Chinese Journal of Oceanology and Limnology
Vol. 30 No. 1, P. 118-123, 2012
DOI: http://dx.doi.org/10.1007/s00343-012-1014-1

Effects of dietary menadione on the activity of antioxidant enzymes in abalone, *Haliotis discus hannai* Ino*

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Received Jan. 19, 2011; accepted in principle Mar. 9, 2011; accepted for publication May 11, 2011

Abstract A 240-day growth experiment in a re-circulating water system was conducted to investigate the effects of dietary menadione on the growth and antioxidant responses of abalone *Haliotis discus hannai* Ino. Triplicate groups of juvenile abalone (initial weight: 1.19±0.01 g; shell length: 19.23±0.01 mm) were fed to satiation with 3 semi-purified diets containing 0, 10, and 1 000 mg menadione sodium bisulfite (MSB)/kg, respectively. Results show that there were no significant differences in the rate of weight gain or in the daily increment in shell length of abalone among different treatments. Activities of superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione S-transferase (GST) and glutathione reductase (GR) in viscera were significantly decreased with dietary menadione. However, activities of these enzymes except for GPX in muscle were increased. Therefore, antioxidant responses of abalone were increased in muscle and decreased in viscera by dietary menadione.

Keyword: menadione; antioxidant enzymes; *Haliotis discus hannai*; mollusc

1 INTRODUCTION

It has been estimated that about 1%–3% of O_2 consumed by animals is converted to reactive oxygen species (ROS), including the superoxide anion radical ($O^{2-}\cdot$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH\cdot$). These can occur in tissues and may damage DNA, proteins, carbohydrates and lipids (Halliwell and Gutteridge, 1999). As a defense against ROS attacks, cells have developed different antioxidant systems during evolution. The primary defense system consists of antioxidant compounds including ascorbic acid, vitamin E and thiols (Chiou and Tzeng, 2000), along with a variety of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) (Michiels et al., 1994), glutathione reductase (GR) (Ngo and Nutter, 1994), and glutathione S-transferase (GST) (Zaidi and Banu, 2004).

Menadione (2-methyl-1,4-naphthoquinone, vitamin K_3) is quinine, which is cytotoxic and mutagenic and causes DNA damage in vitro (Tampo and Yonaha, 1996). It may undergo one electron reduction leading to the formation of unstable free radical substances. The latter can react rapidly with oxygen, resulting in ROS formation and causing oxidative stress (Kappus and Sies, 1981; Livingstone et al., 1989). Menadione can oxidize Mytilus edulis protein thiol groups (McDonagh and Sheehan, 2007). On the other hand, menadione has also been extensively studied as an effective inhibitor of lipid peroxidation in microsomes. Various mechanisms by which menadione suppresses lipid peroxide formation have been proposed, often including a relationship with single-electron transfer enzymes (Tampo and Yonaha, 1996). Talcott et al. (1985) suggested that one of the reduced forms of menadione (containing hydroquinone) may be an active antioxidant.

* Supported by National Natural Science Foundation of China (No. 30972262)

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On the other hand, menadione can act as a nutrient for animals. It has been reported that 10 mg menadione sodium bisulfite (MSB)/kg in the diet is sufficient for the maintenance of a steady-state tissue concentration in juvenile abalone *Haliotis discus hannai*; one of the most commercially important gastropods in aquaculture (Tan and Mai, 2001). Dietary MSB is converted to menaquinone-4 (MK-4) in abalone. Moreover, either MK-4 or phylloquinone (PK) deposited in the body is derived from food (Tan and Mai, 2001). At present, however, most studies regarding menadione are limited to quinines, which have been widely used to investigate oxidative stress in cells and tissues. Little information is available on the effects of dietary menadione on antioxidant responses in animals. The aim of this study was to investigate the effects of dietary menadione on the growth and antioxidant enzyme activities in different tissues of abalone, *H. discus hannai*.

2 MATERIAL AND METHOD

2.1 Experimental diet

Haliotis discus hannai were fed with a casein-gelatin-based diet as shown in Table 1. The dietary crude protein level was 29.53%, which is considered to be sufficient to maintain optimum growth in *H. discus hannai* (Mai et al., 1995a). A mixture of soybean oil and menhaden fish oil (1:1) was used as a basal lipid source. The dietary lipid level was 3.26%, which was sufficient to support optimum growth and provide enough essential fatty acids (EFA) for abalone (Mai et al., 1995b). Three levels (0, 10 and 1 000 mg/kg) of menadione sodium bisulfite (Sigma Chemical, St. Louis, MO, USA) were used to prepare the diets.

2.2 Animal rearing

The abalone juveniles used in the present study were spawned at Maidao Fisheries, Qingdao, China. Prior to the initiation of the experiment, the abalones were conditioned to the basal diet for 2 weeks in a re-circulated water system. Juvenile abalones of similar size, with initial weight of 1.19±0.01 g and shell length of 19.23±0.01 mm, were assigned to the rearing system using a completely random design with 3 replicates per treatment. Each tank (150 L) was stocked with 35 abalones. Test diets equaling 5%–10% of abalone wet body weight were hand-fed once in a day at 17:00. Feces and uneaten feed were removed to maintain the water quality every morning.

Table 1 Ingredients and proximate composition of the basal diet (%, dry-weight basis)

Ingredient	Content (%)
Casein (vitamin free) (Sigma, St. Louis, MO, USA)	25.00
Gelatin (Shanghai Chemical, Shanghai, China)	6.00
Dextrin (Shanghai Chemical)	34.00
CM-cellulose (Shanghai Chemical)	5.00
Sodium alginate (Shanghai Chemical)	20.00
Vitamin mix[1]	2.00
Mineral mix[2]	4.00
SO/MFO[3] (Food grade)	3.50
Choline chloride (Shanghai Chemical)	0.50
Proximate analysis, $n=3$	
Crude protein	29.53
Crude lipid	3.26
Ash	10.28

[1] Menadione-free vitamin mix, each 1 000 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, 4 000 mg; Biotin, 12 mg; Ascorbic acid, 4 000 mg; Vitamin B_{12}, 180 μg; Vitamin A, 100 000 IU ; Vitamin D, 2 000 IU; Vitamin E, 450 mg.
[2] Mineral mix, each 1 000 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, 12.8 g; $Ca(H_2PO_4)_2 \cdot H_2O$, 8.0 g; Fe-citrate, 1.0 g; Ca-lactate, 1.4 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.
[3] SO/MFO=1: 1 (Soybean oil: Menhaden fish oil=1:1).

During the 240-d feeding trial, water exchange was 150 L/h, water temperature was maintained at 17.5°C–19.0°C, salinity ranged from 31 to 34, the pH value was maintained between 7.4 and 7.9 and dissolved oxygen was not less than 7.0 mg/L.

2.3 Sample collection and analysis

At the termination of the feeding trial, animals were fasted for 3 d. Then all abalone were counted, weighed and measured. The viscera and muscle of abalone were collected and stored at -70°C for subsequent analysis. Growth was reported as the weight gain rate (WGR, %) and the daily increment in shell length (DISL, μm/d). The calculation formulas are as follows:

$$\mathrm{WGR}=[(W_t-W_i)/W_i]\times 100$$

$$\mathrm{DISL}=[(\mathrm{SL}_t-\mathrm{SL}_i)/t]\times 1\,000$$

where W_t and W_i were final and initial mean weight (g), respectively; SL_t and SL_i were final and initial mean shell length (mm), respectively and t was the feeding trial period (days).

Viscera and muscles were homogenized in ice-cold normal saline. Subsequently the homogenates

were centrifuged at 10 000 r/min for 30 min at 4°C. The supernatants were collected for enzyme activity analysis. Catalase (CAT) activity analysis for the viscera and muscle of abalone was performed as described by Góth (1991). Superoxide dismutase (SOD) activity was analyzed according to the method described by Marklund and Marklund (1974). Glutathione peroxidase (GPX) activity was measured using the method of Bell et al. (1985). Glutathione S-transferase (GST) activity was measured according to the method of Habig and Jakoby (1981). The method of Di Ilio et al. (1983) was used to analyze the activity of glutathione reductase (GR).

2.4 Statistical analysis

All percentage data were square-root arcsine transformed before analysis. Data were subject to one-way ANOVA using the SPSS 11.0 package. When overall differences were significant at less than the 5% level, Tukey's test was used to compare the means.

3 RESULT

3.1 Growth analysis of abalone

Growth performances are shown in Table 2. There were no significant differences in WGR, DISL or survival among all treatments. This indicated that dietary menadione had no significant effect on the growth or survival of abalone.

3.2 Analysis of antioxidant enzyme activity

3.2.1 Catalase (CAT) activity analysis

Activities of CAT in viscera and muscle are presented in Tables 3 and 4, respectively. The activities of CAT for the 10 mg/kg dietary MSB treatment were significantly lower than the other two treatments in viscera ($P<0.05$) (Table 3). However, the homogenates prepared from the muscles showed significantly higher CAT activity at a concentration of 10 mg/kg dietary MSB ($P<0.05$), compared with other treatments (Table 4).

Table 2 Effect of dietary menadione on the growth performance of abalone, *Haliotis discus hannai* after 240 d

	Dietary MSB[1] (mg/kg)			ANOVA	
	0	10	1000	*F*	*P*
Initial weight (g)	1.19±0.01	1.21±0.00	1.19±0.01	1.921	0.227
Initial shell length (mm)	19.23±0.01	19.31±0.01	19.15±0.01	0.840	0.477
Final weight (g)	4.14±0.20	4.33±0.08	4.19±0.31	0.318	0.745
Final shell length (mm)	31.79±0.41	31.59±0.42	32.52±0.67	0.893	0.458
WGR[2] (%)	247.25±14.25	258.63±7.05	249.77±12.93	0.230	0.805
DISL[3] (μm/d)	52.34±1.90	51.14±1.70	55.27±2.53	1.047	0.407
Survival (%)	79.05±0.95	77.14±1.65	77.14±2.86	0.308	0.746

[1] MSB: menadione sodium bisulfite; [2] WGR: weight gain rate; [3] DISL: daily increment in shell length.

Table 3 Effects of dietary menadione on the activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione S-transferase (GST) and glutathione reductase (GR) in viscera of the abalone, *Haliotis discus hannai* after 240 d

	Dietary MSB (mg/kg)			ANOVA	
	0	10	1 000	*F*	*P*
CAT activity (U/mg pr)	4.96±0.05[a]	2.81±0.34[b]	4.44±0.17[a]	21.083	0.002
SOD activity (U/mg pr)	3.02±0.10[a]	1.25±0.10[b]	0.51±0.08[c]	202.719	0.000
GPX activity (U/mg pr)	3.75±0.28[a]	2.58±0.20[b]	0.34±0.06[c]	73.025	0.000
GST activity (U/mg pr)	65.34±0.46[a]	48.24±0.39[c]	56.77±1.04[b]	151.528	0.000
GR activity (U/g pr)	10.25±0.03[a]	8.38±0.01[c]	9.34±0.06[b]	552.745	0.000

[a, b, c] Means in the same line, not sharing a common superscript letter were significantly different ($P<0.05$) as determined by Tukey's test. Values are means±SE, $n=3$; MSB: menadione sodium bisulfite.

Table 4 Effects of dietary menadione on the activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione S-transferase (GST) and glutathione reductase (GR) in muscle of the abalone, *Haliotis discus hannai* after 240 d

	Dietary MSB (mg/kg)			ANOVA	
	0	10	1 000	*F*	*P*
CAT activity (U/mg pr)	3.20±0.09[c]	8.74±0.21[a]	4.60±0.06[b]	446.545	0.000
SOD activity (U/mg pr)	4.01±0.09[c]	10.91±0.25[a]	6.00±0.19[b]	353.785	0.000
GPX activity (U/mg pr)	6.33±0.46[a]	4.24±0.39[c]	5.77±1.04[b]	35.538	0.001
GST activity (U/mg pr)	35.19±2.73[c]	53.21±3.32[a]	44.13±3.98[b]	65.137	0.001
GR activity (U/g pr)	5.08±0.14[c]	15.63±0.03[a]	12.33±0.07[b]	218.545	0.000

[a, b, c] Means in the same line, not sharing a common superscript letter were significantly different ($P<0.05$) as determined by Tukey's test. Values are means±SE, $n=3$; MSB: menadione sodium bisulfite.

3.2.2 Superoxide dismutase (SOD) activity analysis

Dietary menadione significantly influenced the activity of SOD in both viscera and muscles ($P<0.05$) (Tables 3 and 4). SOD activity significantly decreased in viscera with increased concentration of dietary MSB (Table 3). In contrast, SOD activity was elevated in muscles as the concentration of dietary MSB increased (Table 4) ($P<0.05$).

3.2.3 Glutathione peroxidase (GPX) activity analysis

Dietary menadione significantly decreased the activities of GPX in both viscera and muscles ($P<0.05$) (Tables 3 and 4). The highest values of GPX activity 3.75±0.28 U/mg and 6.33±0.46 U/mg were observed in viscera and muscles, respectively, with a diet containing 0 mg/kg dietary MSB supplement.

3.2.4 Glutathione S-transferase (GST) activity analysis

The GST activities in muscles were significantly elevated by dietary menadione ($P<0.05$) (Table 4), but significantly decreased in viscera ($P<0.05$) (Table 3). The highest value of GST activity 65.34±0.46 U/mg was observed in viscera within the control treatment (0 mg/Kg MSB).

3.2.5 Glutathione reductase (GR) activity analysis

Activities of GR in viscera were significantly decreased by dietary menadione (Table 3). But those in muscles were significantly increased, by as much as 2–3 times, by dietary menadione ($P<0.05$) (Table 4).

4 DISCUSSION

Toxic effects of high doses of menadione and its analogues have been observed in mammals (Suttie, 1991). However, high dietary levels of MSB (100–2 400 mg MSB/kg diet) had no significant effects on the growth rates of brook trout (Poston, 1971). In the present study, three levels of MSB were used to investigate the effects of dietary MSB: deficient (0 mg/kg), normal (10 mg/kg) and excessive (1 000 mg/kg), on the growth and antioxidant enzyme activities in abalone.

Dietary menadione had no significant effects on the growth or survival of abalone, which was consistent with the results from Tan and Mai (2001). However, the enzyme activities in different tissues were significantly different. SOD, which is considered an important antioxidant enzyme system in invertebrate species (Livingstone, 2001), showed different responses to dietary menadione between viscera and muscles. It was significantly inhibited in viscera and elevated in muscles at a concentration of 10 mg and 1 000 mg MSB/kg after 240 d of feeding. Because of the small size of the abalone used in the present study, the analyzed viscera was mainly hepatopancreas. In molluscs, hepatopancreas is known to be a primary organ reducing the impacts of environmental pollutants, toxins and heavy metals on the organism (Livingstone et al., 1992). Dietary pollutants may be first processed by digestive glands before being distributed to other tissues (e.g., muscle), thus any toxic effects should be more pronounced in the viscera in this study. If antioxidant defenses are effective in detoxifying ROS, then no harmful consequence should result in the tissues. However, if the ROS attack is severe, then the antioxidant defense systems may be overwhelmed, resulting in the inhibition of antioxidant enzymes and oxidative damage to lipid, protein, DNA and other key molecules (Ritola et al., 2002). Compared with those in treatment without dietary MSB supplement, in present study, antioxidant enzyme

activities in viscera were inhibited in groups with dietary MSB supplements (10 mg/kg and 1 000 mg/kg). It is suggested that that ROS production was probably more active than ROS detoxification in viscera, ultimately leading to enzyme inactivation. However, further research is necessary to determine oxidative damage to lipids, proteins, DNA and other key molecules. Nonetheless, certain properties of muscle may render it especially susceptible to free radical injury (Chan and Decker, 1994). During contraction, there are rapid changes in energy supply and oxygen flux through the mitochondrial respiratory chain, which results in the generation of ROS. This electron flux and ROS may predispose muscles to oxidative injury (Jackson and O'Farrell, 1993). Menadione is a strong redox cycler (Livingstone et al., 1990; Stohs and Bagghi, 1995) and is thought to generate the superoxide anion ($O^{2-}\cdot$) radical, which enhances the activities of SOD to change $O^{2-}\cdot$ to H_2O_2 (Halliwell and Gutteridge, 1999). On the basis of the above findings, it is possible that the effective antioxidant defenses of muscles against oxidative damage are due to the elevated activity of SOD.

Apart from the SOD antioxidant system, CAT and GPX are considered as a secondary defense against ROS (Barata et al., 2005). CAT and GPX have complementary roles in hydrogen peroxide detoxification. CAT is induced by high levels of H_2O_2 while GPX is inhibited and vice versa (Halliwell and Gutteridge, 1999). In the present study, the activity of CAT was induced while that of GPX was inhibited in muscles by menadione. These results were in agreement with Livingstone et al. (1990) and Stephensen et al. (2002), who found that menadione induced CAT and inhibited GPX in the digestive glands of common mussel and rainbow trout liver after two days of exposure. High CAT activity in muscles may reflect increased levels of $O^{2-}\cdot$ resulting in more H_2O_2 in muscles, due to the susceptibility of muscles to free radical injury. Although a complementary relationship was exhibited in muscles, CAT and GPX activities did not show the same pattern in viscera. Further work is necessary to elucidate it.

Glutathione S-transferases (GST) catalyses the conjugation of glutathione (GSH) with various electrophilic substances and plays a role in oxidative damage prevention by conjugating breakdown products of lipid peroxides to GSH (Ketterer et al., 1983). Menadione, which is a potent redox cycler can affect the GSH pool by both conjugation and oxidation, caused elevated GST activity in rainbow trout liver after 5 days exposure (Stephensen et al., 2002). Barata et al. (2005) also demonstrated that menadione increased GST activity in the freshwater cladoceran *Daphnia magna*. In the present study, menadione caused increased activity of GST in muscles, which was in accordance with the above mentioned findings. However, decreased activity of GST was observed in viscera with 10 and 1 000 mg MSB/kg diet. This phenomenon may reflect the possibility that a better protection mechanism may be afforded if the conjugation of menadione to GSH can be avoided (Chiou and Tzeng, 2000).

The activity of GR was also induced to a high level in muscles and inhibited in viscera by menadione after 240 days of feeding. Elevated GR activity was observed in rainbow trout liver after 2 or 5 days exposure of menadione (Stephensen et al., 2002). GR has been previously been suggested as a promising biomarker for redox cycling compounds (including menadione) with an increase in GR activity possibly indicating oxidative stress in rainbow trout (Stephensen et al., 2002). The increased activity of GR in muscles of abalone in the present study might indicate oxidative stress caused by menadione towing to the properties of muscles mentioned above.

5 CONCLUSION

In the present study, dietary menadione did not significantly influence the growth and survival of the abalone *Haliotis discus hannai*. However, it decreased the activities of SOD, GST and GR in viscera and increased these activities in muscles. It is suggested that antioxidant responses of abalone were increased in muscle and decreased in viscera by dietary menadione. Further study is needed to elucidate oxidative damage of menadione to lipid, protein, DNA and other key molecules in abalone.

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