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Chronic stress effects of high doses of vitamin D_3 on *Megalobrama amblycephala*

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ABSTRACT

Dietary vitamin D_3 plays an important role in the growth of aquatic animals, but long-term excessive feeding has potential hazards. In this study, *Megalobrama amblycephala* specimens were fed different experimental diets with 2000 IU/kg or 200,000 IU/kg of vitamin D_3 for 90 days, in order to evaluate chronic stress effects of high doses of vitamin D_3 on growth, immunity, and structural damage to enterohepatic tissues. The results showed that high doses of vitamin D_3 did not have a significant influence on the growth performance of *M. amblycephala* ($P > 0.05$), but it significantly reduced the survival rate after infection by *Aeromonas hydrophila* ($P < 0.05$). Serum albumin, alkaline phosphatase, and insulin levels, as well as hepatic total antioxidant capacity, were also significantly reduced ($P < 0.05$). Serum cortisol levels and hepatic heat stress protein 70 expression in *M. amblycephala* showed that high doses of vitamin D_3 significantly inhibit the anti-stress ability of *M. amblycephala* ($P < 0.05$). Paraffin tissue sections and electron microscopy showed that high doses of vitamin D_3 could cause different degrees of structural damage to enterohepatic tissues of *M. amblycephala*. Our results indicate that, although *M. amblycephala* can tolerate high doses of dietary vitamin D_3 over a long period, its glycolipid metabolism, immune function, anti-stress function, and resistance to pathogenic infections are adversely affected.

1. Introduction

Vitamin D is a fat-soluble protein, and it is involved in a variety of physiological functions in animals: it plays an important role in calcium and phosphorus metabolism and bone development, and it is an essential nutrient required for the normal growth of aquatic animals [1]. There are many studies on the synthesis and effects of vitamin [2–5], Recently, scientists have started to study its effects on the immune function of aquatic animals [6]. Unlike mammals, fish cannot produce vitamin D_3 from sunlight, and they acquire vitamin D_3 only from food [7]. Therefore, vitamin D_3 supplements in feed plays an important role on the growth of fish. There are two main methods used to add vitamin D to fish feed; low dose continuous addition and high dose intermittent addition. Excess vitamin D can lead to metabolic disturbances, and is potentially toxic. Hypervitaminosis D, which is characterized by hypercalcemia, extensive deposition of calcium salts in arteries, calcinosis, and bone damage, can result from excess vitamin D [8,9]. However, to the best of our knowledge, the potential for excess vitamin D_3 to cause metabolic disturbances in, or chronic stress to, fish has been little studied. Therefore, in the study reported here, we fed *Megalobrama amblycephala* with high doses of vitamin D_3, analyzed their physiological, biochemical and immune responses, and studied the structural damage caused to their livers and intestines using TEM technology. In addition to determining the toxic effects of high doses of vitamin D_3 to *M. amblycephala*, we also sought to define the safe upper limit of vitamin D_3 which can be fed to *M. amblycephala*, in order to provide a theoretical basis for the development and use of artificial feeds for *M. amblycephala*.

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M. amblycephala is one of the main varieties of freshwater fish farmed in China. Aquaculture production of *M. amblycephala* was up to 780,000 tons in 2014, and it ranks as the No. 8 among all varieties of freshwater fish in China. Systematic studies have been performed on the nutrition demand of *M. amblycephala* for proteins, fats, carbohydrates, vitamins, and minerals [10–14]. In this study, chronic nutritional stress effects of high doses of vitamin D_3 diet on the growth, immunity, and tissue structure of *M. amblycephala* specimens were evaluated.

2. Materials and methods

2.1. Diet preparation

Formulation and proximate composition of the basal diet are shown in Table 1. The basal diet was supplemented with 2000 IU (control) and 200,000 IU of vitamin D_3 kg^{-1} dry diet. Vitamin D_3 (code: C9756; purity >98%) was supplied by Sigma Co., Ltd. (St. Louis, MO, US). For preparation of experimental diets, ingredients were ground into a fine powder and passed through a 60-mesh sieve. Diets were prepared by thoroughly mixing the powdered ingredients, followed by the addition of oils and water. Vitamin D_3 was first dissolved in ethanol, then dissolved in soybean oil, and added to the feed [15]. The dough was pelletized using a laboratory pelletizer (die diameter, 2 mm; Xinchang Machinery Ltd, China) and dried in a ventilated oven at 35 °C. After drying, the diets were packed into airtight plastic bags and stored at 4 °C until required. The actual amounts of vitamin D_3 in the feed were determined using high-performance liquid chromatography as 1837.4 IU/kg and 197,000 IU/kg.

2.2. Feeding and management

M. amblycephala specimens were obtained from the Nanquan Fish Farm, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. Healthy fish of similar sizes (initial body weight, 17.65 ± 0.65 g; mean ± SE) were selected and restocked in eight tanks at a stocking density of 20 per tank. Four tanks were randomly used for each of the following two fish groups: a control group fed with 2000 IU/kg of vitamin D_3 and a high-dose group fed with 200,000 IU/kg of vitamin D_3, which is 100 times the quantity fed to the control group. The experimental diets were fed to the fish by hand to near satiation, three times a day (0800, 1300, and 1700) for 90 days. The feeding trial was conducted in an indoor freshwater recirculating system composed of 8 fiberglass tanks (300 L) associated with mechanical filtration units. All tanks were supplied with equal supplemental aeration and water flow (approximately 3 L/min). During the experimental period, the water temperature was monitored using a data logger, and water quality parameters were recorded throughout the study period (temperature, 26.0 ± 1.5 °C, DO ≥ 6 mg L^{-1}; NH_3 ≤ 0.1 mg L^{-1}; pH 6.8–7.0).

Table 1
Formulation and approximate composition of the basal diets for *M. amblycephala*.

Ingredients (g/kg diet)		Proximate composition (%)	
Casein[a]	320	Crude protein	35.47
Gelatin[b]	80	Crude fat	6.18
Dextrin[c]	300	Phosphorus	0.76
MCC[d]	160	Calcium	0.56
CMC-Na[b]	25	Lysine	2.29
$Ca(H_2PO_4)_2$	20	Methionine	0.88
Zeolite flour	20	Threonine	1.35
Vitamin and mineral mix[e]	15		
Soybean oil	60		

[a] Purchased from Lin Xia Huaan Biological Products Co., Ltd.
[b] Purchased from Shanghai Zhan Yun Chemical Co., Ltd.
[c] Provided by Tongwei Feed Group Co., Ltd.
[d] Purchased from Linghu Xinwang Chemical Co., Ltd.
[e] Vitamin (Vitamin D_3 free, IU or mg/kg feed) and mineral premixes (g/kg feed): Vitamin A, 1000 IU; Vitamin B_1, 10 mg; Vitamin B_2, 20 mg; Vitamin B_6, 30 mg; Vitamin B_{12}, 110 mg; Vitamin C, 100 mg; Vitamin E, 100 mg; Vitamin H, 0.5 mg; Vitamin K, 10 mg; Calcium pantothenate, 50 mg; Choline, 2500 mg/kg; Folic acid, 5 mg; Inositol, 200 mg; Niacin, 100 mg; $MgSO_4$, 15 g; $FeSO_4$, 2.5 g; $CuSO_4$, 0.031 g; $MnSO_4$, 0.162 g; $ZnSO_4$, 0.353 g; KIO_3, 0.003 g; Na_2SeO_3, 0.003 g; $CoSO_4$, 0.001 g.

2.3. Sampling and measurement of growth performance

At the end of the 90-day feeding trial, the fish were fasted for 24 h before sampling. Total weight of the fish per tank and number of fish were measured, and weight gain rate (WGR), specific growth rate (SGR), and feed conversion ratio (FCR) were calculated. Then, three fish were collected from each tank (12 for each group). The fish were immediately euthanized using MS-222 (200 mg/L). Blood samples were obtained from the caudal vein and centrifuged at 3000 rpm for 10 min at 4 °C after maintaining them for 1 h at 4 °C in a refrigerator. The serum was collected and stored at −70 °C until analysis. The liver was stripped immediately after blood sampling. Part of the liver was quick-frozen with liquid nitrogen and stored at −80 °C for determining the expression of hepatic heat stress protein 70 (HSP70), and the other part of the liver was used to prepare 10% liver homogenate and then stored at −20 °C for determining the hepatic antioxidant indexes. Finally, foregut and liver tissue blocks were washed with physiological saline and fixed with Bouin's solution and 2.5% glutaraldehyde solution for 24 h in order to prepare paraffin sections and sections for transmission electron microscopy (TEM), respectively.

2.4. Challenge experiment

After the feeding experiment, challenge tests were conducted in quadruplicate with 10 fish per replicate. The gram-negative bacterium *Aeromonas hydrophila* was originally isolated from an infected *M. amblycephala* fingerling. LC_{50} was determined by intraperitoneally injecting 48 fish with graded concentrations of *A. hydrophila* (10^6, 10^7, 10^8, 10^9, and 10^{10} CFU/ml) at 25 °C, and the results showed that the LC_{50} on day 7 was 5×10^7 CFU/ml. *A. hydrophila* was diluted using sterile normal saline, and the final concentration was set to 5×10^7 CFU/ml. The bacterial suspension (1.0 ml per 100 g of body weight) was injected into the abdominal cavity. No diet was administered to the fish during the test, and mortality in each tank was observed at 0 h, 12 h, 24 h, 36 h, 48 h, and 96 h.

2.5. Sample determination

2.5.1. Determination of blood biochemistry and hepatic antioxidant indexes

Serum albumin (ALB), aspartate aminotransferase (AST), alkaline phosphatase (ALP), insulin (INS), glucose (GLU), total cholesterol (TC), and triglyceride (TG) levels were determined using a colorimetric method (Beckman Cx-4 Automatic Biochemical Analyzer, Fullerton, CA, US) and kits purchased from Shanghai Mingdian Bioengineering Co, Ltd (China). Serum cortisol level was determined using radioimmunoassay (RIA), and the kit was purchased from the Beijing North Institute of Biological Technology (China). Peroxidase (POD), superoxide dismutase (SOD) and total antioxidant capacity (T-AOC) levels in the liver were determined using commercial kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).