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Toxic Dinoflagellate *Alexandrium tamarense* Induces Oxidative Stress and Apoptosis in Hepatopancreas of Shrimp (*Fenneropenaeus chinensis*)

LIANG Zhongxiu[1), 2)], LI Jian[2), *], LI Jitao[2)], TAN Zhijun[2)], REN Hai[1)], and ZHAO Fazhen[2)]

1) *College of Fisheries and Life Science*, *Shanghai Ocean University*, *Shanghai* 201306, *P.R. China*
2) *Key Laboratory of Sustainable Development of Marine Fisheries of Ministry of Agriculture*; *Yellow Sea Fisheries Research Institute*, *Chinese Academy of Fishery Sciences*, *Qingdao* 266071, *P. R. China*

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Abstract This study investigated the inductive effect of *Alexandrium tamarense*, a toxic dinoflagellate producing paralytic shellfish poison, on oxidative stress and apoptosis in hepatopancreas of Chinese shrimp, *Fenneropenaeus chinensis*. The individuals of *F. chinensis* were exposed to 200 and 1000 cells mL^{-1} of *A. tamarense* with their superoxide dismutase (SOD), glutathione S-transferase (GST) activities, malonyldialdehyde (MDA) concentration, and caspase gene (*FcCasp*) expression in hepatopancreas determined at 12, 24, 48, 72 and 96 h. In addition, apoptosis in hepatopancreas of *F. chinensis* at 96 h after exposure was determined through terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The hepatopancreatic SOD and GST activities of *F. chinensis* exposed to 1000 cells mL^{-1} of *A. tamarense* showed a bell-shaped response to exposure time. The hepatopancreatic MDA concentration of *F. chinensis* exposed to 1000 cells mL^{-1} of *A. tamarense* increased gradually from 48 to 96 h, and such a trend corresponded to the decrease of GST activity. The hepatopancreatic *FcCasp* transcript abundance of *F. chinensis* exposed to 1000 cells mL^{-1} of *A. tamarense* was positively and linearly correlated to MDA concentration. Results of TUNEL assay showed that exposure to 1000 cells mL^{-1} of *A. tamarense* induced apoptosis in the hepatopancreas of *F. chinensis*. Our study revealed that *A. tamarense* exposure influenced the antioxidative status of *F. chinensis* and caused lipid peroxidation and apoptosis in the hepatopancreas of shrimp.

Key words *Alexandrium tamarense*; oxidative stress; apoptosis; *Fenneropenaeus chinensis*

1 Introduction

Harmful algal blooms can damage shrimp due to their oxygen consumption and toxin production (Alonso-Rodríguez and Páez-Osuna, 2003). The toxic dinoflagellate *Alexandrium tamarense* is known to produce paralytic shellfish poisoning toxins (PSTs) and non-PSP toxic substances such as hemolytic toxin (Yan *et al*., 2003; Tan, 2006). These toxins impose a far-reaching impact on the health of aquatic organisms and humans (García *et al*., 2004). In natural environments, aquatic organisms are exposed to toxic *A. tamarense* during bloom formation and decay mainly through the ingestion of *Alexandrium* cells and/or the bioaccumulation of water-borne toxins (Tan *et al*., 2007; Costa *et al*., 2010).

The Chinese shrimp *Fenneropenaeus chinensis* is widely cultured in China (Wang *et al*., 2011). This species is ideally suited for culture because of its rapid growth, resistance to low temperature, excellent nutritional properties, and easiness of adaptation to artificial culture conditions (Li *et al*., 2006). Concomitant with the growth of this industry has been an increase in the importance of looking at diseases, mostly those provoked by infectious agents. Previous research has focused on the most important diseases of cultured crustacean associated with viral, bacterial, and fungal infections. In recent decades, the effects of environmental stress and toxins have gained increasing research attention (Stephan *et al*., 2005; Wang *et al*., 2011). In ponds where prawns are cultured, dinoflagellate blooms frequently occur because of the shallow water and high nutrient loading (Su *et al*., 1993; Lin, 1996). The issue of the potential hazard of *A. tamarense* to prawns arose in 1989 when Su *et al*. (1993) suspected that *A. tamarense* could cause mortality in prawns. In shrimp farms in some areas of China, the observed effects are morbidity and a delay in the growth of shrimp that turns into an economic loss (Zhu, 1993).

Reactive oxygen species (ROSs) and the resulting oxidative stress to living organisms have been reported to be involved in the pollution-mediated mechanism of toxicity, which can damage macromolecules such as DNA and proteins and cell membrane (Skulachev, 1998; Living-

* Corresponding author. Tel: 0086-532-85830183
E-mail: lijian@ysfri.ac.cn

stone, 2001). Antioxidant enzymes are able to eliminate highly reactive intermediate ROSs induced by pollutants to maintain cell homeostasis. Superoxide dismutase (SOD) and glutathione S-transferases (GST) are among the major antioxidant enzymes. Malonyldialdehyde (MDA) is an end point of lipid peroxidation (LPO), whose formation is regarded as a general indicator of LPO (Winston, 1991). ROS can be involved in apoptosis which is well conserved in terms of both morphological features and genes controlling the process (Armstrong, 2006; Fleury *et al*., 2002). Caspases are the critical central molecules of apoptotic pathways (Thornberry, 1998). Previous results indicated that overexpression of caspase gene resulted in apoptotic morphological features in shrimp cells (Leu *et al*., 2008; Wang *et al*., 2011).

The impact of toxic strains of *A. tamarense* on the survival, growth, fecundity and nutritional status of shrimp has been studied (Tan *et al*., 2002). In addition, the toxic dinoflagellate may induce oxidative stress as shown by enhanced LPO and depressed antioxidant defense system of aquatic organisms (Estrada *et al*., 2007). However, few studies have dealt with *A. tamarense*-induced apoptosis via the production of free radicals in *F. chinensis*.

The aim of this study was to evaluate the toxicity of *A. tamarense* to *F. chinensis*. First, *A. tamarense*-induced oxidative stress to the shrimp was measured through determination of the antioxidant enzyme activities and MDA concentration. Second, *A. tamarense*-induced apoptosis in hepatopancreas of the shrimp was evaluated by determining *FcCasp* transcript abundance with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The results will aid to gaining valuable information on shrimp antioxidative system and the physiology of hepatopancreas under *A. tamarense* stress.

2 Materials and Methods

2.1 Shrimp Culture

Chinese shrimp (*F. chinensis*), 6.8 cm± 0.3 cm in length and 3.6 g± 0.2 g in weight, were collected from Baorong Aquatic Product Technology Co., Ltd. (Qingdao, China). Prior to the experiment, the shrimp were reared in 200-L polyvinyl chloride polymer tanks containing aerated seawater (salinity 30 and temperature 23℃± 1℃) for seven consecutive days to acclimate them to laboratory conditions. During acclimation, the shrimp were fed with commercial prawn pellets, three times each day, and seawater was exchanged by 30% every day.

2.2 Algal Preparation

The toxic dinoflagellate *A. tamarense* ATHK was obtained from Institute of Oceanology, Chinese Academy of Sciences, and cultured in *f/2* medium prepared with filtered (0.45 μm) seawater. Strain ATHK was grown in monoalgal culture in 5-L glass flasks on a 14 h:10 h light:dark photocycle at 20℃. Algal cells at exponential growth phase were harvested and used for the experiments. According to Tan *et al*. (2007), the PSTs produced by strain ATHK were mainly composed of N-sulfocarbamoy1 toxins (C, B1) and gonyautoxin (GTX1/4, GTX2/3). The GTX toxins accounted for the highest proportion of the total toxins (54.84%). The total concentration of PSPs produced by ATHK was 5.538 pg STX Equal/cell.

2.3 *A. tamarense* Exposure and Sampling

After acclimation, 270 shrimp were randomly put into nine 100-L plastic containers, 30 each. The experimental shrimp were randomly divided into three groups, three replicates each. Diluted culture of *A. tamarense* with fresh seawater was used for the exposure treatment, and fresh seawater was used as control. According to preliminary experimental results, the 96-h median lethal concentration of *A. tamarense* was 10000 cells mL^{-1}. In order to get stressful response with low mortality rate, the concentration of *A. tamarense* was adjusted to be 200 and 1000 cells mL^{-1} in this experiment. During the testing period, the shrimp were fed commercial prawn pellets, three times each day, and each test solution was renewed daily based on a static renewal method for toxicity test (Buikema *et al*., 1982). Hepatopancreases were collected from six shrimp each group after 12, 24, 48, 72 and 96 h of exposure and separately preserved in liquid nitrogen for RNA extraction and biochemical determinations. In addition, hepatopancreases were collected from two shrimp each group at the end of the exposure period and fixed with Davidson's solution (95% ethanol 330 mL, formalin 220 mL, acetic acid 115 mL and H_2O 335 mL) for 24 h, then transferred to 70% ethyl alcohol for storage until further analysis.

2.4 Biochemical Analysis

Hepatopancreas samples were prepared as 10% (w:v) homogenates with normal saline. The homogenates were centrifuged at 4000 r min^{-1} and 4℃ for 10 min. The supernatant was recovered and frozen at −80℃ until biochemical determinations. The SOD and GST activities and MDA concentration were determined with commercial test kits (Jiancheng, Ltd, Nanjing, China) following the manufacturer's instructions. The protein concentration was determined with a Coomassie Brilliant Blue protein assay kit (Jiancheng, Ltd, Nanjing, China).

2.5 RNA Extraction and cDNA Synthesis

Total RNA was extracted from hepatopancreas using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's protocol. Contaminant DNA was removed from RNA extract using RQ1 RNase-Free DNase (Promega, Madison, WI, USA). First-strand cDNA synthesis was performed using M-MLV reverse transcriptase (Promega Madison, WI, USA) according to the manufacturer's instructions.

2.6 Quantification of mRNA Abundance of *FcCasp* Gene

FcCasp gene-specific primers (FcCAS-F and FcCAS-R) were designed to amplify a 127-bp product, and 18S rRNA gene-specific primers (18S-F and 18S-R) were designed to amplify a 218-bp fragment (Table 1). Nuclease-free water was used instead of cDNA templates as PCR control.

The mRNA abundance of *FcCasp* in the hepatopancreas of *F. chinensis* after *A. tamarense* exposure was measured with quantitative real-time PCR (qRTPCR) on an ABI 7500 real-time PCR machine (Applied Biosystems, Foster City, CA, USA) as described by Marisa and Juan (2005). All PCR reactions were repeated in triplicate and the 20-μL qRTPCR reaction mixture contained 10 μL of 2×SYBR Premix Ex Taq α (TaKaRa), 1 μL of cDNA (1:10 diluted with PCR-grade H_2O), 0.8 μL each of 10 μmol L^{-1} forward and reverse primers, 0.4 μL of Rox Reference Dye, and 7 μL of PCR-grade water. The qRTPCR was set as 95℃ for 30 s, followed by 40 cycles of 95℃ for 5 s and 60℃ for 34 s. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. The fluorescent real-time PCR data were analyzed using the 7500 System SDS Software (Applied Biosystems, Foster City, CA, USA).

Table 1 Primers used in this study

Primer	Sequence (5'–3')
FcCAS-F	GCTTGTTATGTGGGCTTCCTAC
FcCAS-R	CACTTCCCTGCTGACTTTGAG
18S-F	AGTAGCCGCCCTGGTTGTAGAC
18S-R	TTCTCCATGTCGTCCCAGT

Note: F and R stand for forward and reverse primer, respectively.

2.7 TUNEL Assay

DNA fragmentation analysis was conducted using the DeadEnd™ Colorimetric TUNEL System (Promega, USA) according to manufacturer's instructions. The tissue sections were deparaffinized, rehydrated, and treated with protein digestion buffer at room temperature for 15 min, followed by incorporation of biotinylated nucleotide mix in the presence of working-strength terminal deoxynucleotidyl transferase (TDT) at 37℃ for 60 min. The reactions were terminated by immersing the slides in 2×saline sodium citrate (SSC) at room temperature for 15 min. Then, the tissue samples were developed with dimethylaminoazobenzene at room temperature for 5 min, and the slides were mounted in an aqueous mounting medium followed by examination under a light microscope. One positive control test was included in this experiment. The rehydrated paraffin sections of hepatopancreas tissues were incubated with 10 U mL^{-1} DNase (Promega, Madison, WI, USA) for 10 min and then washed thoroughly with phosphate buffered saline prior to TUNEL assay. A negative control was prepared by immersing a rehydrated paraffin tissue section into the labeling solution without TDT and then directly used for TUNEL assay.

2.8 Statistical Analysis

Data are expressed as means ± standard error. After testing the homogeneity of variance, statistical difference between treatments and controls were determined by single-factor one-way ANOVA. LSD multiple comparison tests were carried out when the variances were homogeneous. Values were calculated using SPSS 16.0 software. For all tests, the level of significance was set at $P<0.05$.

3 Results

3.1 Hepatopancreatic SOD and GST Activities and MDA Concentration

The hepatopancreatic SOD activity of shrimp exposed to 1000 cells mL^{-1} of *A. tamarense* was significantly ($P<0.05$) higher than that of control at all sampling points and showed a bell-shaped response to the increase of exposing time. The maximum hepatopancreatic SOD activity (3.9-folds of control) was observed at 48 h post treatment (Fig.1a). However, the hepatopancreatic SOD activity of shrimp exposed to 200 cells mL^{-1} of *A. tamarense* was similar to each other ($P>0.05$) except for those observed at 48 h.

Likewise, the hepatopancreatic GST activity of shrimp exposed to 1000 cells mL^{-1} of *A. tamarense* showed a bell-shaped response to the increase of exposing time and reached the maximum (2.8-folds of control) at 48 h post treatment. Thereafter, the hepatopancreatic GST activity decreased to the minimum (0.6-fold of control) at 96 h post treatment (Fig.1b). Similar to the hepatopancreatic SOD activity, the hepatopancreatic GST activity of shrimp exposed to 200 cells mL^{-1} of *A. tamarense* did not change significantly throughout the experiment ($P>0.05$). The hepatopancreatic MDA concentration of shrimp exposed to 1000 cells mL^{-1} of *A. tamarense* increased gradually from 48 to 96 h (Fig.1c), whereas no significant differences were observed in hepatopancreatic MDA concentrations between 200 cells mL^{-1} of *A. tamarense* treatment and the control group ($P>0.05$).

3.2 Expression Profile of *FcCasp* Gene

As compared to that of control, the hepatopancreatic *FcCasp* transcript abundance of shrimp exposed to 1000 cells mL^{-1} of *A. tamarense* was significantly inhibited in the first 12–48 h ($P<0.05$). Thereafter, the hepatopancreatic *FcCasp* transcript abundance increased immediately and peaked at 96 h (1.6-folds of control) post treatment (Fig.2). However, the hepatopancreatic *FcCasp* transcript abundance of shrimp exposed to 200 cells mL^{-1} of *A. tamarense* showed no significant increase compared with that of control ($P>0.05$).

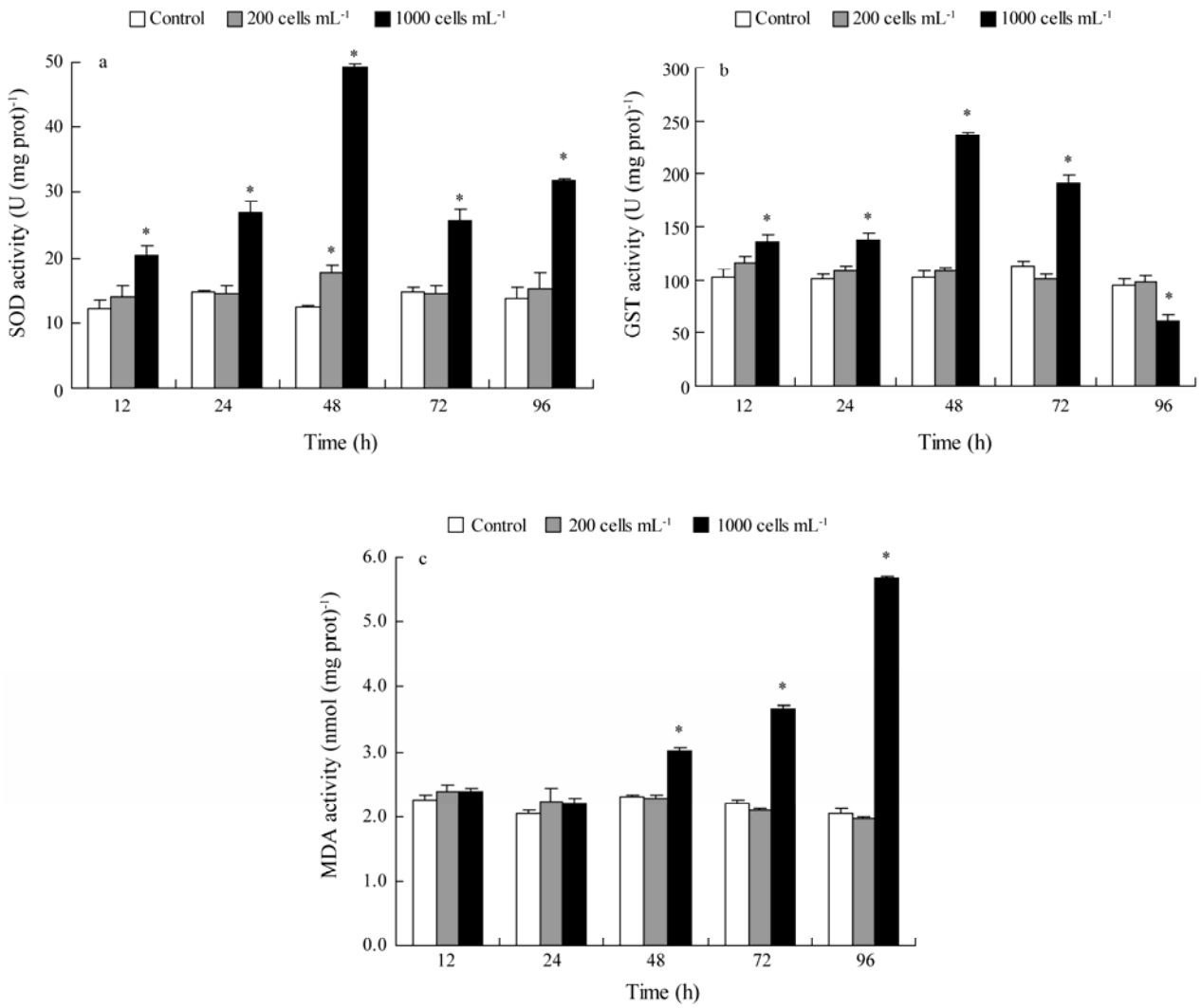

Fig.1 The hepatopancreatic superoxide dismutase (SOD, a) and glutathione S-transferase (GST, b) activities and malonyldialdehyde (MDA, c) concentration of *F. chinensis* exposed to *A. tamarense*. * Means are significantly different from that of control.

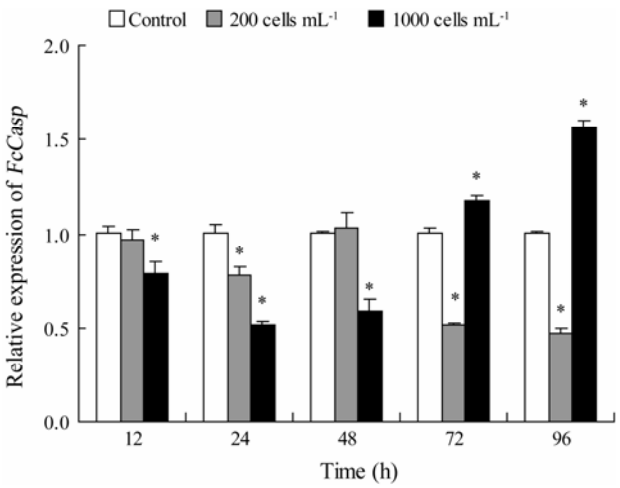

Fig.2 The expression profile of *FcCasp* in hepatopancreas of *F. chinensis* exposed to *A. tamarense.*

3.3 TUNEL Observation

TUNEL-stained cells showed the characteristic ultrastructural features of DNA damage (Fig.3). Apoptotic cells were stained brown by TdT. Brownish nuclei were observed in hepatopancreas of 1000 cells mL^{-1} of *A. tamarense* treatment (Fig.3D), being similar to the TUNEL-positive nuclei found in positive control (Fig.3B). Conversely, TUNEL-positive nuclei were not observed in control (Fig.3C) or 200 cells mL^{-1} of *A. tamarense* group (Fig.3E).

3.4 Correlation Between MDA Concentration and *FcCasp* Transcript Abundance in Hepatopancreas

As clear apoptosis was shown in hepatopancreas of shrimp under 1000 cells mL^{-1} of *A. tamarense* treatment (Fig.3), the correlation between MDA concentration and *FcCasp* transcript abundance in hepatopancreas of *F. chinensis* in 1000 cells mL^{-1} group was analyzed. Results showed that the *FcCasp* transcript abundance had a positive correlation with MDA concentration in hepatopancreas of *F. chinensis* in 1000 cells mL^{-1} of *A. tamarense* treatment ($R^2 = 0.8606$) (Fig.4).

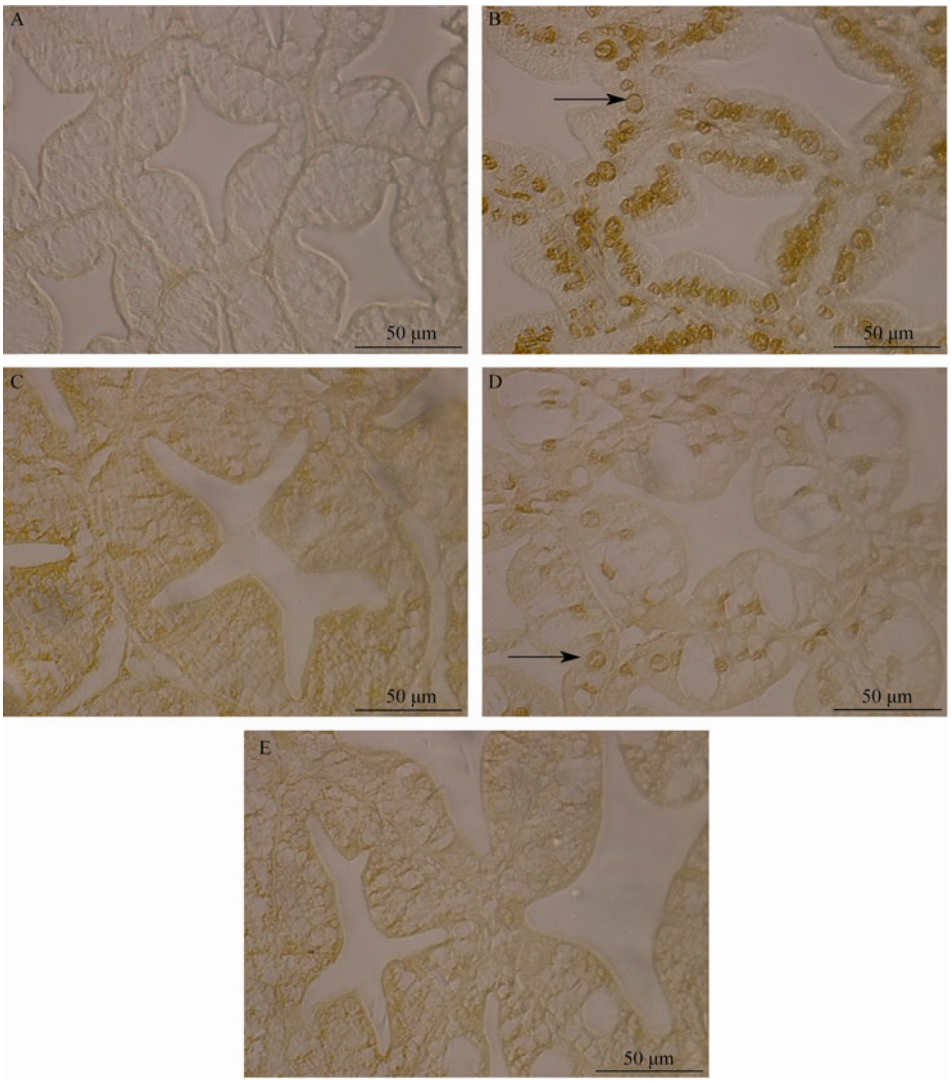

Fig.3 Microscopic images (×40 magnification) of TUNEL assay of hepatopancreas in *F. chinensis* after exposure to 1000 cells mL^{-1} of *A. tamarens* for 96 h. A, negative control; B, positive control, indicating DNA cleavage; C, control group; D, 1000 cells mL^{-1} of *A. tamarense* treatment; E, 200 cells mL^{-1} of *A. tamarense* treatment. Arrows indicate apoptotic cell (brown).

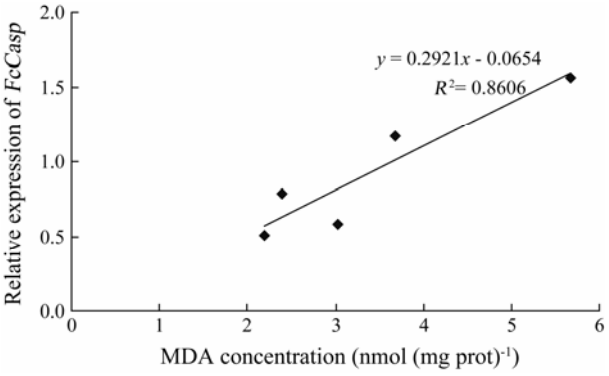

Fig.4 Correlation between malonyldialdehyde (MDA) concentration and *FcCasp* transcript abundance in hepatopancreas of *F. chinensis* exposed to 1000 cells mL^{-1} of *A. tamarense.*

4 Discussion

There is a dynamic balance between the production and elimination of ROSs. Cells possess antioxidant defense systems to prevent the formation of ROSs, and the SOD activity plays an important role in antioxidant protection of invertebrates (Livingstone, 2001; Livingstone, 2003). It is known that superoxide anion (O_2^-) is one of the major ROSs. SOD is the first enzyme to respond to O_2^- and offers the greatest response to oxidative stress (Trezado *et al*., 2006). The increase in hepatopancreatic SOD activity of shrimp exposed to 1000 cells mL^{-1} of *A. tamarense* indicated that O_2^- was formed during the exposure treatment. In contrast, the hepatopancreatic SOD activity of shrimp under 200 cells mL^{-1} of *A. tamarense* treatment was not effectively stimulated within the exposure time except at 48 h. This observation indicated that the level of O_2^- produced under 200 cells mL^{-1} of *A. tamarense* was not sufficient to effectively increase the SOD activity. The hepatopancreatic SOD activity of shrimp under 1000 cells mL^{-1} of *A. tamarense* treatment decreased at 72 h post treatment, probably due to the poisonous effect of excessive O_2^- (Sun *et al*., 2007). The bell-shaped response pattern of hepatopancreatic SOD activity of *F.*

chinensis in the present study was consistent with the reported in perch *Lateolabrax japonicus* (Tan, 2006).

GSTs are a multicomponent family of phase II biotransformation enzymes that detoxify diverse electrophilic endogenous and xenobiotic substrates by means of conjugation with GSH to produce less toxic and more water-soluble compounds (Ron *et al*., 2003), each of which plays a vital role in protecting tissues from oxidative stress (Wang and Ballatori, 1998). The increase in hepatopancreatic GST activity of shrimp exposed to 1000 cells mL^{-1} of *A. tamarense* indicated that the presence of *A. tamarense* generated stress to the cellular metabolism of shrimp. The toxic dinoflagellate *A. tamarense* ATHK is known to produce PSTs, mainly N-sulfocarbamoyl toxins (C, B1) and Gonyautoxin (GTX1/4, GTX2/3) (Tan *et al*., 2007). The GSTs catalyze the conjugation of PSTs with GSH, promoting the excretion of those compounds (Costa *et al*., 2012). The transformation of C-11 sulfated carbamate analogues (*e.g*., GTX2+3 and GTX1+4) into STX and NEO involved the GSH conjugation using GST as catalyst (Sakamoto *et al*., 2000; Sato *et al*., 2000). Induction of the GST activity has also been reported in Atlantic salmon and puffer fish exposed to PSTs (Gubbins *et al*., 2000; Jeon *et al*., 2008; Costa *et al*., 2012). The GST activity under 1000 cells mL^{-1} of *A. tamarense* first increased and then decreased with the increase of exposing time, indicating that more ROSs were produced and that the GST activity was more effectively enhanced at higher dose. Meanwhile, extra ROSs inactivated the GST activity by interacting with the enzyme (Wang and Ballatori, 1998). The relatively altered GST activity at the lowest level under 200 cells mL^{-1} of *A. tamarense* within the exposure time might have been caused by the low level of oxidative stress that could not effectively induces GST activity in shrimp.

Because of the presence of antioxidants including SOD and GST, the hepatopancreatic MDA concentration of shrimp under 1000 cells mL^{-1} of *A. tamarense* did not vary significantly within 24 h. However, the antioxidant response was not sufficient to prevent oxidative damage with increasing exposure time. The hepatopancreatic MDA concentration under 1000 cells mL^{-1} of *A. tamarense* treatment was significantly greater than that of control from 48 to 96 h, and displayed a time-response relationship. The increasing trend of hepatopancreatic MDA concentration was in accordance with the decreasing trend of GST activity. Thus, it is predicted that the MDA concentration would increase while the GST activity would be inhibited if shrimp are chronically exposed to 1000 cells mL^{-1} of *A. tamarense*. The increased hepatopancreatic MDA concentration of shrimp exposed to 1000 cells mL^{-1} of *A. tamarense* indicated that the critical balance between oxidants and antioxidants was disrupted and that the defense mechanism of antioxidants was declined in shrimp under *A. tamarense* stress. Similarly, a previous study reported that *Alexandrium* could induce oxidative stress as shown by the enhancement of MDA production (Chen, 2008). Estrada *et al*. (2007) revealed that the toxic dinoflagellate *Gymnodinium catenatum* could induce oxidative stress as shown by enhanced LPO production and depressed antioxidant defense system of giant lions-paw scallop *Nodipecten subnodosus*.

The peroxidation products accumulated in hepatopancreas could result in changes in cellular ultrastructure and induce apoptosis (Xu *et al*., 2009). Caspases are the critical central molecules of apoptotic pathways (Thornberry, 1998). Leu *et al* (2008) noted that overexpression of *Penaeus monodon* caspase resulted in apoptotic morphological features in SF-9 cells. In the present study, the hepatopancreatic *FcCasp* transcript abundance of shrimp under 1000 cells mL^{-1} of *A. tamarense* was significantly induced from 72 to 96 h and apoptotic cells were observed at 96 h post treatment. These observations demonstrated that *A. tamarense* induced apoptosis in hepatopancreas. A positive correlation was observed between the hepatopancreatic MDA concentration and the *FcCasp* transcript abundance, which confirmed that LPO is related to the development of apoptosis in hepatopancreas of shrimp (Buttke and Sandstrom, 1994).

5 Conclusions

Results of the present study showed that exposure to 1000 cells mL^{-1} of *A. tamarense* influenced the antioxidative status of *F. chinensis,* and caused LPO and apoptosis in the hepatopancreas of shrimp. Future studies are needed to analyze the ROS level for directly proving the oxidative stress caused by *A. tamarense*. The current results couldn't prove that PSTs were the main cause of oxidative stress and apoptosis in the hepatopancreas of shrimp. Therefore, further studies are necessary to examine the effect of PSTs on above-mentioned indices.

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