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# Effect of Dietary Vitamin E Supplementation on Oxidative Stress Response of Acute Nitrite Exposure in Sea Cucumber (*Apostichopus japonicus* Selenka) Juveniles

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**Abstract:** Different amounts of vitamin E supplementation were added to feed sea cucumber (*Apostichopus japonicus* Selenka) juveniles (2.5±0.15 g) testing to reduce nitrite stress. Commercial feed was used as the control diet, 3 experimental diets containing vitamin E supplementation (150, 250 and 350 mg $kg^{-1}$ diet), respectively was designed to experiment for 45 days. The specimens were exposed to three different concentrations (0.5, 1.0 and 1.5 mg $L^{-1}$) of nitrite for 4, 8 and 12 h at four different time points (0, 15, 30 and 45 days). Hydroxyl free radical (-OH), Malondialdehyde (MDA), Total Antioxidant Capacity (T-AOC), Superoxide Dismutase (SOD) and Catalase (CAT) were measured. The control group' level of -OH and MDA turned higher whereas T-AOC turned lower and SOD and CAT activity elevated after exposed to nitrite solution. The experimental group' level of -OH and MDA turned lower whereas T-AOC turned higher. The results suggested vitamin E supplementation diets could reduce nitrite stress response and increase the antioxidant capacity. The optimal amount of vitamin E supplementation ranged from 180-260 mg $kg^{-1}$ and number of feeding days ranged from 33-35 days were obtained according regress equations.

**Key words:** Sea cucumber (*Apostichopus japonicus* Selenka), vitamin E, nitrite stress, antioxidant enzyme, Reactive Oxygen Species (ROS), Response Surface Methodology (RSM)

## INTRODUCTION

The sea cucumber *Apostichopus japonicus* (Selenka) is an important holothurian species (Liao, 1980) and is considered to be the most valuable species in many parts of Asia (Sun *et al*., 2004; Okorie *et al*., 2008). Recently, the aquaculture of sea cucumber in China has grown rapidly (Wang *et al*., 2007; Dong *et al*., 2008). Annual output of *A. japonicus* exceeded 10,000 tons in 2010 which result in direct economic benefit of >20 billion US dollars (Ma *et al*., 2013). However, the fast expansion also brought frequent epidemic diseases (Ma *et al*., 2006; Deng *et al*., 2009; Li *et al*., 2010).

Nitrite stress is an important potential factor that can lead to diseases and its toxicity is considered to be the primary concern in aquaculture (Hong *et al*., 2009). In culture systems, sea cucumber juveniles are reared in high stocking density (Pei *et al*., 2012) and are fed by formulated feed during the winter indoor nursery (Wang *et al*., 2007). The presence of feces, excretory products and leftover feed in the tanks and low amounts of water exchange (to maintain sea water temperature) mean that the ammonia and nitrite levels are chronically very high which can severely damage the health of sea cucumber juveniles. The level of nitrite has been reported to reach 0.5-3.0 mg $L^{-1}$ which is far above the water quality standard for fisheries (GB 11607-89, China).

The toxicity mechanism and physiological effects of nitrite have been extensively studied in many aquatic organisms (Stormer *et al*., 1996). The major outcome of nitrite poisoning is the oxidation of haemoglobin to methaemoglobin in erythrocytes (Bodansky, 1951) and consequently blood oxygen transport is compromised since methaemoglobin does not bind oxygen. In addition, nitrites disrupt multiple physiological functions including ion regulatory, respiratory, cardiovascular, endocrine and excretory processes (Jensen, 2003). The exposure to stress in aquatic ecosystems can enhance the intracellular formation of Reactive Species of Oxygen (ROS) such as hydrogen peroxide, superoxide and the hydroxyl radical which induce oxidative damage to biological systems. Some ROS can initiate lipid peroxidation, a self-propagating process in which a peroxyl radical is formed when a ROS has sufficient reactivity to abstract a

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hydrogen atom from an intact lipid and the reaction of ROS with lipids is considered one of the most prevalent mechanisms of cell damage (Halliwell and Gutteridge, 1999). Malondialdehyde (MDA) is the most important index which reflecting the lipid peroxidation. Additionally, changes in the activities of antioxidant enzymes like catalase and superoxide dismutase can be used as possible stress biomarkers in different aquatic organisms (Akhtar *et al*., 2010). To date, no studies about the responses of ROS related indexes and antioxidant levels in sea cucumber exposed to nitrite stress.

Vitamin E (tocopherols) is an important antioxidant in natural. Vitamin E has been known for protective actions against free radicals (Lee and Dabrowski, 2004). There are a few research about dietary vitamins supplementation to reduce stress in fish (Henrique *et al*., 1998; Montero *et al*., 1999; Ortuno *et al*., 2003; Trenzado *et al*., 2008; Li *et al*., 2013) in shrimp (Liu *et al*., 2007) and so on. However, no data for sea cucumbers exist. Under culture conditions, sea cucumber juveniles are easily stressed, especially by nitrite and this can lead to great economic losses. Therefore, understanding the mechanisms that cause nitrite stress and identifying substances that can mitigate such stress are urgently needed to improve sea cucumber aquaculture systems. In the present study, researchers aim to investigate the sea cucumber *Apostichopus japonicus* (Selenka) about:

- How do the ROS-related indexes and the antioxidant activities respond to different levels of nitrite stress at exposure durations of 4, 8 and 12 h?
- Can different amounts of vitamin E supplementation reduce or diminish the nitrite stress responses after a period of days?
- What is the optimal amount and duration of vitamin E supplementation for better growth and reduced stress response in *A. japonicus*?

## MATERIALS AND METHODS

**Experimental diets:** Commercial powder feed (Rui zi feed Co., Ltd. Qingdao) was used as the control diet for the sea cucumber juveniles. Powdered vitamin E (50%) was supplemented to the control diet to obtain experimental diets containing vitamin E supplementation (150, 250 and 350 mg $kg^{-1}$ diet), respectively. Then, the feed was stored at -4°C until use. Table 1 shows the formulation and chemical composition of the four types of feed.

**Experimental animal and stress trial:** The experiments were conducted at Qingdao Ruizi Aquatic Seeding and Breeding Farm, Shandong Province, China. Twelve blue plastic aquaria (100×100×60 $cm^3$) were used for triplicate treatments of each of the four diet experiments. Two hundred healthy juvenile sea cucumbers with initial body weights of 2.5±0.15 g were randomly assigned to each aquarium. The selected sea cucumbers were acclimatized for 7 days and fed with the control diet before the experiments began. When the experiment was started, animals in each group were fed their respective diet twice a day at 8:00 and 14:00. Feeding rate was based on 1% of body weight. The leftover food and feces on the bottom of the aquaria were removed daily 12 h after the 14:00 feeding. Water was continuously aerated and 30% of the water was exchanged daily. The mean water quality parameters were as follows: temperature 15±2°C, pH 8.01±0.02, salinity 30±0.5 PSU, dissolved oxygen>5 mg $L^{-1}$, ammonia-N<0.1 mg $L^{-1}$ and nitrite-N<0.02 mg $L^{-1}$.

Table 1: The formulation and chemical composition of experimental diets feeding for sea cucumber *A. japonicus* containing different amounts of vitamin E supplementation

| Ingredient (%) | Control | A | B | C |
|---|---|---|---|---|
| Fish meal | 6.00 | 6.00 | 6.00 | 6.00 |
| Yeast protein | 6.00 | 6.00 | 6.00 | 6.00 |
| Shrimp shell powder | 6.00 | 6.00 | 6.00 | 6.00 |
| Fermented soybean meal | 8.00 | 8.00 | 8.00 | 8.00 |
| Sargasso | 30.00 | 30.00 | 30.00 | 30.00 |
| Kelp | 10.00 | 10.00 | 10.00 | 10.00 |
| Ulva latuca | 15.00 | 15.00 | 15.00 | 15.00 |
| Corn meal | 10.00 | 10.00 | 10.00 | 10.00 |
| Calcium dihydrogen phosphate | 1.00 | 1.00 | 1.00 | 1.00 |
| Shell powder | 5.00 | 5.00 | 5.00 | 5.00 |
| Spirulina powder | 2.00 | 2.00 | 2.00 | 2.00 |
| Premix[a, b] | 1.00 | 1.00 | 1.00 | 1.00 |
| Vitamin E supplementation | 0.00 | 150.00 | 250.00 | 350.00 |
| **Chemical composition** | | | | |
| Dry matter | 90.21 | 90.31 | 90.88 | 90.09 |
| Crude protein (N×6.25, DM%) | 20.56 | 20.16 | 20.22 | 20.98 |
| Crude fat (DM%) | 2.11 | 2.01 | 2.12 | 2.39 |
| Ash (DM%) | 33.55 | 33.25 | 33.76 | 33.73 |

[a]Mineral mixture (mg $kg^{-1}$ diet): cobalt sulfate, 0.4; copper sulfate, 5.0; ferric citrate, 40; magnesium oxide, 100; manganous sulfate, 10; [b]Vitamin mixture (mg $kg^{-1}$ diet): alpha tocopherol, 100; Na menadione bisulfate, 5; thiamin, 5; riboflavin, 5; calcium pantothenate, 10; nicotinic acid, 100; pyridoxine, 5; folic acid, 2; cyanocobalamin, 0.05; biotin, 0.5; ascorbic acid, 400; p-aminobenzoic acid, 50; inositol, 200; choline chloride, 500; UI $kg^{-1}$ diet: retinol, 10,000; cholecalciferol, 2000

The effects of three nitrite concentrations were tested in this experiment. Nitrite solutions were made by adding sodium nitrite to the fresh sea water to attain 0.5, 1.0 or 1.5 mg $L^{-1}$ in glass aquaria (40×40×50 $cm^3$). Before use, the sea water was aerated and adjusted to 30 PSU. After 24 h starvation, 90 specimens from each group were randomly chosen for the nitrate exposure experiment (i.e., 30 specimens for each nitrate concentration). The specimens were exposed to the respective nitrite concentrations for 4, 8 and 12 h and every time point 6 specimens were removed for samples collecting. During the 45 days of the feeding experiment, the nitrite exposure experiment was conducted on days 0 (i.e., the control), 15, 30 and 45.

**Samples and measurements**

**Sampling procedure:** For each time point in the experiment (0, 15, 30 and 45 days), the body wall tissues were excised when specimens were removed from the nitrite solutions after 4, 8 and 12 h of exposure. Tissues were immediately frozen in liquid nitrogen and stored in the -80°C until determined. The body wall samples were processed in a cold saline solution (sodium chloride, 0.9%) using tissue grinder. Then, they were centrifuged for 15 min, 2500~3000 rpm. The supernatant was transferred to new Eppendorfs and the total amount of protein were calculated using the Bradford Method (Bradford, 1976) then frozen at -20°C immediately for further analysis.

**Measurements methods:** Because every 15 days, 30 sea cucumbers of each group had to get out to be exposed to nitrite stress, growth performance was evaluated by two indexes: Accumulation Weight (AW), Accumulation Weight Gain Ratio (AWGR), Average Special Growth Rate (ASGR) which were calculated as following equations:

$$AW\ (g) = W_{01}+W_{02}+W_{03}+W_{04}$$

$$AWGR\ (\%) = \frac{100\times(AW-W_1)}{W_1}$$

$$ASGR\ (\%\ day^{-1}) = \frac{100\times(\ln AW-\ln W_1)}{T}$$

Where:

$W_1$ = The initial body weight of sea cucumbers in each aquarium

$W_{01}$-$W_{03}$ = The body weights of the sea cucumbers chosen for exposure to nitrite in each aquarium on days 15, 30 and 45, respectively

$W_{04}$ = The body weight of the sea cucumbers remaining in the aquarium on the final day

T = The duration of the experiment (45 days)

The equations were made a few changes according Li (1994). Hydroxyl free radical (-OH) was measured by a spectrophotometer method based on the reaction of $H_2O_2$ and Fenton and turn red with griess reagents and the OD value of the samples was measured 550 nm (Reagent kit A018, Jiancheng Bioengineering Institute, Nanjing, China) Malondialdehyde (MDA) was assayed according Thiobarbituric Acid Reactive Substances (TBARS) protocol (Uchiyama and Mihara, 1978). Total Antioxidant Capacity (T-AOC) was measured according deoxygenizing $Fe^{3+}$ to $Fe^{2+}$ and the OD value of the samples was measured 520 nm (Reagent kit A015, Jiancheng Bioengineering Institute, Nanjing, China).

**Determination of catalase activity:** A Spectrophotometer Method was used according to the measurement of absorbance at 240 nm (Li and Schellhorn, 2007), the catalase activity was defined during every second the amount of decomposition the peroxide in substrate which absorbance between 0.50~0.55 as a unit which was express as U/g protein (Reagent kit A007-2, Jiancheng Bioengineering Institute, Nanjing, China).

**Determination of superoxide dismutase activity:** Superoxide Dismutase (SOD) activity was determined according by Ji (1991) with the assay kit (Reagent kit A001-1, Jiancheng Bioengineering Institute, Nanjing, China). Assay conditions were 65 μmol phosphate buffer, pH 7.8, 1 μmol hydrochloric hydroxylamine, 0.75 μmol xanthine and $2.3\times10^{-3}$ IU xanthine dismutase. The 50 μL of the supernatant given no blank reaction were incubated in the system for 40 min at 37°C and terminated with 2 mL 3.3 g $L^{-1}$ p-aminobenzene sulfonic acid and 10 g $L^{-1}$ naphthylamine. An SOD unit was defined as the amount of enzyme that inhibits the superoxide induced oxidation (monitored at 550 nm) by 50% and was expressed as U/mg protein.

**Statistical analysis**

**One way ANOVA:** The growth performance, ROS indexes and enzyme activity data were analyzed using SPSS 19.0 Statistical Software. Values are presented as means±standard deviations. The enzyme activity data were tested for homogeneity of variance and then compared among time intervals using one-way ANOVA followed by a Duncan multiple comparison test. Differences were considered significant among treatment groups at a probability level of $p<0.05$ and extremely significant at $p<0.01$.

**Response surface analysis:** Because the ROS-related indexes and the enzyme activities were affected by four factors (nitrite concentration, exposure time, vitamin E supplementation level and number of feeding days), RSM was used to analyze the effects of these factors. Design-Expert Software (Version 8.0.7.1, Stat-Ease Inc., Minneapolis, MN, USA) was used to conduct the analysis and Box-Behnken design and quadratic regression equations were used to analyze the data. Differences were considered significant among treatment groups at a probability level of $p<0.05$ and extremely significant at $p<0.01$. The optimal amount of vitamin E that should be added to the diet under nitrite stress was analyzed and the growth performance was taken into account in this analysis.

## RESULTS AND DISCUSSION

**Growth performance:** The growth performance parameters displayed significant differences among the

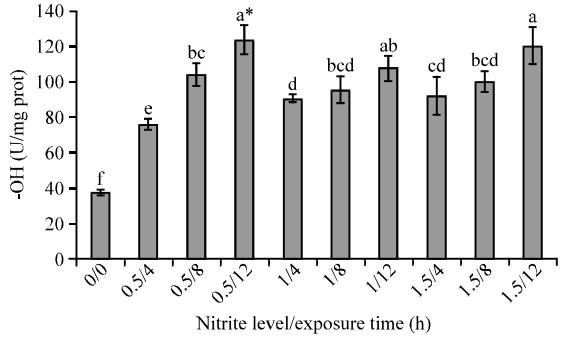


Fig. 1: Changes in the concentration of -OH in *A. japonicus* fed with the control diet under different nitrite levels and exposure times. *Values without same letters differ significantly ($p<0.05$)

Table 2: Effect of vitamin E dietary supplementation on growth performance of *A. japonicus* (mean±SE; n = 3)

| | Diets treatments | | | |
|---|---|---|---|---|
| Parameters | Control | A | B | C |
| AW (g) | 661.78±9.01$^{b}$ | 695.07±20.87$^{a}$ | 717.48±10.70$^{a}$ | 700.53±9.11$^{a}$ |
| AWGR (%) | 32.36±1.80$^{b}$ | 39.01±4.17$^{a}$ | 43.50±2.14$^{a}$ | 40.11±1.82$^{a}$ |
| ASGR(% day$^{-1}$) | 0.62±0.03$^{b}$ | 0.73±0.07$^{a}$ | 0.80±0.03$^{a}$ | 0.75±0.03$^{a}$ |
| Survival rate (%) | 95.12 | 94.32 | 95.09 | 96.76 |

Values in the same row with different superscripts differ significantly ($p<0.05$). Diets treatments: control, commercial feed; vitamin E supplementation groups (mg kg$^{-1}$): A: 150; B: 250; C: 350

diets treatments ($p<0.05$) as is shown in Table 2. The parameters of control diet are significant lower than those of other groups ($p<0.05$). According to the AWGR and the amount of vitamin E supplementation, the quadratic regress equations were obtained; vitamin E $y = 1.51E\text{-}06x^2+0.0077x+32.07$, $R^2 = 0.72$. The equations suggest the best amounts of vitamin E supplementation are 256.30 mg kg$^{-1}$ and the correspondence values of AWGR are 41.98%. There were no differences in survival rate among the four experimental groups.

**Effect of vitamin E supplementation on -OH level under different levels of nitrite stress, exposure time and number of feeding days**

**Changes in -OH level of the control group:** The 0 h value of -OH was significant lower than that of the other exposure time ($p<0.05$) (Fig. 1). The -OH values increased as the exposure time extended. Nitrite levels also increased the hydroxyl free radicals but not significantly after exposure >8 h ($p> 0.05$).

**Differences in -OH level among the vitamin E supplementation groups:** Figure 2 shows -OH changes with the factors of nitrite levels, exposure time, the amounts of vitamin E supplementation and numbers of feeding days, Table 3 shows significant analysis. The

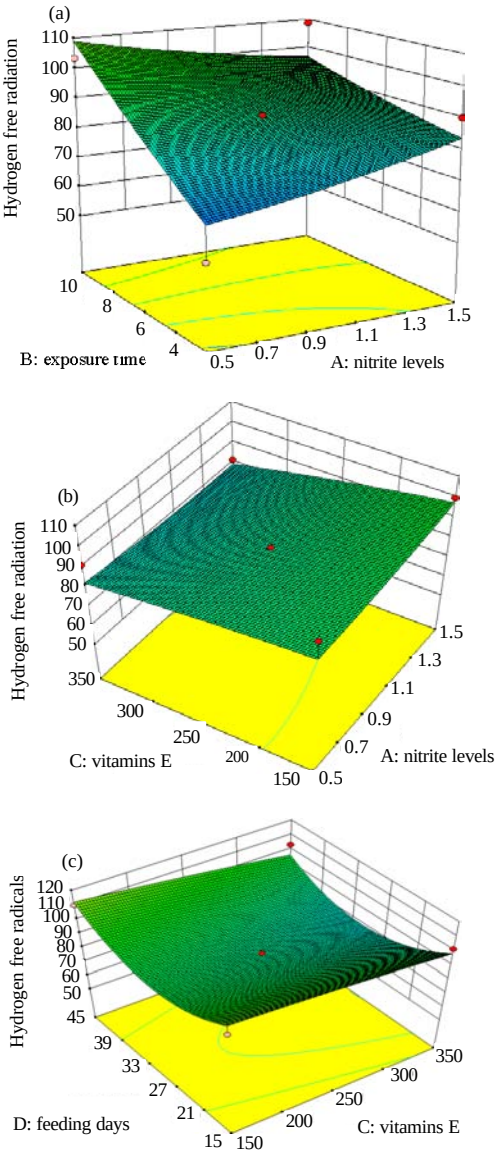


Fig. 2: Response surface plots of -OH level in *A. japonicus* generated using nitrite levels, exposure time, the amounts of vitamin E supplementation and number of feeding days; a) Interaction effect of exposure time and nitrite levels on production of -OH at fixed 250 mg kg$^{-1}$ vitamin E supplementation and 30 feeding days; b) interaction effect of the amount of vitamin E and nitrite levels on production of -OH at fixed 8 h exposure time and 30 feeding days; c) interaction effect of feeding days and the amount of vitamin E on -OH at fixed 1.00 mg nitrite L$^{-1}$ and 8 h exposure time

production of -OH increased following exposure time extending and nitrite levels increasing, significantly with

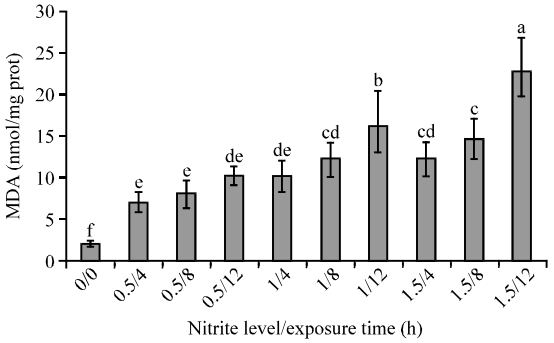


Fig. 3: Changes in the concentration of MDA in *A. japonicus* fed with the control diet under different nitrite levels and exposure times. Values without same letters differ significantly ($p<0.05$)

Table 3: Response surface quadratic model analysis of the -OH level of *A. japonicus* at different nitrite levels, exposure times, amounts of vitamin E supplementation and number of feeding days using ANOVA

| Sources | Sum of squares | df | Mean square | F-values | p-values |
|---|---|---|---|---|---|
| Model | 5678.410 | 14 | 405.600 | 3.66 | 0.0105 |
| A-nitrite levels | 7.010 | 1 | 7.010 | 0.063 | 0.8051 |
| B-exposure time | 2132.920 | 1 | 2132.920 | 19.25 | 0.0006 |
| C-vitamin E | 545.610 | 1 | 545.610 | 4.92 | 0.0435 |
| D-feeding days | 0.043 | 1 | 0.043 | 3.92E-04 | 0.9845 |

Table 4: Response surface quadratic model analysis of the MDA level of *A. japonicus* at different nitrite levels, exposure times, amounts of vitamin E supplementation and number of feeding days using ANOVA

| Sources | Sum of squares | df | Mean square | F-values | p-values |
|---|---|---|---|---|---|
| Model | 153.68 | 14 | 10.98 | 6.26 | 0.0008 |
| A-nitrite levels | 13.69 | 1 | 13.69 | 7.80 | 0.0144 |
| B-exposure time | 59.07 | 1 | 59.07 | 33.66 | <0.0001 |
| C-vitamin E | 18.68 | 1 | 18.68 | 10.64 | 0.0057 |
| D-feeding days | 16.84 | 1 | 16.84 | 9.60 | 0.0079 |

exposure time time ($p<0.05$) but not the nitrite levels ($p>0.05$), may due to the vitamin E supplementation reduce the production of -OH significantly ($p<0.05$). The numbers of feeding days have effects on -OH values but not significantly ($p>0.05$).

**Effect of vitamin E supplementation on MDA concentration under different levels of nitrite stress, exposure time and number of feeding days**

**Changes in MDA level of control group:** As shown in Fig. 3, MDA values of without nitrite stress (i.e., 0 h) was significant lower than that of all the other groups ($p<0.05$). MDA values increased as the exposure time extended and nitrite levels increased and was significantly affected by nitrite levels ($p<0.05$).

**Differences in the MDA concentration among the vitamin E supplementation groups:** All the four factors have significant effects on MDA ($p<0.05$) as shown in Fig. 4 and Table 4. MDA increased as exposure time extending and nitrite levels increasing, decreased while number of

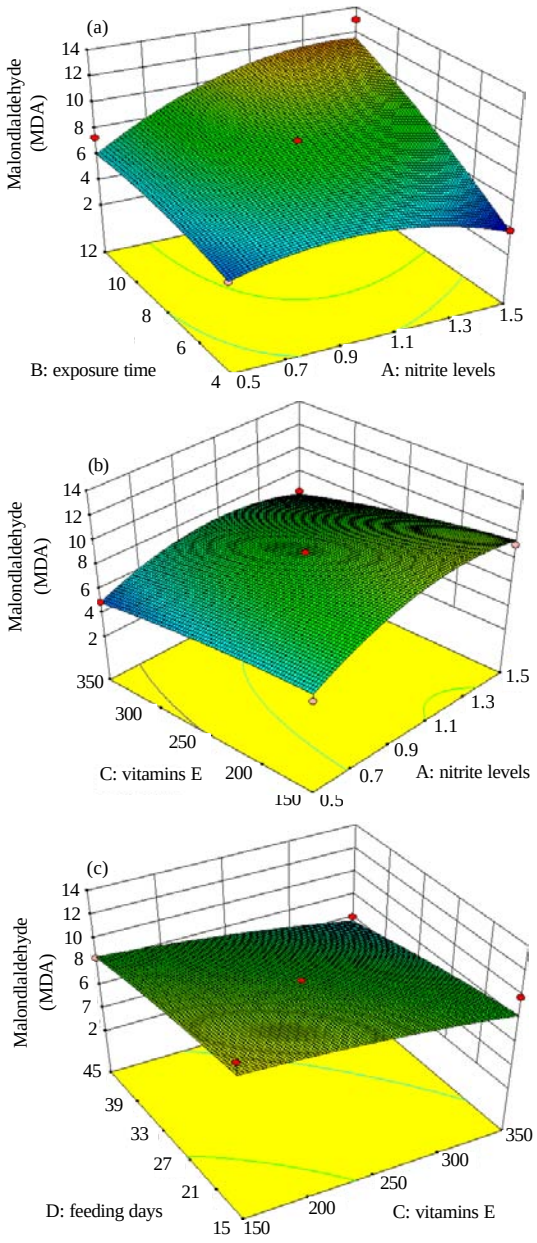


Fig. 4: Response surface plots of MDA level in *A. japonicus* generated using nitrite levels, exposure time, the amounts of vitamin E supplementation and number of feeding days; a) interaction effect of exposure time and nitrite levels on production of MDA at fixed 250 mg $kg^{-1}$ vitamin E supplementation and 30 feeding days; b) interaction effect of the amount of vitamin E and nitrite levels on production of MDA at fixed 8 h exposure time and 30 feeding days; c) interaction effect of feeding days and the amount of vitamin E on MDA at fixed 1.00 mg nitrite $L^{-1}$ and 8 h exposure time

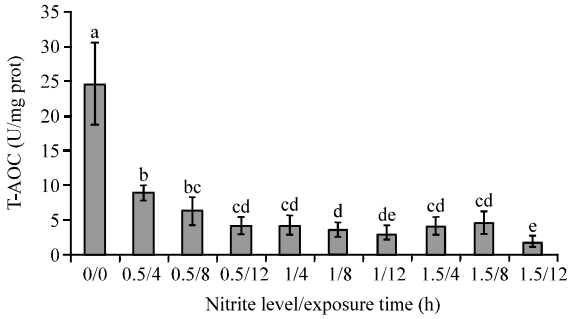


Fig. 5: Changes in the concentration of T-AOC in *A. japonicus* fed with the control diet under different nitrite levels and exposure times. Values without same letters differ significantly ($p<0.05$)

feeding days prolonging and vitamin E supplementation increasing and all the factor extremely significantly effected on MDA values ($p<0.01$) except nitrite levels while which was significantly ($p<0.05$). Probably because the vitamin E is a strong antioxidant and could eliminate lipids peroxide effectively and MDA values reduced obviously.

**Effect of vitamin E supplementation on T-AOC under the different levels nitrite stress, exposure time and number of feeding days**

**Changes in T-AOC level in the control group:** At the 0 h of the nitrite stress, T-AOC value remained at a significant higher level than those of all the other groups ($p<0.05$) as shown in Fig. 5. The values dropped sharply after exposure to the nitrite >4 h both exposure time and nitrite levels have important effect on MDA values and the nitrite 1.5 mg $L^{-1}$ and exposure 12 h was the lowest and significant lower than those of other groups except nitrite 1.0 mg $L^{-1}$ and exposure 12 h may suggest the body T-AOC being ruined seriously and lead to death.

**Differences in the T-AOC among the vitamin E supplementation groups:** As shown in Fig. 6, T-AOC decreased while exposure time extended and nitrite levels increased and increased following vitamin E supplementation increasing and number of feeding days prolonging. All the four factors had a significant correlation with T-AOC ($p<0.05$) (Table 5). Suggesting that the body T-AOC was gradually destroyed under the nitrite stress and the vitamin E accumulated after feeding days >15 days and increased the T-AOC to protect body from oxidative damage.

**Effect of vitamin E supplementation on SOD under the different levels nitrite stress, exposure time and number of feeding days**

**Changes in SOD activity in the control group:** As shown in Fig. 7, SOD values went up at first then go down as the

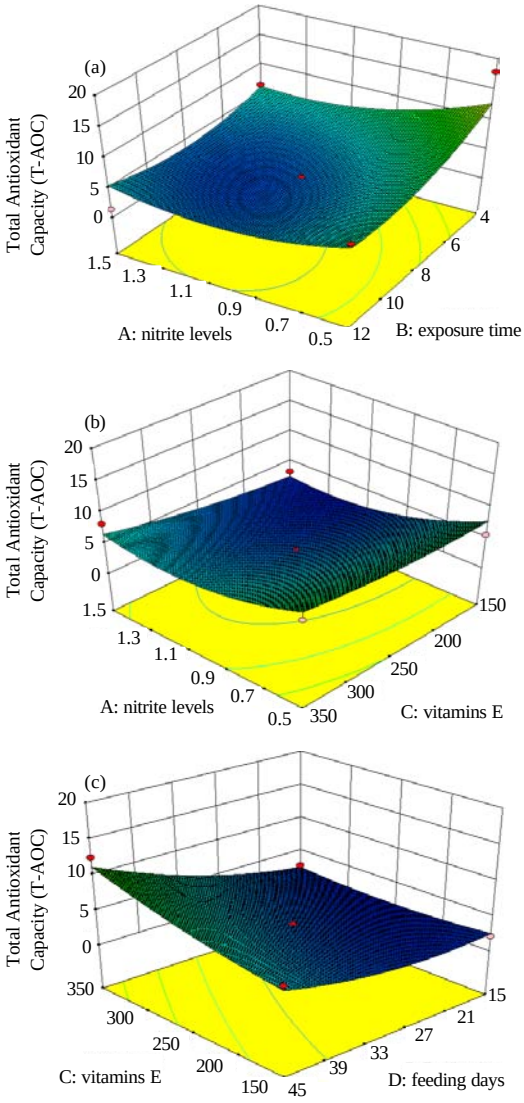


Fig. 6: Response surface plots of T-AOC level in *A. japonicus* generated using nitrite levels, exposure time, the amounts of vitamin E supplementation and number of feeding days; a) interaction effect of exposure time and nitrite levels on production of T-AOC at fixed 250 mg $kg^{-1}$ vitamin E supplementation and 30 feeding days; b) interaction effect of the amount of vitamin E and nitrite levels on production of T-AOC at fixed 8 h exposure time and 30 feeding days; c) interaction effect of feeding days and the amount of vitamin E on T-AOC at fixed 1.00 mg nitrite $L^{-1}$ and 8 h exposure time

exposure time extended and decreased as the nitrite level increased, especially at 1.5 mg $L^{-1}$ and 12 h exposure time, the SOD value was significant lower than all the other

groups except group 0 h ($p<0.05$). Suggesting at the first of stress, SOD activities increased to reduce ROS and as the exposure time extended and nitrite levels increased, the synthesis of SOD may be hindered and the activities went down.

**Differences in the SOD activity among the vitamin E supplementation groups:** The response surface model of SOD was not significant with the four factors ($p>0.05$) as shown in Fig. 8 and Table 6, only exposure time impacted the values of SOD significantly ($p<0.05$). SOD values increased as the exposure time extended while firstly increased and then went down as the nitrite levels increased. May suggest SOD went up first to reduce the stress and when the exposure time too long and the nitrite levels too high, the strong stress response ruined the immune system and the SOD activities exhausted. The supplementation of vitamin E kept the SOD activities and following a few feeding days, the SOD values kept stable and active.

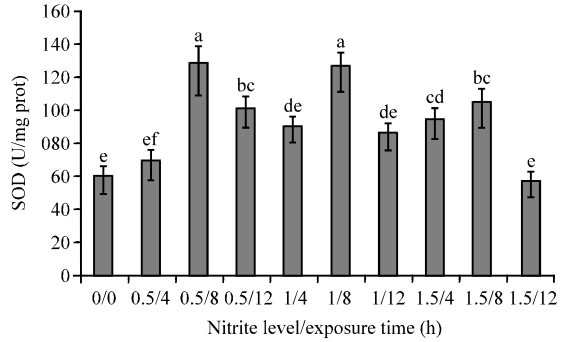


Fig. 7: Changes in the concentration of SOD in *A. japonicus* fed with the control diet under different nitrite levels and exposure time. Values without same letters differ significantly ($p<0.05$)

Table 5: Response surface quadratic model analysis of the T-AOC level of *A. japonicus* at different nitrite levels, exposure times, amounts of vitamin E supplementation and number of feeding days using ANOVA

| Sources | Sum of squares | df | Mean square | F-values | p-values |
|---|---|---|---|---|---|
| Model | 300.21 | 14 | 21.44 | 4.50 | 0.0063 |
| A-nitrite levels | 48.73 | 1 | 48.73 | 10.24 | 0.0085 |
| B-exposure time | 52.55 | 1 | 52.55 | 11.04 | 0.0034 |
| C-vitamin E | 10.59 | 1 | 10.59 | 2.22 | 0.0488 |
| D-feeding days | 102.17 | 1 | 102.17 | 21.46 | 0.0006 |

**Effect of vitamin E supplementation on CAT under the different levels nitrite stress, exposure time and number of feeding days**

**Changes in CAT activity in the control group:** At the 0.5 mg $L^{-1}$ nitrite level, CAT values increased as the exposure time extended but at 1.0 and 1.5 mg $L^{-1}$ nitrite level, the values decreased significantly ($p<0.05$). CAT values of 0.5 mg $L^{-1}$ exposure time 8 h and 1.5 mg $L^{-1}$ exposure time 4 h were significant higher the those of other groups ($p<0.05$) as shown in Fig. 9.

**Differences in CAT activity among the vitamin E supplementation groups:** As shown in Fig. 10 and Table 7, the model of the four factors with CAT was not significant ($p>0.05$) only feeding days impacted CAT significantly ($p<0.05$). CAT values increased as the vitamin E supplementation and nitrite levels increased but not significantly ($p>0.05$) while feeding days increasing CAT values significantly ($p<0.05$).

**Optimization of the amount of vitamin E supplementation and number of feeding days needed to reduce stress:** The RSM Models of SOD and CAT were not significant ($p>0.05$) and therefore were not taken into account during the optimization analysis. According to design-expert, the quadratic regression equations for −OH, MDA and T-AOC were as follows:

Table 6: Response surface quadratic model analysis of the SOD level of *A. japonicus* at different nitrite levels, exposure times, amounts of vitamin E supplementation and number of feeding days using ANOVA

| Sources | Sum of squares | df | Mean square | F-values | p-values |
|---|---|---|---|---|---|
| Model | 3919.70 | 14 | 279.98 | 2.14 | 0.0836 |
| A-nitrite levels | 41.62 | 1 | 41.62 | 0.32 | 0.5818 |
| B-exposure time | 2082.03 | 1 | 2082.03 | 15.90 | 0.0013 |
| C-vitamin E | 0.39 | 1 | 0.39 | 0.00 | 0.9572 |
| D-feeding days | 32.66 | 1 | 32.66 | 0.25 | 0.6252 |

$$-OH = 87.39+0.76\times A+13.33\times B-6.74\times C+0.060\times D-6.88\times A\times B-2.28\times A\times C-2.19\times A\times D+2.75\times B\times C+12.07\times B\times D-1.17\times C\times D+1.12\times A^2+0.92\times B^2-0.75\times C^2+17.56\times D^2$$

$$MDA = 8.85+1.07\times A+2.22\times B-1.25\times C-1.18\times D+1.67\times A\times B-0.48\times A\times C-1.16\times A\times D-1.37\times B\times C-0.12\times B\times D-0.15\times C\times D-1.75\times A^2-0.47\times B^2-0.30\times C^2-0.21\times D^2$$

$$T\text{-}AOC = 3.41-2.04\times A-1.57\times B+1.44\times C+2.92\times D+1.22\times A\times B+0.66\times A\times C-1.79\times A\times D+0.30\times B\times C-1.50\times B\times D+1.50\times C\times D+2.44\times A^2+2.14\times B^2+0.67\times C^2+1.33\times D^2$$

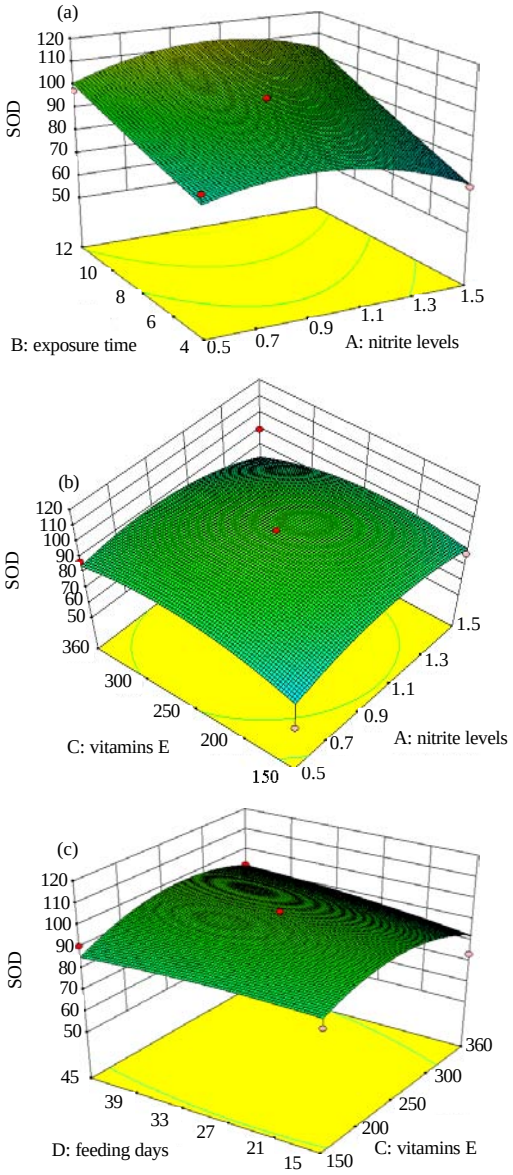


Fig. 8: Response surface plots of SOD level in *A. japonicus* generated using nitrite levels, exposure time, the amounts of vitamin E supplementation and number of feeding days; a) interaction effect of exposure time and nitrite levels on production of SOD at fixed 250 mg $kg^{-1}$ vitamin E supplementation and 30 feeding days; b) interaction effect of the amount of vitamin E and nitrite levels on production of SOD at fixed 8 h exposure time and 30 feeding days; c) interaction effect of feeding days and the amount of vitamin E on SOD at fixed 1.00 mg nitrite $L^{-1}$and 8 h exposure time

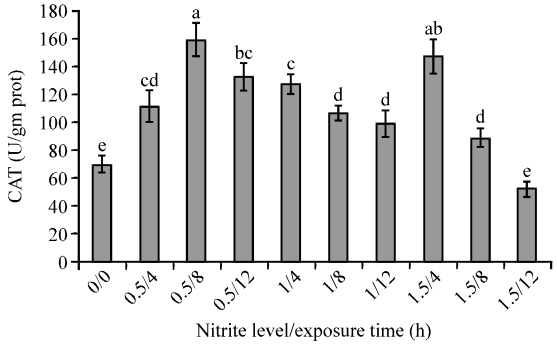


Fig. 9: The Catalase (CAT) changes of *A. japonicus* fed with control diet under different nitrite levels and exposure time. Values without same letters differ significantly ($p<0.05$)

Table 7: Response surface quadratic model analysis of the CAT level of *A. japonicus* at different nitrite levels, exposure times, amounts of vitamin E supplementation and number of feeding days using ANOVA

| Sources | Sum of squares | df | Mean square | F-values | p-values |
|---|---|---|---|---|---|
| Model | 1049000.00 | 14 | 74902.04 | 2.45 | 0.053 |
| A-nitrite levels | 7635.08 | 1 | 7635.08 | 0.25 | 0.630 |
| B-exposure time | 4492.07 | 1 | 4492.07 | 0.15 | 0.710 |
| C-vitamin E | 52258.44 | 1 | 52258.44 | 1.71 | 0.210 |
| D-feeding days | 297100.00 | 1 | 297100.00 | 9.70 | 0.010 |

Where:
A = Nitrite level
B = Exposure time
C = Vitamin E concentration
D = Number of feeding days

According to the equations in order to minimize the levels of −OH and MDA and maximize the T-AOC, the amounts of vitamin E and number of feeding days would be 200.63 mg $kg^{-1}$ and 33.68 days, 182.68 mg $kg^{-1}$ and 34.32 days and 202.11 mg $kg^{-1}$ and 33.15 days, respectively. When growth performance was taken into account to reduce nitrite stress in the tested range (0.5-1.5 mg $L^{-1}$) at exposure time ≤12 h, the optimal amount of vitamin C supplementation would approximately be 180-260 mg $kg^{-1}$ for 33-35 days.

**Effect of vitamin E supplementation on the growth performance:** The growth parameters of vitamin E supplementation groups significantly higher than control group ($p<0.05$) and 250 mg $kg^{-1}$ vitamin E supplementation group higher than those of all the other groups but not significantly than that of the other two vitamin E supplementation groups. According the regress equation to attain best growth performance, the best amounts of vitamin E supplementation was 256.30 mg $kg^{-1}$

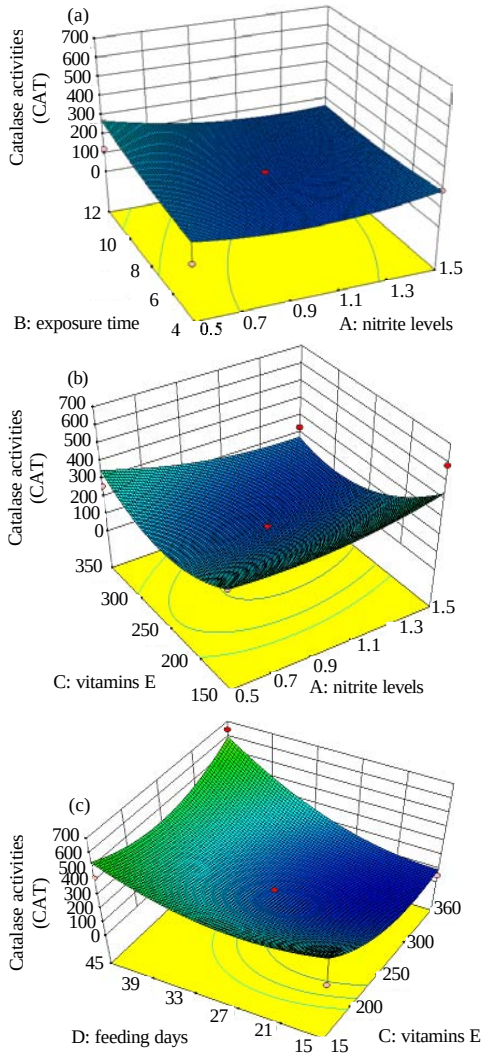


Fig. 10: Response surface plots of Catalase (CAT) of *A. japonicus* with the factors of nitrite levels, exposure time, the amounts of vitamin E supplementation and feeding days; a) interaction effect of exposure time and nitrite levels on CAT at fixed 250 mg $kg^{-1}$ vitamin E supplementation and 30 feeding days; b) interaction effect of the amount of vitamin E and nitrite levels on CAT at fixed 8 h exposure time and 30 feeding days; c) interaction effect of feeding days and the amount of vitamin E on CAT at fixed 1.00 nitrite levels and 8 h exposure time

diet. Vitamin Eisessential for aquatic organism and insufficient vitamin E will retard growth performance (Sau *et al*., 2004). Vitamin E diary requirement had been demonstrated in a number of fish which include 120 mg $kg^{-1}$ diet (Hamre and Lie, 1995) for Atlantic salmon and 200-300 mg $kg^{-1}$ diet for common carp (Watanabe *et al*., 1977). Few studies reported vitamin E requirement in echinoderms, Zhang *et al*. (2011) reported dietary 250 mg vitamin E $kg^{-1}$ and 600 mg selenoyeast $kg^{-1}$ diet for sea cucumber was considered to be the amounts to attain best growth performance and immunity. But vitamin E requirements have been found to vary based on various factors such as the dietary lipid (especially, Polyunsaturated Fatty Acid-PUFA) level in diets (Schwarz *et al*., 1988; Shiau and Shiau, 2001). The requirement may also vary with the environmental conditions and the developmental stage of the fish. In the present study, dietary vitamin E supplementation enhanced the growth performance of *A. japonicus* and the amounts of supplementation was similar by Zhang *et al*. (2011) report.

**The mechanism of nitrite stress:** In current study, −OH and MDA values of control group turned up significantly after exposed to nitrite solution for >4 h ($p<0.05$) while T-AOC turned down sharply. The results may suggest that when the sea cucumbers were suffered nitrite stress, the poisoning of nitrite led to oxidation of haemoglobin to methaemoglobin in erythrocytes and during the abnormal state much Reactive Oxygen Species (ROS) were produced. In certain circumstances of ROS overproduction the protection afforded by antioxidant defense mechanisms might be overcome there by leading to oxidative damage to tissue macromolecules including DNA, proteins and lipids (Gomez-Mendikute and Cajaraville, 2003). The elevated content of −OH after exposure to nitrite manifested the accumulation of ROS and the elevated content of MDA were the result of lipids peroxidation which was induced by ROS and MDA was considered to be the biomarker of lipid oxidation (Lykkesfeldt, 2007). T-AOC of sea cucumber body dropped significantly ($p<0.05$) under the assaulted by ROS. SOD and CAT was the main enzyme of antioxidant defense system which acted on $O_2^-$ and $H_2O_2$ separately.

In the present study, SOD and CAT activity went up significantly ($p<0.05$) after exposure to nitrite >4 h and maintained at a high level as the nitrite concentration increase and exposure time extends but dropped at the nitrite concentration of 1.5 mg $kg^{-1}$ and exposure time at 12 h. The results may suggest that when the *A. japonicus* exposed to nitrite, the ROS was generated and then the activity of SOD and CAT started to grow up and scavenged $O_2^-$ and $H_2O_2$ to reduce oxidant damage. As the nitrite concentration increasing and exposure time extending, over produced ROS broke down the Antioxidant Defense System causing the decrease activity of SOD and CAT. A significant increase of SOD and

CAT was reported after evisceration in *A. japonicus* (Zang *et al*., 2012). Wang *et al*. (2008) found that the activity of SOD in *V. natans* was initially up-regulated under ammonia-N stress but it decreased with a lengthened exposure or at an ammonia-N concentration >1.2 mM. A similar result was reported in *P. crispus* (Cao *et al*., 2004).

**Effect of vitamin E supplementation on ROS indexes and antioxidant enzyme activities:** The antioxidant defense system of living organisms can be subdivided into enzymatic antioxidants such as Superoxide Dismutase (SOD), CAT and Glutathione Peroxidase (GPX) and nonenzymatic antioxidants such as glutathione, vitamin E, ascorbate, b-carotene and urate (De Zwart *et al*., 1999). Vitamin E was widely used as an antioxidants to reduce stress in rainbow trout (Trenzado *et al*., 2008), seabream (Ortuno *et al*., 2003), shrimp (Liu *et al*., 2007) and so on. To date there are no previous reports about vitamin E supplementation to reduce nitrite stress in *A. japonicus*.

In the present study, the results suggest that vitamin E supplementation can decrease the content of −OH and MDA and increase the content of T-AOC in *A. japonicus* and the contents of −OH, MDA and T-AOC were also significantly impacted by the amounts of vitamin E, nitrite concentration, exposure time and number of feeding days, −OH, MDA turned lower as the amount of vitamin E supplementation and number of feeding days higher while T-AOC turned higher. SOD and CAT activity was significantly impacted by exposure time ($p<0.05$) and the activity turned lower as the exposure time longer and increased by vitamin E supplementation. The results suggest that feeding dietary supplementation vitamin E for a >15 days could decrease the body over produced ROS and enhance the body T-AOC and antioxidant system. Dietary 400 mg $kg^{-1}$ vitamin E and 2% linseed oil fed on darkbarbel catfish *Pelteobagrus vachelli* can mitigate the harmful effects of ammonia and enhance SOD and CAT activity (Li *et al*., 2013). In tilapia, vitamin E was reported to inhibit tissue lipid peroxidation (Huang and Huang, 2004) and similar results were found in *Atlantic salmon* (Onibi *et al*., 1996) and sea bass (Gatta *et al*., 2000). In shrimp, dietary chitosan oligosaccharide and N-acetyl-D-glucosamine were reported that they might have induced an increase in SOD to neutralize oxidative stress damage such as lipid peroxidation (MDA) (Niu *et al*., 2012). Few studies have evaluated the effects of vitamin E supplementation reducing stress in sea cucumbers. Zhang *et al*. (2011) report the effect of vitamin E supplementation on growth performance, immunity and disease resistance of sea cucumber but she did not investigate the effect of vitamin E supplementation on stress.

**Optimization of amount of vitamin E supplementation and number of feeding days:** In the present study, the regression equations for growth performance and ROS indexes revealed that the optimal amount of vitamin E supplementation ranged from 180-260 mg $kg^{-1}$ and the optimal number of feeding days ranged from 33-35 days. These values would produce the best growth performance and effectively reduce the stress response at the nitrite levels (0.5-1.5 mg $L^{-1}$) and exposure times (4-12 h) tested. Few studies have reported the optimal amount of vitamin E supplementation and number of feeding days needed to minimize stress in marine organisms, Montero *et al*. (1999) reported that vitamins C and E supplementation of 250 mg $kg^{-1}$ for 9 weeks could reduce the crowding stress in sea bream juveniles. Zhang *et al*. (2011) reported dietary 250 mg vitamin E $kg^{-1}$ and 600 mg selenoyeast $kg^{-1}$ diet for sea cucumber was considered to be the amounts to attain best growth performance, immunity and disease resistance.

## CONCLUSION

Researchers evaluated the changes in ROS indexes and antioxidant enzyme activities that occurred in *A. japonicus* under nitrite stress and explained the mechanism of nitrite stress in this organisms, researchers also found that vitamin E supplementation was effectively to reduce nitrite stress and got the best amount of vitamin E supplementation and the number of feeding days. In the future, changes in gene expression levels should be studied.

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