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Effects of dietary thiamin on the physiological status of the grouper *Epinephelus coioides*

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Abstract This study was conducted to evaluate the effects of dietary thiamin on the physiological status of the juvenile grouper, *Epinephelus coioides*. Graded levels of thiamin (0.08, 0.50, 2.12, 3.15, 4.63, 12.37 mg thiamin kg^{-1} diet) were fed to grouper juveniles (mean weight: 16.97 ± 0.14 g) for 10 weeks. Although fish fed the thiamin-deficient (TD) diet showed no obvious symptoms of thiamin deficiency or increased mortality, those fed the lowest doses of thiamin (0.08 and 0.50 mg thiamin kg^{-1} diet) had significantly decreased transketolase activity in the liver. In addition, the level of liver thiobarbituric acid reactive substances in fish fed the TD diet was 33–67% higher than that in fish with the thiamin-supplemented diet. There were no significant differences in superoxide dismutase activity between the different groups of fish.

Keywords *Epinephelus coioides* · Grouper · Superoxide dismutase (SOD) · Thiamin · Thiobarbituric acid reactive substances (TBARs) · Transketolase

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Introduction

The water-soluble vitamin, thiamin, is an essential nutrient in the diet of humans and other animals, and the detrimental effects of thiamin deficiency originating from alcoholism and brain diseases have been reported under in vitro and in vivo conditions (Ba et al. 2005; Hazell et al. 2003; Lonsdale 2006; Martin et al. 2003). In addition, the requirements for thiamin and the symptoms of thiamin deficiency have been determined in a number of fish species (Amcoff et al. 2002; Hashimoto et al. 1970; Lehmitz and Spannhof 1977; Masumoto et al. 1987; Morito et al. 1986; Murai and Andrews 1978). Severe thiamin deficiency in mammals is often associated with neurological disturbances (Ciccia and Langlais 2000; Mulholland 2006; Nakagawasai et al. 2000; Pannunzio et al. 2000) and is strongly related to impaired carbohydrate metabolism in the form of thiamin pyrophosphate (TPP) (Martin et al. 2003).

TPP is an active form of thiamin, and it acts as an important coenzyme for transketolase and several other enzymes involved in the conversion of carbohydrate and fat into energy, including pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, which are important in carbohydrate metabolism and intracellular redox homeostasis (Blair et al. 1999; Shangari et al. 2003). Transketolase activity is very sensitive to thiamin

status and has been shown to be the rate-limiting enzyme in the nonoxidative portion of the pentose phosphate pathway (Berthon et al. 1992). A thiamin deficiency-induced impairment of carbohydrate metabolism can lead to cellular oxidative stress (Calingasan et al. 1999; Langlais et al. 1997; Todd and Butterworth 1999), while a number of studies in the rat have suggested that thiamin has antioxidative properties (Langlais et al. 1997; Lukienko et al. 2001).

Wilson (1994) pointed out that fish cannot utilize carbohydrate efficiently; this is in contrast to the situation in mammals where carbohydrate is one of the main sources of fuel/energy. This has raised the question of whether there would be a difference between the rat and fish in terms of their degree of dependence on thiamin. However, to date very little physiological research has focused on the role of thiamin in fish. In Asia, the grouper, *Epinephelus coioides*, is considered to be an important commercial maricultured carnivorous fish (Boonyaratpalin 1997), but very little information has been published on the role of the B-family vitamins in this fish; the exception being pyridoxine (Huang et al. 2005). Our previous study (Huang et al. 2005) reported that symptoms of pyridoxine deficiency in the grouper resemble those reported in mammals (Baxter 2003). Because pyridoxine and thiamin are tightly correlated to protein and carbohydrate metabolisms, the aim of this study was to determine some of the physiological and biochemical alterations that occur in grouper as a result of thiamin deficiency, including changes in the levels of transketolase, superoxide dismutase (SOD), and oxidative stress, in order to compare the differences in thiamin-related metabolic processes between fish and mammals.

Materials and methods

Diet preparation

The formulation of the basic purified diet (45.8% protein and 14.18 kJ g^{-1} gross energy) – excluding the thiamin components – is shown in Table 1. The vitamin-free casein was supplied by Sigma-Aldrich (product no. C3400; St. Louis, Mo.); all other ingredients were obtained from companies located in the People's Republic of China. Thiamin hydrochloride (Roche) was added to the diets at the expense of cellulose to provide concentrations of 0, 1, 2.5, 5, 10, and 20 mg kg^{-1} dry diet. The diets were analyzed for thiamin by the fluorometric method (AOAC 1990) and found to contain 0.08, 0.50, 2.12, 3.15, 4.63, and 12.37 mg kg^{-1} dry diet, respectively. The diets were prepared by first mixing the dry ingredients with oil and water, followed by pelleting the wet mixture into 2.5-mm pellets and air-drying in an air-conditioned room for two nights (2000–0800 hours). The air-dried pellets were stored at –20°C until use.

Experimental procedure

Grouper juveniles were obtained from a local fish fry dealer. Upon arrival, they were acclimatized to laboratory conditions for 4 weeks. The fish were fed the thiamin-deficient (TD) diet for 2 weeks prior to the trial to deplete the body of all stored thiamin; subsequently, the experimental diets were fed to triplicate groups of 20 fish, with an individual initial weight 16.97 ± 0.14 g. The experiments were conducted in flow-through seawater tanks (300 l), provided with continuous oxygen by an air stone connected to the air pump. The water temperature was maintained between 27.3°C and 31.9°C, dissolved oxygen content was ≥6 mg l^{-1}, and the salinity was 28–30‰. A 12:12-h photoperiod was maintained (light: 0700–1900 hours). The trial lasted for 10 weeks. For the duration of the trial, any abnormal behavior of the fish was observed and recorded daily.

Assays for biochemical enzymes

At the end of the experiment, the fish were first subjected to a 24-h fasting period, following which three fish were sacrificed and their livers removed for the enzyme assays. Transketolase (EC 2.2.2.1) activity was measured using the orcinol reaction determination of the sedoheptulose (colorimetric assays; Horecker 1957; Warnock 1970). Liver samples were homogenized and the supernatants incubated with D-ribose-5-phosphate (0.012 *M*) in a 37°C water bath for 1 h. A trichloroacetic acid

Table 1 Composition of the basal diet, with the exception of thiamin

Ingredients	(g kg^{-1} dry weight)
Vitamin-free casein	300
Gelatin	75
Corn starch	200
Fish oil	45
Corn oil	45
Amino acid mixture[a]	120
Choline chloride	5
Ascorbic acid phosphate ester	5
Vitamin mixture[b]	20
Mineral mixture[c]	40
α-Cellulose	145
Protein	458
Carbohydrate	197
Lipid	86
Energy (kJ g^{-1})	14.18

[a] The composition of the amino acid mixture in grams per kilogram dry diet is: leucine, 15.48; valine, 11.5; phenylalanine, 10.24; tyrosine, 9.68; isoleucine, 9.18; threonine, 8.97; aspartic acid, 7.56; glutamic acid, 7.56; serine, 7.51; proline, 7.46; arginine-HCl, 7.06; lysine-HCl, 5.55; histidine, 4.74; cysteine, 2.57; methionine, 2.47; tryptophan, 2.47 (modified from Shaik Mohamed 2003)

[b] The composition of the vitamin mixture in terms of milligrams per gram mixture is: riboflavin, 6.67; calcium pantothenate, 9.33; nicotinic acid, 26.67; pyridoxine hydrochloride, 2.5; folic acid, 0.5; inositol, 133.33; menadione, 1.33; alpha-tocopheryl acetate, 13.33; retinol acetate, 0.403; cholecalciferol, 0.0025; biotin, 2; vitamin B_{12}, 0.003. All ingredients were diluted with alpha-cellulose to 1 g

[c] The composition of the mineral mixture in terms of milligrams per gram mixture is: calcium lactate, 333.3; KCl, 233.3; $Ca(H_2PO_4)_2{\cdot}H_2O$, 133.3; $MgSO_4{\cdot}7H_2O$, 133.3; $NaH_2PO_4{\cdot}H_2O$, 83.3; $FeSO_4{\cdot}H_2O$, 33.3; $ZnSO_4{\cdot}7H_2O$, 20; $MnSO_4{\cdot}4H_2O$, 6.7; KI, 6.7; $AlCl_3{\cdot}6H_2O$, 6.7; $CuSO_4{\cdot}5H_2O$, 10

solution (15%) was then added to terminate the enzymatic reaction. A fixed volume of the solution was centrifuged at 10000 rpm for 10 min, following which ferric chloride-orcinol reagent was added to the supernatant and the mixture placed in a boiling water bath for 40 min. The optical densities were then measured at 580 and 670 nm using a UV/VIS spectrophotometer. Transketolase activity was calculated using the method of Warnock (1970), and the activity of this enzymey was expressed as micromoles sedoheptulose per gram wet tissue. Liver SOD activity was determined using a commercial kit (Jiancheng Bioengineering Institute, Nanjing, P.R. China). The results are expressed as the percentage inhibition of color formation, with high values indicating high SOD activity.

Assay for liver thiobarbituric acid reactive substances (TBARs)

The susceptibility of liver to induced lipid peroxidation (LPO) was assayed by the TBARs test using a commercial kit (Jiancheng Bioengineering Institute). The results are expressed as nanomoles of malondialdehyde (MDA) per milligram protein. Protein measurements were carried out by the Bradford method using a commercial kit (Beyotime Biotechnology, Jiangsu, P.R. China).

Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) using the SPSS ver. 12.0 statistical software (SPSS, Chicago, Ill.). Significant differences between means were analyzed using Duncan's new multiple range test at a 95% interval of confidence ($P < 0.05$).

Results

During the 10-week experimental period, no obvious signs of thiamin deficiency were observed. As shown in Fig. 1, there was no significant difference in terms of the survival of *E. coioides* (90.0–98.3%; $P > 0.05$) between the groups fed the different diets.

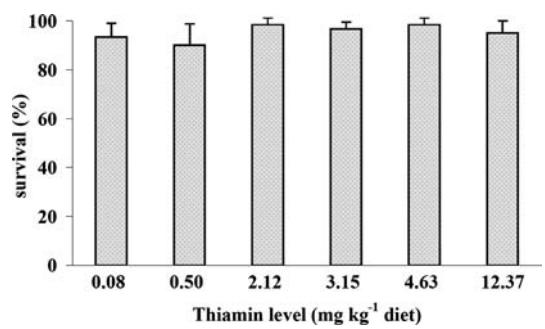

Fig. 1 Survival of *Epinephelus coioides* fed diets containing different levels of thiamin for 10 weeks. Values are shown as the mean ± standard deviation (SD) ($n = 3$)

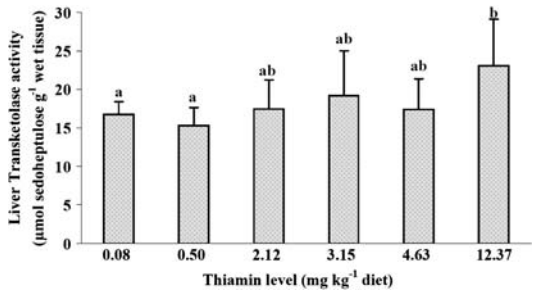

Fig. 2 Liver transketolase activity of *E. coioides* fed diets containing different levels of thiamin for 10 weeks. Values are shown as the mean ± SD ($n = 3$). Statistical group differences ($P < 0.05$) are marked with *different letters*

The activity of liver transketolase in *E. coioides* was significantly affected by dietary thiamin level only between the lowest doses (0.08 and 0.50 mg kg^{-1} diet) and the maximum dose (12.37 mg kg^{-1} diet) ($P < 0.05$). There was no significant difference when the diet contained thiamin at levels between 0.08 to 4.63 mg kg^{-1} (Fig. 2).

Levels of TBARs in the liver decreased from 8.82 to 5.29 nmol MDA mg^{-1} protein as the dietary thiamin increased from 0.08 to 12.37 mg kg^{-1} diet (Fig. 3a). TD fish had 33–67% higher TBARs levels

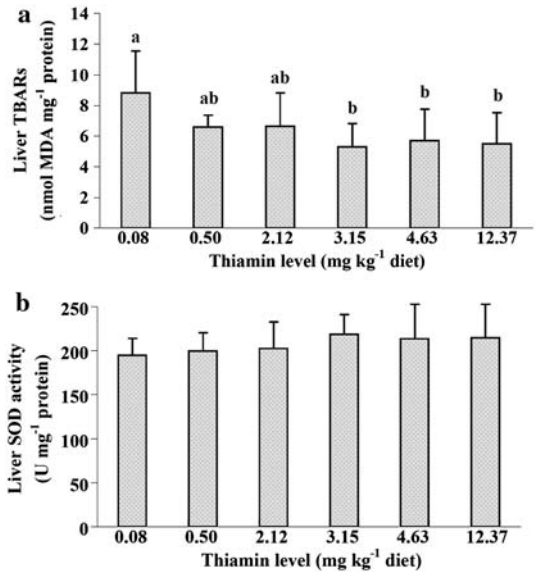

Fig. 3 Content of liver thiobarbituric acid reactive substances (TBARS) (**a**) and superoxidase dismutase (SOD) activity (**b**) of *E. coioides* fed diets containing different levels of thiamin for 10 weeks. Values are shown as the mean ± SD ($n = 3$). Statistical group differences ($P < 0.05$) are marked with *different letters*

than the fish fed the thiamin-supplemented diets; significant difference was detected between the TD fish and those fed 3.15 to 12.37 mg kg^{-1} of thiamin ($P < 0.05$). There was no significant difference among all the thiamin-supplemented groups ($P > 0.05$). Figure 3b shows that thiamin supplemented to the diet had no effect on liver SOD activity ($P > 0.05$).

Discussion

There were no obvious signs of thiamin deficiency in the 10-week experiment reported here. A similar result was also reported for turbot fed a TD diet for 16 weeks (Cowey et al. 1975). Murai and Andrews (1978) reported the absence of neurological symptoms in channel catfish fed a TD diet for 20 weeks, although other signs of thiamin deficiency, such as anorexia, dark coloration of the skin, extremely poor growth, and increased mortality rates, did appear. Neurological symptoms, such as ataxia, were reported on rainbow trout fed with a TD diet for 30 weeks (Masumoto et al. 1987). On the basis of our resuts and those reported earlier, it would appear that TD symptoms, especially neurological symptoms, are not easy to induce in fish. When these results on thiamin deficiency in fish are compared to the thiamin deficiency symptoms reported in rats, it is interesting to note that fish may be much less susceptible to thiamin deficiency (at least in terms of the more obvious symptoms) than rats. For example, neurological symptoms appeared in rats fed a TD diet for only about 4 weeks (Ciccia and Langlais 2000); furthermore, when treated with a thiamin antagonist, pyrithiamine, rats showed gross neurological symptoms after only 14 days of treatment (Gibson et al. 1984). Thiamin plays an important role in carbohydrate metabolism, and the energy derived from the oxidation of glucose is highly dependent upon the availability of TPP (Lonsdale 2006). The ingestion of carbohydrate automatically increases the need for dietary thiamin (Elmadfa et al. 2001; Lonsdale 2006). Compared to mammals, fish utilize carbohydrate relatively poorly (Wilson 1994). Therefore, fish may need less thiamin and may be less susceptible to thiamin deficiency than mammals.

Alterations in the cellular carbohydrate metabolism has been observed to be a common cause of neurological lesions in the TD rat brain (Singleton and Martin 2001). However, fish – especially carnivorous fish – have a remarkable capacity to utilize amino acids both as a metabolic fuel and as precursors for protein, lipid, and carbohydrate synthesis (Wood 1993). In other words, carbohydrate is not the most important source of energy in carnivorous fish (Wilson 1994), and once carbohydrate metabolism is impaired under extreme conditions (such as in thiamin deficiency), fish can still live for a relatively long time. Taking into account that our previous study (Huang et al. 2005) found that grouper fish were very susceptible to pyridoxine deficiency, we suggest that our results emphasize the fact that carbohydrate is relatively less important than protein in carnivorous fish.

Although no clear-cut signs of thiamin deficiency were observed in the present study, liver transketolase activity had been affected by thiamin deficiency. Transketolase activity is regarded as being the most sensitive indicator of thiamin status, and a substantial decline in transketolase activity resulting from thiamin deficiency has been found to occur in the brain of mammals prior to the onset of severe brain damage (Giguère and Butterworth 1987). The decrease in transketolase activity in the present study was similar to that observed in the rat (Rains et al. 1997) and other aquatic animals, such as turbot (Cowey et al. 1975), abalone (Zhu et al. 2002) and shrimp (Chen et al. 1994). Sheu et al. (1996) suggested that the decrease in transketolase activity is due to a decrease in the enzyme protein. This loss of transketolase protein was demonstrated to be due to a decrease in its synthesis rate and mRNA steady state levels but not to an increase in the degradation rate, as reported by Pekovich et al. (1998).

NADPH can be produced to combat oxygen radicals in the transketolase-mediated pentose phosphate pathway (Berthon et al. 1992). As such, the disruption in the metabolism of transketolase can destroy the homeostasis of cellular redox potential (Sheu et al. 1996) and can further lead to oxidative stress (Langlais et al. 1997). Oxidative stress is considered to be one of the major factors underlying the deleterious effects of thiamin deficiency. The present results indicate that the highest TBAR values occurred in fish fed the TD diet (see Fig. 3a). Similarly, high levels of free radicals or lipid peroxidation (LPO) products were found in TD rats (Calingasan et al. 1999; Langlais et al. 1997). However, the addition of thiamin to the diet at concentrations of 10^{-4}–10^{-6} *M* can inhibit LPO in rat liver microsomes, as reported by Lukienko et al. (2001), who subsequently suggested that the antioxidant effects of thiamin result from thiamin being able to interact directly with free radicals and hydroperoxides by successive transfer ($2H + +2e^{-}$) from the NH_2 group of the pyrimidine ring to radicals. However, whether the grouper was fed with a TD diet or not, we found no significant difference in the activity of liver SOD, which is a free radical scavenging enzyme. We suggest the possibility that SOD is not a main factor affecting oxidative stress in grouper, but further research on the antioxidant effects of thiamin need to be carried out.

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