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Effects of graded levels of dietary methionine hydroxy analogue on immune response and antioxidant status of immune organs in juvenile Jian carp (*Cyprinus carpio* var. Jian)

Sheng-Yao Kuang [a,d], Wei-Wei Xiao [a], Lin Feng [a,b,c], Yang Liu [a,b,c], Jun Jiang [a,b,c], Wei-Dan Jiang [a,b,c], Kai Hu [a,b,c], Shu-Hong Li [a], Ling Tang [a,d], Xiao-Qiu Zhou [a,b,*]

[a] *Animal Nutrition Institute, Sichuan Agricultural University, Ya'an 625014, China*
[b] *Fish Nutrition and Safety Production University Key Laboratory of Sichuan Province, Sichuan Agricultural University, Ya'an 625014, China*
[c] *Key Laboratory for Animal Disease-resistance Nutrition of China Ministry of Education, Ya'an 625014, China*
[d] *Animal Nutrition Institute, Sichuan Academy of Animal Science, Chengdu 610066, China*

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ABSTRACT

Immune response and antioxidant status of immune organs in juvenile Jian carp (*Cyprinus carpio* var. Jian) fed graded levels of methionine hydroxy analogue (MHA) (0, 5.1, 7.6, 10.2, 12.7, 15.3 g kg^{-1} diet) for 60 days were investigated. Results indicated that head kidney index, spleen index, red and white blood cell counts significantly increased with increasing MHA levels up to a point ($P < 0.05$), whereupon decreased ($P < 0.05$). Glutathione reductase activity in head kidney and spleen, anti-hydroxy radical and glutathione-S-transferase activities in spleen, catalase activity and GSH content in head kidney significantly increased by MHA supplement, while malondialdehyde content, anti-superoxide anion, superoxide dismutase, glutathione peroxidase activities in head kidney and spleen, protein carbonyl content and catalase activity in spleen, anti-hydroxy radical activity in head kidney significantly decreased by MHA supplement. However, protein carbonyl content and glutathione-S-transferase activity in head kidney, GSH content in spleen remained unaffected. After 60-day feeding trial, a challenge study was conducted by injection of *Aeromonas hydrophila* for 17 days. Results showed that survival rate, leukocytes phagocytic activity, lysozyme activity, acid phosphatase activity, total iron-binding capacity, haemagglutination titre, complement 3, 4 and immunoglobulin M contents significantly increased by optimal dietary MHA supplement ($P < 0.05$). These data suggested that MHA affected antioxidant status of immune organs and promoted immune response in juvenile Jian carp.

1. Introduction

Methionine is an essential dietary nutrient for normal growth of fish [1,2]. The fish growth rate is often related to the diseases resistance [3]. Studies indicated that methionine deficiency led to depressed survival rate, as well as reduced growth performance in Jian carp (*Cyprinus carpio* var. Jian) [4], rainbow trout (*Oncorhynchus mykiss*) [5] and juvenile red drum (*Sciaenops ocellatus*) [6]. Supplement methionine hydroxy analogue (MHA), a common used synthetic methionine source, to methionine-deficient diets increased survival rate and also improved growth performance of rainbow trout [5] and juvenile red drum [6]. Animal disease resistance in general is associated with immune organs growth [7] and immune response [8]. However, little is known concerning the effect of MHA on immune organs growth and immune response in fish. A few studies found that MHA improved spleen weight, serum lysozyme activity and phagocytosis of peripheral blood lymphocyte in broiler chicken [9], and increased antibody titres to sheep red blood cells in white leghorn layer [10]. Furthermore, studies had demonstrated that MHA was converted into L-methionine for effective utilization in chicken liver, rat liver and hog kidney [11]. Our laboratory previous study found that dietary DL-methionine could improve serum lysozyme activity, total iron-binding capacity, haemagglutination titre, complement 3, 4 and immunoglobulin M (IgM) contents in Jian carp [4]. These appear that MHA can also affect fish immune response, which needs investigation.

* Corresponding author. Animal Nutrition Institute, Sichuan Agricultural University, Ya'an 625014, China. Tel.: +86 835 2885157; fax: +86 835 2885968.
E-mail addresses: xqzhouqq@tom.com, zhouxq@sicau.edu.cn (X.-Q. Zhou).

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Head kidney and spleen are two important immune organs in carp [12]. Head kidney is the principal immune organ responsible for phagocytosis, antigen processing, formation of IgM and immune memory through melanomacrophagic centres [13], while spleen is mainly responsible for antibody formation and B cells differentiation [14]. Thus the normal structure and function of immune organs is correlated with fish immunity. Nevertheless, oxidative injury often led to alteration of structure and function in many organs [15]. The higher percentage of polyunsaturated fatty acids in membranes and frequently exposed to ROS produced as part of normal function lead to sensitively oxidative stress in immune cells [16], which may also result in sensitively oxidative stress in head kidney and spleen. It is, therefore, very important to keep normal antioxidant status in head kidney and spleen, but no studies about the effect of MHA on antioxidant status in immune organs had been conducted. Our previous study found that MHA improved antioxidant defense in intestine and hepatopancreas of Jian carp [17]. It appears that MHA can also affect antioxidant status in immune organs to regulate fish immunity, which warrants further investigation.

This study was a part of further study that involved in the determination of the effects of MHA on growth performance in Jian carp using the same growth trial as the previous study [18]. The study was performed to investigate the possible effects of MHA on fish immune response and further investigate antioxidant status in immune organs.

2. Materials and methods

2.1. Fish

Hatchery-reared juvenile Jian carp were obtained from the TongWei Hatchery (Sichuan, China). Before starting the experiment, the fish were acclimatized to the experimental conditions for 4 weeks. A total of 900 fish with an initial weight of 8.24 ± 0.03 g were randomly assigned to each of 18 experimental aquaria (90 L × 30 W × 40 H cm). Each experimental diet was randomly assigned to triplicate aquaria. All the aquaria system, operation of the culture system, and water quality were the same as our previous study [18]. The fish were hand-fed with the respective diet to apparent satiation six times daily from day 1 to 30 and four times daily from day 31 to 60. Thirty minutes after the feeding, uneaten feed were removed by syphoning and then air dried. The experimental units were under a natural light and dark cycle. The water temperature, pH value and dissolved oxygen were 25 ± 1 °C, 7.0 ± 0.3 and 5 mg L^{-1}, respectively.

2.2. Rations

The formulation of the basal diet is presented in Table 1. Except methionine, dietary components (amino acids, vitamins and minerals) were supplemented to meet the requirements of juvenile Jian carp according to our previous studies [19–27] and reported nutritional requirements for common carp [28]. Six experimental diets were formulated according to MHA supplementation: 0 (control), 5.1, 7.6, 10.2, 12.7 and 15.3 g MHA kg^{-1} diet. Liquid MHA product (measured with 879 g kg^{-1} active substance) (Sumitomo-chemical, Tokyo, Japan) was added to the test diets to provide different concentrations, and the amount of corn starch was reduced to compensate final amount. The methionine concentration in the basal (control) diet was 6.9 g kg^{-1} diet, which was determined by the method of Spindler et al. [29]. Experimental diets and the procedures for diet preparation and storage (−20 °C) were the same as was previously reported in Ref. [18].

Table 1
Ingredients and composition of the basal diet.[a]

Ingredients	g kg^{-1}
Fish meal	75.0
Soybean meal	75.0
Rice gluten meal	100.0
Cottonseed meal	147.8
Rapeseed meal	295.6
Wheat flour	165.7
Fish oil	22. 5
Soybean oil	7.2
Vitamin premix[b]	10.0
Trace mineral premix[c]	10.0
Ca $(H_2PO_4)_2$	26.7
Choline chloride (50%)	1.3
Carboxymethyl cellulose	20.0
Ethoxyquin (30%)	0.5
Threonine (98.5%)	5.9
Lysine (78.8%)	6.8
MHA premix[d]	–
Proximate composition	
Moisture (%)	11.70
Crude protein (% dm)	32.14
Crude fat (% dm)	5.44
Crude ash (% dm)	8.79
Cysteine (%)	0.79
Methionine (%)	0.69

[a] Fish meal, soybean meal, rapeseed meal, cottonseed meal and rice gluten meal were used as dietary protein sources. Fish oil, soybean oil and wheat flour were used as dietary lipid and carbohydrate source respectively. Lysine, threonine, available phosphorus, n-3 and n-6 calculated contents were 20.0, 17.0, 6.0, 10.0 and 10.0 g kg^{-1} diet respectively according to NRC (1993) [28] and Bell (1984) [67].

[b] Per kilogram of vitamin premix (g kg^{-1} diet): retinyl acetate, 0.80 (500,000 IU g^{-1}); cholecalciferol, 0.48 (500,000 IU g^{-1}); DL-α-tocopherol acetate, 20.00 (50%); menadione, 0.20 (50%); cyanocobalamin, 0.01 (10%); D-biotin, 0.50 (20%); folic acid, 0.52 (96%); thiamin nitrate, 0.10 (98%); ascorhyl acetate, 7.23 (92%); niacin, 2.86 (98%); meso-inositol, 52.86 (98%); calcium-D-pantothenate, 2.51 (98%); riboflavine, 0.63 (80%); pyridoxine hydrochloride, 0.76 (98%). All ingredients were diluted with corn starch to 1 kg.

[c] Per kilogram of mineral premix (g kg^{-1} diet): $FeSO_4 \cdot 7H_2O$, 69.70 (19.7% Fe); $CuSO_4 \cdot 5H_2O$, 1.20 (25.0% Cu); $ZnSO_4 \cdot 7H_2O$, 21.64 (22.5% Zn); $MnSO_4 \cdot H_2O$, 4.09 (31.8% Mn); KI, 2.90 (3.8% I); $NaSeO_3$, 2.50 (1.0% Se). All ingredients were diluted with $CaCO_3$ to 1 kg.

[d] MHA premix (30 g kg^{-1} diet) was added to obtain graded level of MHA, and the amount of corn starch was reduced to compensate. Six MHA premix were elaborated according to different proportion liquid MHA (g) and corn starch (g): 0/1000.0, 193.3/806.7, 290.0/710.0, 386.7/613.3, 483.3/516.7 and 580.0/420.0.

2.3. Red and white blood cell counts assay

After the feeding trial, blood was collected from the caudal vein by the syringe with heparin as the anticoagulant from 3 fish of each aquarium for blood cell count. The red and white blood cell counts were determined by using Neubauer haemocytometer and diluting the blood sample with Hayem's and Turke's solution, respectively [30].

2.4. Antioxidant-related parameters assay

2.4.1. Sample and tissue preparation

At the end of the feeding trial, fish were anaesthetized with benzocaine (50 mg L^{-1}) 12 h after the last feeding. Head kidney and spleen of 15 fish from each aquarium were quickly removed, weighed and frozen in liquid nitrogen, and then stored at −70 °C until analyzed. Tissue samples of 6 fish per tank were homogenized on ice in 10 volumes (w v^{-1}) of ice-cold physiological saline (0.7 g mL^{-1}) and centrifuged at 6000 *g* for 20 min at 4 °C respectively, and then supernatants were stored at −20 °C for antioxidant parameters analysis.