression was up-regulated at 3 h after exposure to low density of *P. lima*. However, the expression of KEAP1 and Nrf2 did not exhibit significant changes at 6 h and 96 h, indicating that the low density of *P. lima* did not activate the Nrf2 signaling pathway in the gill tissue of mussel. The up-regulation of ABCB1 and MRP indicated that the mussel might be mainly dependent on ABC transporter family for detoxification of DSP toxins when the mussel was exposed to low density of *P. lima*. ABCB1 is also known as multidrug resistance protein 1 (MDR1) or P-glycoprotein (P-gp). P-gp, and MRP mediate most multi-xenobiotic resistance (MXR) in aquatic

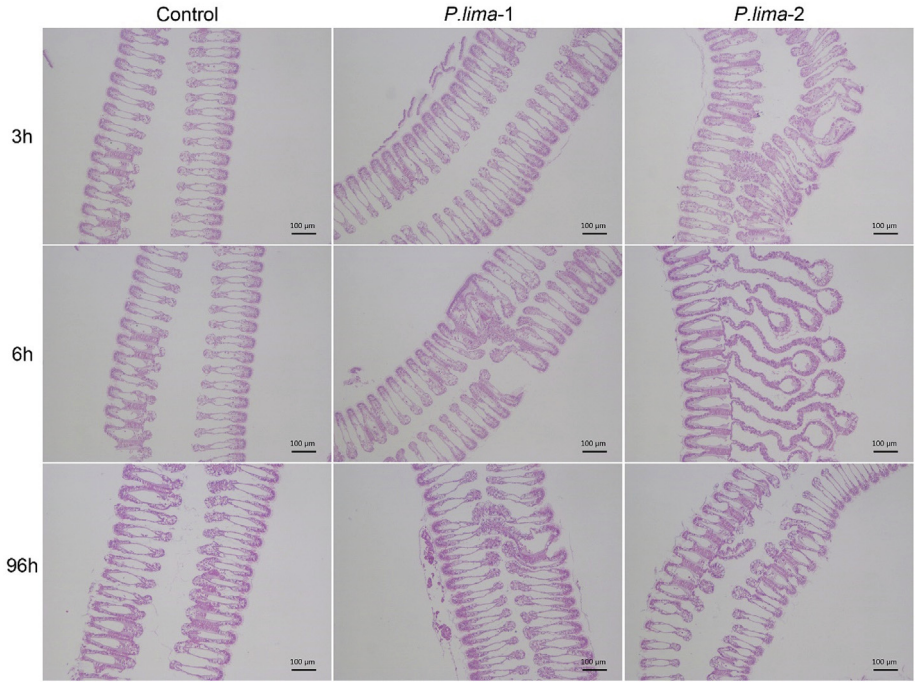

Fig. 3. Histological alterations of gill tissues after exposure to *Prorocentrum lima* revealed by paraffin section stains. *P. lima*-1, 2×10^5 cells/L *P. lima*; *P. lima*-2, 2×10^6 cells/L *P. lima*.

invertebrates, pumping xenobiotics out of cells/organisms, which in turn prevent the accumulation of xenobiotics and associated harmful effects on cells/organisms [36,37]. It has been found that P-gp and MRP were constitutively expressed in larvae and adults of zebra mussel, and that they could be induced as a cellular stress response [38]. So far, many paper have dealt with the role of P-gp in resistance to DSP toxins [12,22]. P-gp and MRP have been suggested to be involved in the resistance mechanisms of DSP toxins in bivalves [9,11,37].

When exposed to high density of *P. lima*, KEAP1 at 3 h and CAT gene at 6 h were up-regulated, while NQO1 at 6 h, and GPx1 at 3 h and 6 h were down-regulated. However, the expression of Nrf2 was found to display no significant changes at 3 h and 6 h. In general, KEAP1 is negatively correlated with Nrf2 expression. Under static states, Nrf2 binds to Keap1 in the cytoplasm, which facilitates the ubiquitination and subsequent proteolysis of Nrf2. The sequestration and further degradation of Nrf2 contribute to the repressive effects of Keap1 on Nrf2 [39,40]. The increase in KEAP1 transcript and decreases in NQO1 and GPx1 after short-term exposure of DSP toxins suggested that the Nrf2 signaling pathway might not be activated, though Nrf2 pathway downstream genes had some changes in the mRNA level after short-term exposure of DSP toxin. In line with our result, Danielli et al. [34] found that curcumin could induce GSH-related antioxidant defenses in the gills of Pacific oysters *Crassostrea gigas* and up-regulate expression of glutamate-cysteine ligase, glutathione reductase (GR), GPx2 and GST-pi when Nrf2 and Keap1 genes did not induced. Pregnane X receptor (PXR) and other nuclear receptors might contribute to the changes due to their crucial roles in regulating phase I and II enzymes and transporters [41].

After longer exposure of high density of *P. lima*, KEAP1 expression was significantly down-regulated and Nrf2 was significantly up-regulated in the gill of the mussel. Correspondingly, expressions of GST-ω, SOD, NQO1 and ABCB1 genes were up-regulated at that time. Combined with increase in SOD activity and restoration of cytoskeleton after longer exposure, we proposed that longer exposure of *P. lima* in high density could activate Nrf2 signaling pathway, thereby reducing the influence of toxin on mussel.

It is of note that MRP transcript was decreased after 96 h exposure of high density of *P. lima*, while ABCB1 was increased distinctly. Lin et al. [42] proposed that there was a compensatory mechanism in P-gp and MRP-mediated resistance, which in turn made up a functional network to defense against xenobiotics [43]. In line with our observation, Valenzuela-Muñoz et al. [44] found that ABCB was up-regulated but ABCC down-regulated in the salmon louse *Caligus rogercresseyi* after exposure to deltamethrin. However, the reason for this should be further studied in the future.

5. Conclusion

Taking all together, we proposed that *P. lima* in some cell density had a negative impact on cytoskeleton of mussel gill tissue, and caused oxidative damage to the gill. However, longer exposure of *P. lima* in high density could activate Nrf2 signaling pathway, thereby reducing the toxicity of DSP toxins on mussel. Our study might provide a novel clue for the resistance mechanism of bivalves to DSP toxins.

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