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Dietary ascorbic acid modulates the expression profile of stress protein genes in hepatopancreas of adult Pacific abalone *Haliotis discus hannai* Ino

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ABSTRACT

This study was conducted to investigate the effects of dietary ascorbic acid (AA) on transcriptional expression patterns of antioxidant proteins, heat shock proteins (HSP) and nuclear factor kappa B (NF-κB) in the hepatopancreas of Pacific abalone *Haliotis discus hannai* Ino (initial average length: 84.36 ± 0.24 mm) using real-time quantitative PCR assays. L-ascorbyl-2-molyphosphate (LAMP) was added to the basal diet to formulate four experimental diets containing 0.0, 70.3, 829.8 and 4967.5 mg AA equivalent kg^{-1} diets, respectively. Each diet was fed to triplicate groups of adult abalone in acrylic tanks (200 L) in a flow-through seawater system. Each tank was stocked with 15 abalone. Animals were fed once daily (17:00) to apparent satiation for 24 weeks. The results showed that the dietary AA (70.3 mg kg^{-1}) could significantly up-regulate the expression levels of Cu/Zn superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), feritin (FT) and heat shock protein 26 (HSP26) in the hepatopancreas of abalone in this treatment compared to the controls. However, the expression levels of Mn-SOD, glutathione peroxidase (GPX), thioredoxin peroxidase (TPx), selenium-binding protein (SEBP), HSP70 and HSP90 were significantly down-regulated. Compared with those in the group with 70.3 mg kg^{-1} dietary AA, the expression levels of CAT, GST and HSP26 were decreased in abalone fed with very high dietary AA (4967.5 mg kg^{-1}). In addition, significant up-regulations of expression levels of Mn-SOD, GPX, TPx, SEBP, FT, HSP70, HSP90 and NF-κB were observed in abalone fed with apparently excessive dietary AA (829.8 and 4967.5 mg kg^{-1}) as compared to those fed 70.3 mg kg^{-1} dietary AA. These findings showed that dietary AA influenced the expression levels of antioxidant proteins, heat shock proteins and NF-κB in the hepatopancreas of abalone at transcriptional level. Levels of dietary AA that appeared adequate (70.3 mg kg^{-1}) reduced the oxidative stress by influencing gene expression of antioxidant proteins, but excessive dietary AA (829.8 and 4967.5 mg kg^{-1}) induced oxidative stress in Pacific abalone *H. discus hannai*.

1. Introduction

Reactive oxygen species (ROS) are generated as part of normal metabolism in animal cells [1]. ROS can stimulate signal transduction pathways and mediate cell growth and apoptosis [2]. However, the excessive accumulation of these toxic by-products causes serious damage to lipids, proteins and nucleic acids when oxidative stresses are induced by radiation, high temperature, malnutrition, chemicals or pathogens [3–5]. In order to protect cells against the toxicity caused by ROS, animals have evolved protective antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and thioredoxin peroxidase (TPx) [6]. In addition, oxidative stresses are known to induce increased production of heat shock proteins (HSPs) (e.g., HSP26, HSP70 and HSP90) [7,8] and other functional proteins (e.g., selenium-binding protein (SEBP), ferritin (FT)) [9,10], which are important components of the cellular protective response.

Ascorbic acid (AA) is an essential nutrient required to maintain normal physiological functions in aquatic animals [11]. It has numerous biological functions, such as increasing anti-oxidative capacity [12], enhancing immunity [13] and protecting cells against toxicity [14]. And many studies also proved that supplementation of dietary AA could raise up the anti-stress ability and

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immune functions of animals possess L-gulonolactone oxidase (GLO, EC 1.1.3.8) that is responsible for the synthesis of AA *de novo*, such as broilers (*Gallus gallus* domesticus), soft-shelled turtle (*Pelodiscus sinensis*) and Siberian sturgeon (*Acipenser baerii*) [15–17]. In addition, previous studies have shown that dietary AA could influence expression level of antioxidant genes, and then improve anti-stress capacities in human and other vertebrates [15,18–21]. However, high level of dietary AA could also exhibit prooxidant activity through inducing cytochrome P4502E1-linked monooxygenases and then generating large amounts of the anion radical superoxide (O^{2-}), which could cause oxidative stress in human and animals depending on the doses [22,23]. Therefore, whether dietary AA could influence the antioxidant responses is an interesting question in invertebrates that may be able to synthesize AA, and there was no published data on effects of dietary AA on antioxidant responses in molluscs.

Pacific abalone *Haliotis discus hannai* Ino are the most commercially important species of large algivorous marine gastropods in aquaculture for Asia [24,25]. However, abalone culture has suffered serious problems of mortality from disease outbreaks, environmental contamination and decreased immunity of abalone [26–28]. It is of important to alleviate stresses and enhance the animal immunity through nutritional methods [29]. Previous studies have shown that optimal vitamins (e.g., vitamin A, vitamin E and pyridoxine) could significantly elevate the activities of antioxidant enzymes and could protect cells of abalone from peroxidation damage in order to maintain maximum growth and antioxidant system [25,30,31]. However, there is no published data on the interactions between dietary AA and anti-oxidative responses in abalone.

As well as a major metabolic organ, the hepatopancreas is also an important defence organ because it is the major localization of microsomal cytochrome P-450 mediating the oxyradical generation system and antioxidant enzymes [32–35]. Considering that the anti-oxidative response at translational level is an important part of innate immune functions, the transcriptional changes in a complete set of antioxidant and heat stress genes in the abalone fed with graded levels of dietary AA were investigated. These genes include SOD, CAT, GPx, GST, TPx, SEBP, FT, HSP26, HSP70, HSP90 and nuclear factor kappa B (NF-κB).

2. Materials and methods

2.1. Animals, experimental diets and treatments

Abalone *H. discus hannai* (initial weight: 74.32 ± 0.43 g) were obtained from farmed stock. There were four dietary AA treatments, and each treatment was conducted in three replicates. Artificial diet flakes with graded levels of AA (0, 100, 1000 and 5000 mg kg^{-1}) were prepared to feed the experimental animals. L-ascorbate-2-monophosphate (LAMP) (35%) was used as the sources of dietary AA. The compositions of the control diet are presented in Table 1. Procedures of the diet preparation were modified from previous studies on the abalone [24]. Final AA concentrations in the four diets were 0.0, 70.3, 829.8 and 4967.5 mg kg^{-1}, respectively, as determined by reverse-phase high-performance liquid chromatography (HPLC, HP 1100, USA) ($n = 3$). All the diets were sealed in sample bags and stored at −20 °C until use.

Prior to initiation of the feeding experiment, the abalone were acclimated to laboratory conditions for 2 weeks. During the acclimation period, all the abalone were fed with the control diet (Table 1). They were then assigned to 12 tanks in a flow-through system using a completely randomized design with 4 triplicate treatments. Each replicate acrylic tank (200 L) was stocked with 15 adult abalone, with a shelter. Each diet was fed to satiation to abalone once daily (17:00) for 24 weeks. Every morning, feces and excess diets were removed to maintain water quality. During the experimental period, water temperature ranged from 12.5 to 21.0 °C, salinity of 30–34, pH 7.6–7.9, and dissolved oxygen was not less than 7.0 mg L^{-1}.

Table 1
Ingredient and proximate composition of the experimental diets (on dry weight basis).

Ingredients	Percents in diet (%, dry weight)
Casein (vitamin-free)[a]	25.00
Gelatin[a]	6.00
Dextrin[b]	33.50
CM-cellulose[b]	5.00
Sodium alginate[b]	20.00
Vitamin mix (Ascorbic acid-free)[c]	2.00
Mineral mix[d]	4.50
Choline chloride[b]	0.50
SO/MFO[e]	3.50
Proximate analyse($n = 3$)	
Crude protein	31.47
Crude lipid	3.38
Ash	7.68

[a] Sigma Chemical, St. Louis, MO, USA.
[b] Shanghai Chemical Co., Shanghai, China.
[c] Vitamin mix (Ascorbic acid-free), each 1000 g of diet contained: thiamin HCl, 120 mg; riboflavin, 100 mg; folic acid, 30 mg; PABA, 400 mg; pyridoxine HCl, 40 mg; niacin, 800 mg; Ca pantothenate, 200 mg; inositol, 4000 mg; biotin, 12 mg; vitamin E, 450 mg; menadione, 80 mg; B12, 0.18 mg; retinol acetate, 100,000 IU; cholecalciferol, 2000 IU; ethoxyquin, 400 mg.
[d] Mineral mix, each 1000 g of diet contained: NaCl, 0.4 g; $MgSO_4 \cdot 7H_2O$, 6.0 g; $NaH_2PO_4 \cdot 2H_2O$, 10.0 g; KH_2PO_4, 20.0 g; $Ca(H_2PO_4)_2 \cdot H_2O$, 8.0 g; Fe-citrate, 1.0 g; $ZnSO_4 \cdot 7H_2O$, 141.2 mg; $MnSO_4 \cdot H_2O$, 64.8 mg; $CuSO_4 \cdot 5H_2O$, 12.4 mg; $CoCl_2 \cdot 6H_2O$, 0.4 mg; KIO_3, 1.2 mg; Na_2SeO_3, 0.4 mg.
[e] Soybean oil and menhaden fish oil (1:1) with 0.001% ethoxyquin.

2.2. Sample collection and synthesis of abalone cDNA

At the termination of the feeding experiment, animals were not fed for 3 days. Ten abalone per tank were randomly collected, and their hepatopancreas were excised and immediately frozen in liquid nitrogen. The ten hepatopancreas samples in a tank were pooled as a replicate for RNA isolation and subsequent analyses.

Total RNA was extracted using the Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions. RNA quality was checked by spectrophotometry and agarose gel electrophoresis to confirm suitability for cDNA synthesis. Ratios of the absorbance at 260 and 280 nm ranged from 1.6 to 1.8, and ratios of the absorbance at 230 nm and 260 nm ranged from 1.8 to 2.0. First-strand cDNA was synthesized from 3 μg of total RNA using 25 μM of anchored oligo dT20 (Invitrogen, USA), 500 μM dNTPs (Takara, Japan), 200 units of SuperScript™ II RT reverse transcriptase with provided buffer (Invitrogen, USA). Reactions were incubated for 1.5 h at 42 °C followed by 70 °C for 10 min and in a final volume of 20 μl cDNA mix was diluted to 1:3 and stored at −80 °C for subsequent SYBR Green fluorescent quantitative real-time PCR (RT-PCR).

2.3. mRNA quantification by real-time quantitative PCR

Changes in the expression of SOD, CAT, GPx, GST, TPx, SEBP, FT, HSP26, HSP70, HSP90 and NF-κB genes were quantified using fluorescence real-time quantitative PCR analysis, performed in triplicate using a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany).

Primers used to amplify the target genes are listed in Table 2. Nucleotide sequences were selected as primers using known sequences for the abalone species *H. discus hannai* (GPx, GST, TPx,

Table 2
The list of real-time quantitative PCR primers for antioxidant enzyme and heat shock protein genes and Ribosomal protein S9 gene of abalone *H. discus hannai* and *H. discus discus*.

Gene	Basic function		Nucleotide sequence (5′–3′)	Reference
Cu/Zn-SOD	Detoxification of O_2^-	F	CCCACTTCAACCCTTTCG	*H. discus discus*
		R	TTGCCCAGGTCATCCACT	(DQ530214)
Mn-SOD	Detoxification of O_2^-	F	CGCCTACGTCACAAACCT	*H. discus discus*
		R	GCTAAGCACCTCCCAGAA	(DQ530210)
CAT	Detoxification of H_2O_2	F	CAGCACAGAGGATGAAG ACAAC	*H. discus discus* (DQ530211)
		R	GCACGCTTCTGGATGAACTC	
GPx	Detoxification of H_2O_2	F	TTCAGCGACTTCCGCAACA	*H. discus hannai*
		R	ACGGCAGCCCTAGAACCAG	(GU254066)
Mu-GST	Detoxification of H_2O_2	F	CGGCAGCAAGGTGACAGT	*H. discus hannai*
		R	GAAGGCTAACAGCGTGGG	(EU734743)
TPx	Detoxification of H_2O_2	F	TCAGACTACAGAGGGAAATA	*H. discus hannai*
		R	CATCCAAGGACCTCACAG	(DQ118780)
SEBP	Detoxification, Antioxidant	F	AGCCCGTCCATTCACAAG	*H. discus hannai*
		R	TCCAACAGGAAGAAGCCAC	(GU014544)
FT	Detoxification of Fe^{3+} Antioxidant	F	GAGAGGTGGGCGCATCGT	*H. discus hannai* (GU479917)
		R	GAAGTCCATCATCTGTG GGTCA	
HSP26	Chaperone, Antioxidant Biomarker of stress	F	TTCGGTTTGATCTGTCC	*H. discus discus* (EF472916)
		R	GTCATCTCGCCCTCTG	
HSP70	Chaperone, Antioxidant Biomarker of stress	F	ATGCCAATGGTATCCTC	*H. discus hannai* (DQ324856)
		R	GTAATTCTCAGCCTCGTT	
HSP90	Chaperone, Antioxidant Biomarker of stress	F	CACTGTGGACCAGAAATGC	*H. discus hannai* (GU014545)
		R	ACAGCAAAGCACGGAACT	
NF-κB	Transcription factor, Biomarker of stress	F	GCCCTGCTATTGTAGTTGTCT	*H. discus discus* (GQ903763)
		R	ATGTTATCGTATCCGTGTCCT	
RP S9		F	CAGAATCCGAAAGTCAGCC	*H. discus discus*
		R	TCATCTTGCCCTCGTCCA	(EU247757)

F: Forward, R: Reverse.

SEBP, FT, HSP26, HSP70 and HSP90) and *H. discus discus* (SOD, CAT and NF-κB). The Ribosomal protein S9 gene was used as an internal control. TE buffer and DEPC-water for the replacement of cDNA template was used as negative control. Real-time PCR amplification was carried out in triplicate in a total volume of 25 μl, containing 12.5 μl of 2 × SYBR Green Real-time PCR Master Mix (Takara, Japan), 2 μl of 1:3 diluted cDNA and 0.2 mmol each of primers. The real-time PCR temperature profile for abalone *H. discus hannai* genes was 95 °C for 2 min followed by 35 cycles of 5 s at 95 °C, 15 s at 59 °C, 20 s at 72 °C. Fluorescent data were acquired during each annealing phase. During the detection, each sample was run in duplicate and PCR-grade water replaced the template was the negative control. The expression levels of genes were calculated by $2^{-\Delta\Delta CT}$ method, and the value stood for a n-fold difference relative to the calibrator [36].

2.4. Statistical analysis

All data were presented as mean ± S.E.M. (standard error of the mean). Data for the relative expression of genes was logarithmically transformed and checked for outliers and heterogeneity of variance. A one-way analysis of variance (using SPSS 16.0) was performed to compare the four treatments, each with three replicate tanks. When overall differences were significant, Tukey's test was conducted to compare the means between individual treatments. For statistically significant differences, $p < 0.05$ was required.

3. Results

3.1. Transcriptional responses of antioxidant enzymes to dietary AA

The relative expression levels of antioxidant enzymes in the hepatopancreas of abalone fed with different levels of dietary AA are presented in Fig. 1. Compared with those treated with AA-free diet, Cu/Zn-SOD, CAT and mu-GST mRNA levels significantly increased in treatments with 70.3 mg kg^{-1} of dietary AA ($p < 0.05$). However, no significant differences were observed in Cu/Zn-SOD, CAT and mu-GST mRNA levels between treatments with 70.3 mg kg^{-1} and 829.8 mg kg^{-1} of dietary AA. Furthermore, CAT and mu-GST expression levels significantly decreased in the groups treated with 4967.5 mg kg^{-1} of dietary AA compared with the groups treated with 70.3 mg kg^{-1} of dietary AA ($p < 0.05$) (Fig. 1(C and E)).

However, different expression patterns were shown in Mn-SOD, GPx and TPx (Fig. 1(B, D and F)). Mn-SOD, GPx and TPx mRNA levels significantly decreased ($p < 0.05$) and reached minimum levels at 70.3 mg kg^{-1} of dietary AA. Then the expression levels significantly increased at 4967.5 mg kg^{-1} of dietary AA compared with that at 70.3 mg kg^{-1} of dietary AA ($p < 0.05$) (Fig. 1(B, D and F)). In addition, no significant differences were found in Mn-SOD and GPx mRNA levels between 70.3 mg kg^{-1} and 829.8 mg kg^{-1} of dietary AA ($p > 0.05$) (Fig. 1(B and D)).

3.2. Transcriptional responses of SEBP and FT to dietary AA

The responses of the antioxidant proteins (SEBP and FT) in the hepatopancreas of abalone fed with graded levels of dietary AA are also shown in Fig. 1. Compared with those treated with AA-free diet, SEBP mRNA levels decreased in the treatment with 70.3 mg kg^{-1} of dietary AA. And then, these levels increased and reached to the maximum at 4967.5 mg kg^{-1} of dietary AA ($p < 0.05$) (Fig. 1(G)). The relative expression levels of FT transcript in the hepatopancreas were up-regulated and reached the maximal level at 4967.5 mg kg^{-1} of dietary AA (Fig. 1(H)). However, there was no significant difference between 829.8 mg kg^{-1} and 4967.5 mg kg^{-1} ($p > 0.05$).

3.3. Transcriptional responses of HSPs and NF-κB to dietary AA

The expression profiles for heat shock proteins (HSP26, HSP70 and HSP90) and NF-κB in abalone *H. discus hannai* fed with graded AA are shown in Fig. 2. The relative expression level of HSP26 significantly increased in the hepatopancreas of abalone fed with 70.3 mg kg^{-1} of dietary AA. This level was 3-fold higher than that in dietary AA-free group (Fig. 2(A)). However, the expression levels of HSP26 dropped at 829.8 mg kg^{-1} of dietary AA, and then significantly increased at 4967.5 mg kg^{-1} dietary AA ($p < 0.05$). There were similar expression profiles for HSP70 and HSP90 in abalone fed with graded levels of AA (Fig. 2(B and C)). Both HSP70 and HSP90 mRNA levels significantly decreased at 70.3 mg kg^{-1} and then increased at 829.8 mg kg^{-1} ($p < 0.05$) of dietary AA, and then dropped to the original level at 4967.5 mg kg^{-1} of dietary AA. Significant differences in the expression levels of HSP70 and HSP90 were both found at 829.8 mg kg^{-1} and 4967.5 mg kg^{-1} of dietary AA compared with those with 70.3 mg kg^{-1} of dietary AA ($p < 0.05$). The expression levels of NF-κB were up-regulated and reached to the maximal level at excessive dietary AA (829.8 mg kg^{-1}) (Fig. 2(D)). However, no significant difference was observed between groups with 0.0 mg kg^{-1} and 70.3 mg kg^{-1} of dietary AA, and between groups with 829.8 mg kg^{-1} and 4967.5 mg kg^{-1} of dietary AA ($p > 0.05$).

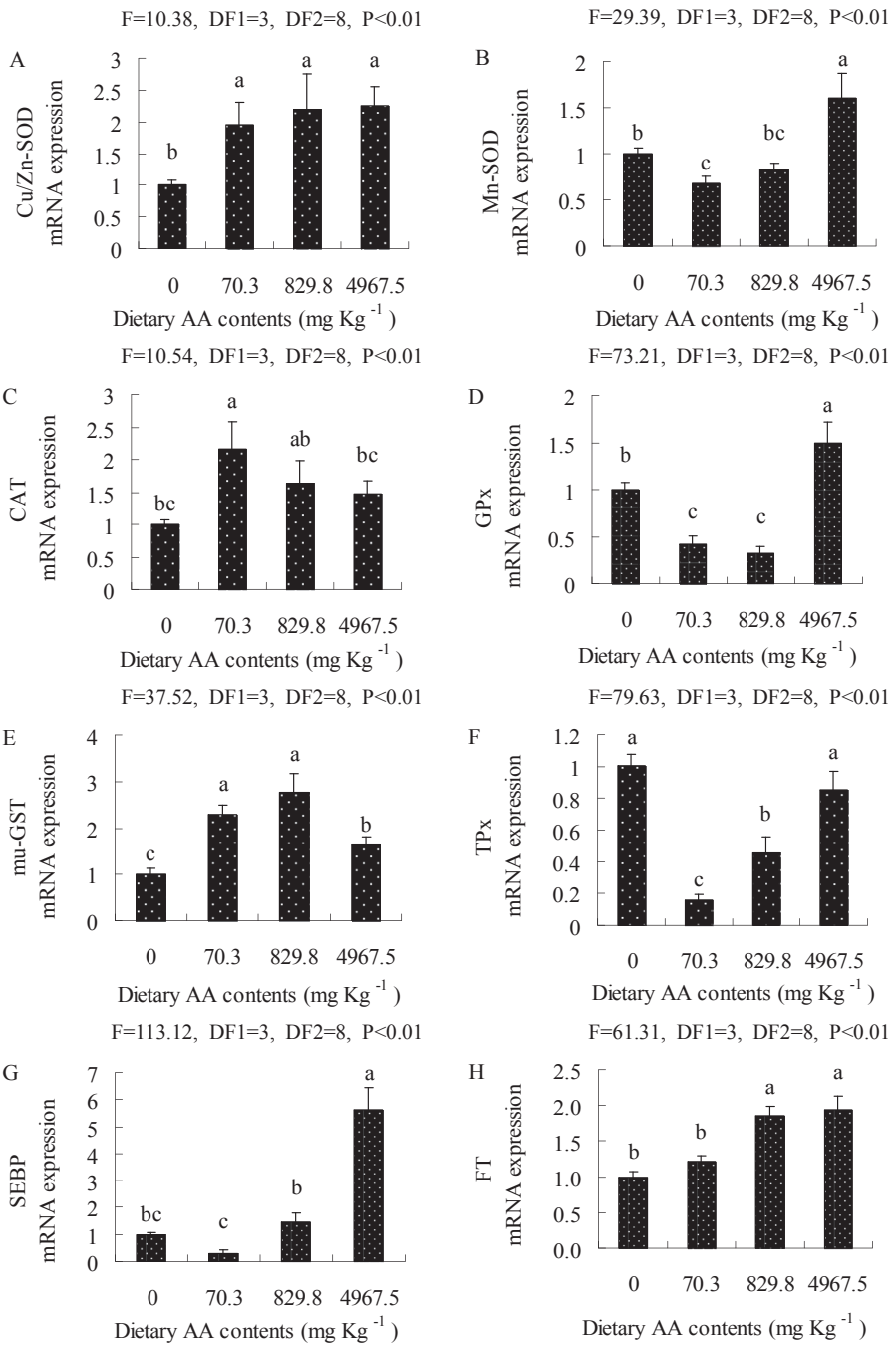

Fig. 1. Relative mRNA expression levels of antioxidant enzymes (SOD, CAT, GPX, GST and TPx), SEBP and FT in the hepatopancreas of abalone *H. discus hannai* after fed to dietary ascorbic acid for 24 weeks, respectively. All values represent the mean ± S.E.M. ($n = 3$). Bars bearing different letters are significantly different ($p < 0.05$; Tukey's test).

4. Discussion

In vertebrates, there is controversy about whether AA is a pro-oxidant or anti-oxidant. On one hand, the production of superoxide anion increased during the autoxidation metabolism of AA and then improved the activities of SOD, CAT and GST, which are the important anti-oxidative enzymes [19,37,38]. Meanwhile, AA was transported into mitochondria via facilitative glucose transporter 1 (Glut1) and then reduced the mitochondrial production of ROS, including superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH·) [39]. On the other hand, it was proposed that AA could exert pro-oxidant effects in some situations, acting to reduce ferric ions with the resulting ferrous species capable of catalysing formation of hydroxyl radical species from H_2O_2 [40]. In addition, AA could significantly increase the content of 8-oxoadenine, which is a marker for oxidative DNA damage [41,42].

In the present study, it was suggested that the roles of AA as anti-oxidant or pro-oxidant are dose-dependent. The expression levels of Cu/Zn-SOD, CAT and mu-GST mRNA significantly increased in the hepatopancreas of abalone fed with 70.3 mg kg^{-1} of dietary AA compared with those in dietary AA-free treatment (Fig. 1). It is well-known that SOD could catalyse the disproportionation of $O_2^{\bullet-}$ to H_2O_2 and oxygen. In turn H_2O_2 was converted by CAT into H_2O and molecular oxygen in animal cells [6]. And SOD and CAT could

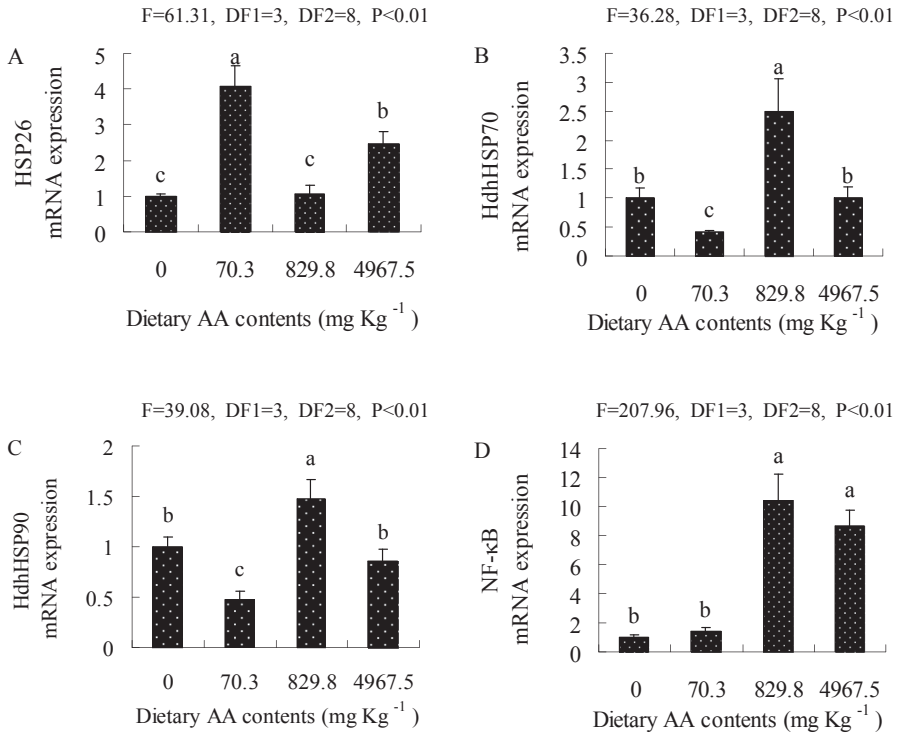

Fig. 2. Relative mRNA expression levels of heat shock protein genes (HSP26, HSP70 and HSP90) and NF-κB in the hepatopancreas of abalone *H. discus hannai* after fed to dietary AA for 24 weeks, respectively. All values represent the mean ± S.E.M. ($n = 3$). Bars bearing different letters are significantly different ($p < 0.05$; Tukey's test).

play as the first defence line either in normal or in stressed cells [43]. However, very high dietary AA (4967.5 mg kg^{-1}) decreased the expression levels of CAT and mu-GST, although up-regulated the expression level of GPx, TPx and SEBP compared with those in treatment with 70.3 mg kg^{-1} of dietary AA (Fig. 1). Up to now, information on the nutritional physiology of AA in abalone is limited. Further studies are needed to clarify if the 70.3 mg kg^{-1} of dietary AA could trigger the expression levels of Cu/Zn-SOD, CAT and mu-GST through stimulating the production of ROS in hepatopancreas of abalone during the AA metabolism. Meanwhile, it would also be valuable to determine if abalone could up-regulate the expression levels of Mn-SOD, GPx, TPx and SEBP to protect cells from oxidative stress caused by excessive dietary AA.

It has been reported that HSPs could serve as cytoplasm antioxidants in cells suffering the oxidative stress [44,45]. Previous studies have found that AA could increase the expression levels of HSP27 in human cells [20] and rat cells [46]. And dietary AA could decrease the expression of HSP70 in broilers *Gallus gallus* domesticus [15]. In addition, Sánchez-Morenoa et al. [14] also found that cells enriched with AA before being exposed to ethanol showed significantly lower concentrations of HSP70. This indicated that AA could act as a strong intracellular antioxidant and these cells were better protected against the alcohol mediated toxicity than AA-free cells. In the present study, 70.3 mg kg^{-1} of dietary AA significantly increased HSP27 mRNA level, but decreased the mRNA levels of HSP70 and HSP90. It was suggested that 70.3 mg kg^{-1} of dietary AA could keep abalone at a better anti-oxidation status.

However, when dietary AA increased to higher level of 829.8 mg kg^{-1}, mRNA levels of HSP26 significantly decreased while HSP70 and HSP90 significantly increased. In consideration of the fact in human that the low HSP27 and the high HSP70 mRNA levels might be a mechanism of adaptation to a low pro-oxidant state [18], it is suggested that the observed expression profiles of HSPs might result from the oxidative stress caused by the pro-oxidant effect of 829.8 mg kg^{-1} of dietary AA. Compared with those in the treatments with 70.3 or 829.8 mg kg^{-1} of dietary AA, the HSPs mRNA levels kept stable when dietary AA level reached to 4967.5 mg kg^{-1}. It is suggested that under the seriously excessive dietary AA situation, HSPs could not play the role of anti-oxidation well any more. Further study is needed to clarify it.

Previous studies in human have found that AA could enhance iron-induced ferritin translation and improve the iron metabolism [42,47,48]. In the present study, it was also showed that the FT mRNA level significantly increased by dietary AA supplement (Fig. 1(H)). However, lots of ROS were also generated during the metabolism of AA, which could cause damage to cellular structures [41]. In addition, iron contents also increased in the hepatopancreas of abalone fed with dietary AA (unpublished data). Excessive iron could also cause oxidative stress and induce the higher expression of FT in abalone [10]. Therefore, it is suggested that abalone could also protected cells or molecules from oxidative stress caused by excessive AA through increasing FT expression.

In conclusion, dietary AA influenced the expression of genes related to antioxidant responses in the hepatopancreas of abalone. The roles of AA on anti-oxidant or pro-oxidant are dose-dependent. These findings revealed coordinate alterations of gene expression in response to dietary AA. And it would be helpful to understand the significance of dietary AA coordinating the oxidation/reduction responses.

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