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Effects of dietary isoleucine on the immune response, antioxidant status and gene expression in the head kidney of juvenile Jian carp (*Cyprinus carpio* var. Jian)

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ABSTRACT

This study was conducted to evaluate the effects of dietary isoleucine (Ile) on the immune response, antioxidant status and gene expression in the head kidney of juvenile Jian carp (*Cyprinus carpio* var. Jian). Six semi-purified isonitrogenous diets (4.2, 7.0, 9.5, 11.9, 13.9 and 16.9 g Ile kg^{-1} diet) were fed to Jian carp (6.9 ± 0.03 g) for 60 days. The results showed that Ile supplementation improved the head kidney index, red and white blood cell counts, anti-hydroxyl radical capacity and the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-*S*-transferase ($P < 0.05$), and decreased the malondialdehyde, protein carbonyl and glutathione contents in the head kidney ($P < 0.05$). After a 60 day feeding trial, an *Aeromonas hydrophila* challenge study was conducted for 17 days. Differences in survival rate, leucocyte phagocytic activity, serum lysozyme activity, acid phosphatase activity, haemagglutination titre, complement components 3 and 4, immunoglobulin M level and *A. hydrophila* agglutination antibody titre followed the same trend as that of the head kidney index ($P < 0.05$). Furthermore, real time polymerase chain reaction revealed that relative mRNA expression of transforming growth factor β2 and target of rapamycin (TOR) in the head kidney significantly increased with increasing Ile levels ($P < 0.05$). Conversely, the relative mRNA expression of tumour necrosis factor α, interleukin 10 and eIF4E-binding protein (4E-BP) in the head kidney showed a downward trend ($P < 0.05$). Collectively, this study indicates that dietary Ile improves the fish immune response, regulates the antioxidant status and cytokine, TOR and 4E-BP gene expression in the head kidney.

1. Introduction

Isoleucine (Ile) is an essential amino acid for the common carp (*Cyprinus carpio* L.) [1]. Our previous study indicates that dietary Ile can improve the growth performance of juvenile Jian carp (*C. carpio* var. Jian) [2]. Fish growth rate is often related to disease resistance [3]. Survival rates after challenge could reflect disease resistance of fish [4]. In mice, Ile deficiency decreased the survival rate after challenge with *Salmonella typhimurium* [5]. However, studies have not been conducted to investigate the effect of dietary Ile on the disease resistance of fish, which warrants investigation.

Disease resistance in fish is dependent on their immune response [4]. To date, information about the effects of dietary Ile on the immune response in fish has not been available. In mammals, Ile is incorporated into human leucocyte cellular proteins and lipids [6] and improves the serum complement component 3 (C3) level in mice [7]. In addition, inflammatory cytokines play an important role in immunity [8]. The expression of tumour necrosis factor α (TNF-α) and interleukin 1β (IL-1β) mRNAs in mouse liver was reduced by branched-chain amino acid (BCAA) (Ile, Leu, Val) supplementation [9]. The above data indicate a possible correlation between Ile and the immune response in fish.

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To the best of our knowledge, the head kidney is the primary immune organ in fish [10]. Fish immunity has been correlated with normal structural and function of the head kidney [11]. In rats, normal kidney structure and function is partly related to oxidative damage and antioxidant status [12]. Fish antioxidant capacity includes enzymatic and non-enzymatic antioxidant defences [13]. However, there is no information about the effect of Ile on oxidative damage and antioxidant status of immune organs in fish. *In vitro,* Ile inhibits lipid peroxidation of ox-brain phospholipids [14]. Katayama and Mine [15] reported that Ile elevates catalase (CAT) and glutathione-*S*-transferase (GST) activities in human intestinal epithelial cells. Thus, Ile may affect the immune function of fish through regulating oxidative damage and antioxidant status of fish head kidney. Accordingly, studies are required to address the effects of Ile on oxidative damage and antioxidant status of the head kidney in fish.

The head kidney contains various immune cells in fish [16]. In mammals, the mammalian target of rapamycin (mTOR) pathway has emerged as a key regulator of the inflammatory response in monocytes, macrophages and peripheral myeloid dendritic cells [17]. Wacyk et al. [18] implied that dietary BCAA increases hepatic TOR gene expression in rainbow trout (*Oncorhynchus mykiss*). Eukaryotic IF4E-binding proteins (4E-BPs), which comprise a family of translational repressors, are the major downstream targets of TOR protein in mammals [19]. Our previous study also shows that dietary Ile regulates the relative gene expression of TOR and 4E-BP in the hepatopancreas and intestine of Jian carp [2]. Therefore, it appears that Ile may be related to the TOR signalling pathway of the head kidney to affect fish immunity. However, there is no information about the effect of Ile on the TOR signalling pathway of the head kidney in fish.

This study is a part of a larger study aimed at determining the effects of Ile on fish growth using the same growth trial as the previous study [2]. The objective of this study was to investigate the effects of Ile on the immune response and further investigate the antioxidant status and gene expression in fish head kidney, which could be used to preliminarily determine the Ile-dependent mechanism of improving disease resistance.

2. Materials and methods

2.1. Experimental diets and feeding management

Experimental diets and diet preparation procedures and storage (−20 °C) were the same as our previous study [2]. Table 1 shows the formulation of the basal diet used in our previous study [2]. Fish meal and gelatine were used as the primary protein sources. Ile was added to the test diets at the concentrations of 4.5 (un-supplemented control), 7.0, 9.5, 12.0, 14.5 and 17.0 g kg^{-1} diet. The diets were made isonitrogenous by supplementation with L-glycine. The Ile concentration in the six experimental diets were determined to be 4.2 (un-supplemented control), 7.0, 9.5, 11.9, 13.9 and 16.9 g kg^{-1} diet.

Feeding was performed as described in our previous study [2]. After an acclimatisation period of 30 days, juvenile Jian carp (mean initial weight 6.9 ± 0.03 g) were randomly assigned to 24 glass aquaria (90 *L* × 30 *W* × 40 *H* cm) for the growth trial (50 fish/tank). The fish in each treatment tank were fed the appropriate diets to apparent satiety for 60 days: six times daily from 1 to 30 days and four times daily from 31 to 60 days. During the experiment, water flow rate in each aquarium was maintained at 1.2 L min^{-1}, and the water was strained through bio-filters to decrease microorganisms, reduce ammonia concentration and remove solid substances in the water. The water temperature and pH were 24 ± 1 °C and 7.0 ± 0.3, respectively. Dissolved oxygen was greater than 5 mg L^{-1}. The experimental units were maintained under a natural light–dark cycle.

Table 1
Composition and nutrient content of the basal diet.

Ingredients	g kg^{-1}	Nutrients content[a]	g kg^{-1}
Fishmeal	132.0	Crude protein	335.8
Gelatin	68.7	Crude lipid	63.2
Amino acid premix[b]	194.1	Crude ash	62.6
Isoleucine–glycine premix[c]	40.0	ω-3	10.0
Alpha-starch	220.0	ω-6	10.0
Corn starch	241.4	Available phosphorus	6.0
Fish oil	17.8	Isoleucine	4.2
Soybean oil	18.9		
Vitamin premix[d]	10.0		
Mineral premix[e]	10.0		
$Ca(H_2PO_4)_2$	25.3		
Choline chloride (50%)	1.3		
Ethoxyquin (30%)	0.5		
Cellulose	20.0		

[a] The values of crude protein, crude fat, crude ash and isoleucine content were measured value. Available phosphorus, ω-3 and ω-6 contents calculated according to Bell (1984) and NRC (1993).

[b] Amino acid mix (g kg^{-1} diet): lysine, 18.974 g; methionine, 8.788 g; tryptophan, 4.184 g; threonine, 13.367 g; arginine, 12.035 g; histidine, 6.724 g; leucine, 23.350 g; phenylalanine, 15.361 g; valine, 17.440 g; cystine, 0.700 g; tyrosine, 12.663 g; glycine, 60.484 g.

[c] Isoleucine–glycine premix (40 g kg^{-1} diet) was added to obtain a graded level of crystalline isoleucine, and the amount of glycine and corn starch was reduced to compensate. Per kilogram of isoleucine premix composition from diet 1 to 6 was as follows (g kg^{-1} diet): L-isoleucine 0.000, 62.500, 125.000, 187.500, 250.000, 312.500 g; glycine 277.218, 241.935, 204.133, 168.851, 133.569, 95.766 g and corn starch 722.782, 695.565, 670.867, 643.649, 616.431, 591.734 g, respectively.

[d] Per kilogram of vitamin premix (g kg^{-1} diet): retinyl acetate (500,000 IU g^{-1}), 0.800 g; cholecalciferol (500,000 IU g^{-1}), 0.480 g; D, L-α-tocopherol acetate (50%), 20.000 g; menadione (50%), 0.200 g; cyanocobalamin (10%), 0.010 g; D-biotin (20%), 0.500 g; folic acid (96%), 0.521 g; thiamin nitrate (98%), 0.104 g; ascorhyl acetate (92%), 7.247 g; niacin (98%), 2.857 g; meso-inositol (98%), 52.857 g; calcium-D-pantothenate (98%) 2.511 g; riboflavine (80%), 0.625 g; pyridoxine hydrochloride (98%), 0.755 g. All ingredients were diluted with corn starch to 1 kg.

[e] Per kilogram of mineral premix (g kg^{-1} diet): $FeSO_4 \cdot 7H_2O$ (19.7% Fe), 69.695 g; $CuSO_4 \cdot 5H_2O$ (25.0% Cu), 1.201 g; $ZnSO_4 \cdot 7H_2O$ (22.5% Zn), 21.640 g; $MnSO_4 \cdot H_2O$ (31.8% Mn), 4.089 g; KI (3.8% I), 2.895 g; $NaSeO_3$ (1.0% Se), 2.500 g. All ingredients were diluted with $CaCO_3$ to 1 kg.

2.2. Red and white blood cell counts

At the end of the feeding trial, blood was collected from the caudal vein using a syringe with heparin as an anticoagulant from 3 fish from each aquarium for red blood cell (RBC) and white blood cell (WBC) counts according to the method described by Harikrishnan et al. [20].

2.3. Antioxidant parameter analysis

2.3.1. Sample collection and tissue preparation

Sample collection procedures were similar to those previously described in another study conducted in our laboratory [11]. At the end of the feeding trial, fish from each aquarium were anaesthetised in a benzocaine bath (50 mg L^{-1}) 12 h after the last feeding, then the head kidney of 15 fish from each aquarium were quickly removed, weighed, frozen in liquid nitrogen, and stored at −80 °C until analysis. Head kidney index (HKI) was calculated according to the following equation: HKI = (wet head kidney weight/wet body weight) × 100%. Head kidney samples were homogenised in 10 volumes (w v^{-1}) of ice-cold physiological saline and centrifuged at 6000 × *g* for 20 min at 4 °C, and supernatants were collected for antioxidant parameters analysis.

2.3.2. Detection of lipid peroxidation, protein oxidation, $O_2^{\cdot-}$-scavenging ability, and $^{\cdot}OH$-scavenging ability

Protein content of samples was measured using the Bradford method [21]. Malondialdehyde (MDA) content was measured using