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Optimal α-lipoic acid strengthen immunity of young grass carp (*Ctenopharyngodon idella*) by enhancing immune function of head kidney, spleen and skin

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ABSTRACT

This study was for the first time to investigate the effects of α-lipoic acid (LA) on growth and immune function of head kidney, spleen and skin in young grass carp (*Ctenopharyngodon idella*). A total of 540 healthy grass carp (with initial body weight at 216.59 ± 0.33 g) were randomly divided into six groups and fed six separate diets with graded dietary levels of LA for 70 days. Un-supplemented group did not find LA and its concentrations in the other five diets were 203.25, 403.82, 591.42, 781.25 and 953.18 mg kg^{-1}, respectively. After the growth trial, fish were challenged with *A. hydrophila* for 14 days. The results showed that, compared with the un-supplemented group, optimal LA improved lysozyme (LZ) and acid phosphatase (ACP) activities, enhanced complement 3 (C3), C4 and immunoglobulin (Ig) M contents and up-regulated hepcidin, liver expressed anti-microbial peptide (LEAP)-2A, LEAP-2B and β-defensin-1 mRNA levels in the head kidney, spleen and skin of young grass carp; meanwhile, optimal LA up-regulated anti-inflammatory cytokines transforming growth factor (TGF)-β1, TGF-β2, interleukin (IL)-4/13A (not IL-4/13B), IL-10 and IL-11 mRNA levels partly related to target of rapamycin (TOR) signaling and down-regulated pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interferon (IFN)-γ2, IL-1β, IL-6, IL-8, IL-12p40 (not IL-12p35), IL-15 (not in the skin) and IL-17D mRNA levels partially associated with nuclear factor-kappa B (NF-κB) signaling in the head kidney, spleen and skin of young grass carp. Above results indicated that optimal LA enhanced the immune function of head kidney, spleen and skin in fish. Interestingly, excessive LA decreased the growth and impaired the immune function of head kidney, spleen and skin in fish. Finally, on the basis of the percent weight gain (PWG), the ability against skin hemorrhage and lesion, the IgM content in the head kidney and the LZ activity in the spleen, the optimal dietary LA levels were estimated to be 315.37, 382.33, 353.19 and 318.26 mg kg^{-1} diet, respectively.

1. Introduction

Modern aquaculture exposes fish to acute stress (such as crowding and handling et al.), resulting in a higher susceptibility to diseases [1,2]. Enhancing immunity of fish can reduce the diseases incidence [3]. Our previous research have indicated that vitamin-like nutrients (such as *myo*-inositol) can strengthen immunity and promote growth of fish [4,5]. α-lipoic acid (LA) is classified as a vitamin-like nutrient in animal [6]. It has been reported that LA improves growth performance of juvenile abalone (*Haliotis discus hannai* Ino) [7]. However, to the best of our knowledge, there is no study to investigate the effects of LA on immunity of fish. It has been confirmed that LA promotes regeneration of vitamin C in rat liver [8]. The earlier study from our laboratory found that increasing vitamin C content enhances the immunity of young grass carp (*Ctenopharyngodon idella*) [9]. Thus, there might be a relationship between LA and immunity of fish, which deserves research.

The immunity of fish is closely linked with the immune function of immune organs [10], which is partly related to innate and adaptive immune responses [11,12]. The LZ, ACP, complements (e.g., C3) and antibacterial peptides (e.g., β-defensin) are important innate immune

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components of fish [13,14]. Meanwhile, the IgM play a critical role in adaptive immune response of fish [15]. Yet, no report has investigated the influence of LA on the innate and adaptive immune responses of fish. According to earlier report, LA increases the glutamine synthesis in C6 astrocyte cells [16]. Elevation of glutamine increases the LZ activity and C3 content in the serum of juvenile Jian carp (*Cyprinus carpio* var. Jian) [17]. In rat, LA can increase the content of brain serotonin [18] which up-regulates the ACP activity in peritoneal macrophages [19]. In addition, LA up-regulates the protein level of insulin like growth factor (IGF)-1 in bovine secondary preantral follicles [20]. Sorensen et al. [21] reported that increased IGF-1 improves the β-defensin gene expression in human keratinocytes. Moreover, LA decreases the content of cortisol in broiler chicken liver [22]. Depressing cortisol level up-regulates the plasma IgM concentration in masu salmon (*Oncorhynchus masou*) [23]. Based on the above researches, we speculate that LA affects the immune function of the immune organs in fish may be associated with the innate and adaptive immune responses, which worth research.

Meanwhile, anti-inflammatory cytokines [e.g., IL-10] and pro-inflammatory cytokines (e.g., IL-6) also play important roles in the immune function of immune organs in fish [24]. Furthermore, previous studies indicated that the TOR signaling and NF-κB signaling modulate inflammatory cytokines in grass carp [25,26]. However, there is no research to investigate the relationship between LA and inflammatory cytokines as well as the possible regulation mechanism in fish. It has been reported that LA elevates the thyroid stimulating hormone (TSH) content in mice plasma [27]. Earlier study found that improving TSH level increases the IL-10 expression in human serum [28]. In human, LA increases the production of T lymphocytes cyclic adenosine 3′, 5′-monophosphate (cAMP) [29] which can inhibit IL-6 synthesis in the peripheral blood mononuclear cells (PBMNC) [30]. It was confirmed that LA promotes the serine/threonine protein kinase (Akt/PKB) phosphorylation in human monocytic THP-1 cells [31]. In rat skeletal muscle cells, Akt phosphorylation activates mTOR [32]. Moreover, in RAW 264.7 cells, LA blunts expression of nitric oxide [33] which induces the activation of NF-κB in rat heart [34]. The above observations indicate that LA influences immune function of the immune organs in fish may be associated with the inflammatory cytokines as well as possible signaling molecules, which deserves investigation.

Grass carp is one of the most important freshwater fish species with the highest aquaculture production all over the world [35]. So far, the optimal level of dietary LA in aquatic animal only investigated in juvenile abalone (*Haliotis discus hannai*) [7]. However, the optimal level of dietary nutrients between abalone and grass carp may be different. For example, the optimal dietary thiamin level of juvenile abalone (51 mg kg^{-1}) [36] is much higher than that in juvenile grass carp (1.3 mg kg^{-1}) [37]. Until now, no study has reported the optimal level of dietary LA in grass carp, which is necessary to be investigated.

Taken together, this study was for the first time to investigate the influence of LA on immunity of fish, which may be partly related to innate and adaptive immune components, inflammatory cytokines as well as the possible TOR and NF-κB signaling. Meanwhile, we were the first to determine the optimal dietary LA levels for young grass carp, which may provide a reference to formulate commercial feed of grass carp.

2. Materials and methods

2.1. Experimental diets preparation

The formulation of the basal diet is shown in Table 1. Casein, gelatin and soybean protein concentrate were used as dietary protein sources. Fish oil and soybean oil were used as dietary lipid sources. Six experimental diets were obtained by supplementing the basal diet with LA (Shan xi Sciphar Hi-Tech Industry Co., Ltd., Purity: 99.0%) at concentrations of 0.00 (un-supplemented group), 200.00, 400.00, 600.00, 800.00 and 1000.00 mg kg^{-1} diet according to Zhang et al. [7] and the amount of corn starch was reduced to compensate according to Jiang et al. [4]. The LA concentrations of diet, tissue and serum were determined by High performance liquid chromatography (HPLC) according to Arshad et al. [38]. Un-supplemented group did not find LA and its concentrations of the other five diets were 203.25, 403.82, 591.42, 781.25 and 953.18 mg kg^{-1}, respectively. The prepared diets were stored at −20 °C according to Liu et al. [39].

Table 1
Composition and nutrient contents of basal diet.

Ingredients	Contents (%)	Nutrient contents	Contents (%)
Casein	13.00	Crude protein[d]	28.49
Gelatin	5.88	Crude lipid[d]	4.53
Soybean protein concentrate	18.23	n-3[e]	1.04
DL-Met (99%)	0.46	n-6[e]	0.96
L-Trp (99.2%)	0.06	Available phosphorus[f]	0.40
Thr (98.5%)	0.03		
α-starch	24.00		
Corn starch	22.02		
Fish oil	2.95		
Soybean oil	1.81		
Cellulose	5.00		
$Ca(H_2PO_4)_2$	1.51		
Vitamin premix[a]	1.00		
Mineral premix[b]	2.00		
LA premix[c]	1.00		
Choline chloride (50%)	1.00		
Ethoxyquin (30%)	0.05		

[a] Vitamin premix (g kg^{-1}): retinyl acetate (500,000 IU g^{-1}), 0.39; cholecalciferol (500,000 IU g-1), 0.40; D, L-a-tocopherol acetate (50%), 23.23; menadione (22.9%), 0.83; thiamine nitrate (98%), 0.09; calcium-D-pantothenate (98%), 3.85; pyridoxine hydrochloride (98%), 0.62; cyanocobalamin (1%), 0.94; niacin (99%), 4.04; D-biotin (2%), 0.75; meso-inositol (98%), 19.39; folic acid (95%), 0.42; riboflavin (80%), 0.73; ascorhyl acetate (95%), 9.77. All ingredients were diluted with corn starch to 1 kg.

[b] Mineral premix (g kg^{-1}): $MnSO_4 \cdot H_2O$ (31.8% Mn), 2.6590; $MgSO_4 \cdot H_2O$ (15.0% Mg), 200.0000; $FeSO_4 \cdot H_2O$ (30.0% Fe), 12.2500; $ZnSO_4 \cdot H_2O$ (34.5% Zn),8.2460; $CuSO_4 \cdot 5H_2O$ (25.0% Cu), 0.9560; KI (76.9% I), 0.0650; Na_2SeO_3 (44.7% Se), 0.0168. All ingredients were diluted with corn starch to 1 kg.

[c] LA premix: premix was added to obtain graded levels of LA.

[d] Crude protein and crude lipid contents were measured value.

[e] n-3 and n-6 contents were referenced to Zeng et al. [97] and calculated according to NRC (2011).

[f] Available phosphorus were referenced to Wen et al. [98] and calculated according to NRC (2011).

2.2. Feeding trial and sample collection

The procedures used in this study were approved by the University of Sichuan Agricultural Animal Care Advisory Committee. As shown in Fig. 1. Young grass carp obtained from fishery (Sichuan, China). Prior to the experiment, fish acclimatized to the experimental environment for 2 weeks, according to Wen et al. [40]. Five hundred and forty fish (mean weight 216.59 ± 0.33 g) were randomly assigned to 18 experimental cages (1.4 L × 1.4 W × 1.4H m), and 30 fish per cage. Each cage was equipped with a disc of 100 cm diameter in the bottom to collect the uneaten feed, according to Wu et al. [41]. Fish fed with their respective diets four times per day for 70 days as referenced by Xu et al. [9]. Thirty minutes after feeding, uneaten feed was collected, dried and weighed to calculate the feed intake (FI), according to study from our lab [42]. The dissolved oxygen was higher than 6.0 mg L^{-1}. The pH value and water temperature were determined to be 7.5 ± 0.3 and 28.5 ± 2.0 °C, respectively. The experiment were under natural light and dark cycle, which was similar to study from our lab [43].

Fish from each cage weighed at the termination of the feeding trial. After the feeding trial, blood of six fish from each treatment were drawn